Université A. Mira Béjaia

Faculté des Sciences de la Nature et de la Vie

Recueil des Publications de la Faculté des Sciences de la Nature et de la Vie

Années 2010/2011

Compilation et Conception

Pr. Riadh MOULAÏ

Vice Doyen P.G.R.S.R.E.

Le présent Recueil, regroupe les principales publications élaborées par les enseignants chercheurs affiliés à la Faculté des Sciences de la Nature et de la Vie.

Nous avons retenu les publications parues durant les années 2010 et 2011. Les publications englobent des articles édités dans des revues internationales ainsi que des actes et proceedings de congrès internationaux.

Les publications sont classées par ordre alphabétique du premier auteur de l'article ou du proceedings.

Sommaire

- **Boucherba** Nawel, Benallaoua Said, Copinet Estelle, Hebal Hakim, Francis Duchiron (2011) - Production and partial characterization of xylanase produced by *Jonesia denitrificans* isolated in Algerian soil

- Abdelazize Franck **Bougaham** , Riadh Moulaï & John O'Halloran (2011) - Breeding biology of Grey Wagtails *Motacilla cinerea* at the southern edge of their breeding range (region of Béjaia, Algeria)

- **HANIFI**-BENHAMICHE Samira, MOULAÏ Riadh, BOUGAHAM A.F and BOUYAHMED H., (2011) - Qualitative study of the vascular flora of three insular environments concerning the west coast of Jijel (Island Grand Cavallo, Island Petit Cavallo and Islet Grand Cavallo) (Algeria). *Proceedings of the International Conference on the Conservation and Sustainable Use of Wild Plant Diversity, 4-8 May 2010, Kolympari, Chania, Crete (Greece). Institute of Theology and Ecology of the Orthodox Academy of Crete (OAC), Eds. L. Andrianos, J.W. Sneep and K. Kenanidis* : 107-115

- R. **Laribi**, F. Laincer, A. Tamendjari S. Keciri L. Arrar S. Venturini P. Rovellini (2011) - Caractérisation de dix variétés d'huile d'olive algérienne: étude du profil en composés phénoliques par HPLC

- KHADRA **MAIZA**, VICTORIA HAMMICHE AND FADILA MAIZA-BENABDESSELAM (2011) - **TRADITIONAL MEDICINE IN NORTH SAHARA: THE"DEFFI"**

- LILA BOULEKBACHE-**MAKHLOUF**, EMMANUELLE MEUDEC, MOHAMED CHIBANE, JEAN-PAULMAZAURIC, SAKINA SLIMANI,MAX HENRY, VERONIQUE CHEYNIER, AND KHODIR MADANI (2010) - Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of *Eucalyptus globulus* Cultivated in Algeria

- Riadh **MOULAÏ** & Lyes AISSAT (2011) - Contribution à l'analyse de la diversité entomologique des milieux insulaires de la région de Jijel (Algérie)

- Riadh **MOULAÏ** et Abdellazize Franck BOUGAHAM (2010) - PLACE DES DECHETS MENAGERS DANS L'ALIMENTATION DU GOELAND LEUCOPHEE, *LARUS MICHAHELLIS* DE LA REGION DE JIJEL (ALGERIE)

- Salim **Ouchemoukh**, Paul Schweitzer, Mostapha Bachir Bey, Hafsa Djoudad-Kadji , Hayette Louaileche (2010) - HPLC sugar profiles of Algerian honeys

- **Oukil** Naima, Bedjou Fatiha*, Maiza-Benabdesselam Fadila and Chibane Mohamed (2011) - Antioxidant activity and separation of phenolic compounds of *Origanum glandulosum* from north Algeria by high performance liquid chromatography (HPLC)

- Malika **Ourari**, Abdelkader Ainouche, Olivier Coriton, Virginie Huteau, Spencer Brown, Marie-Thérèse Misset, Malika Ainouche, and Rachid Amirouche (2011) - Diversity and evolution of the *Hordeum murinum* polyploid complex in Algeria

- Ophelie **Philipot**, Veronique Joliot, Ouardia Ait-Mohamed, Celine Pellentz, Philippe Robin, Lauriane Fritsch, Slimane Ait-Si-Ali (2010) - The Core Binding Factor CBF Negatively Regulates Skeletal Muscle Terminal Differentiation

- **Sobhi** Widad, Khettal Bachra, Belmouhoub Messaoud, Atmani Djebbar, Duez Pierre, Benboubetra Mustapha (2011) -Hepatotoxicity and Langerhans Islets Regenerative Effects of Polar and Neutral Lipids of *Nigella sativa* L. in Nicotinamide/streptozotocin-Induced Diabetic Rats

- Saïda Benbellil-**Tafoughalt**, Mohamed Sahnoune , Annette de Vaufleury & Aissa Moali (2011) - INLUENCE OF SAMPLING DATE ON REPRODUCTION IN THE LAND SNAIL *HELIX APERTA* KEPT UNDER CONTROLLED CONDITIONS OF TEMPERATURE AND PHOTOPERIOD

Go-South Bull. (2011), **8**, 41-43

Nidification du Grand Corbeau *Corvus corax tingitanus* sur un édifice humain à Béjaia (nord-est algérien)

Boubekeur ABERKANE [(1)], Riadh MOULAI [(2)], Leghel TOUAZI [(2)], Yamina HAMIDOUCHE [(2)] et Abdelkrim SI BACHIR [(3)]

[(1)] Faculté des Sciences de la Nature et de la Vie, Université Abderrahmane Mira, Route de Targa Ouzemmour – Bejaia 06000 (Algérie)
aberkaneboubekeur@gmail.com

[(2)] Faculté des Sciences de la Nature et de la Vie, Université Abderrahmane Mira, Route de Targa Ouzemmour – Bejaia 06000 (Algérie)

[(3)] Département de biologie, Université de Batna – Batna 05000 (Algérie)

Disponible en ligne (Available online) : 8 avril 2011

Introduction

Le Grand Corbeau *Corvus corax* est largement répandu dans les zones non-désertiques de l'hémisphère nord. Il utilise une grande variété d'habitats et construit ses nids préférentiellement sur des falaises, parfois sur des arbres ou, plus rarement, sur des constructions humaines (Cramp & Perrins 1994).

C'est une espèce commune en Afrique du Nord. La plupart des cas de reproduction y ont été signalés en falaise ou sur des arbres, parfois sur des pylônes électriques ou sur d'anciens bâtiments (voir les synthèses d'Isenmann & Moali 2000 pour l'Algérie, de Thévenot *et al.* 2003 pour le Maroc, et d'Isenmann *et al.* 2005 pour la Tunisie).

A ce jour, il ne semble pas que des cas de reproduction sur des édifices humains occupés aient été signalés en Algérie. Nous nous proposons de documenter ici la nidification de cette espèce à Béjaia, dans le nord-est algérien.

Matériel et méthodes

La zone d'étude est située dans la ville côtière de Bejaia (36°45'N-5°02'E) située dans le nord-est du pays, à 220 km de la capitale Alger (Fig. 1). Bejaia est limitée à l'est et au sud-est par le massif des Babors, au nord par la Méditerranée et à l'ouest par les crêtes du Djurdjura. La région est caractérisée par la prédominance de zones montagneuses compactes et bosselées ; elle est traversée par le couloir de la vallée de la Soummam.

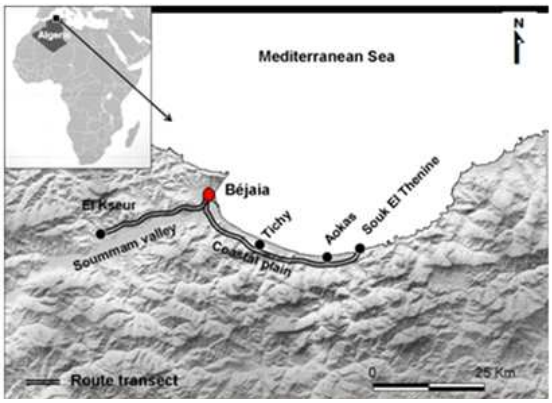


Figure 1. Localisation de Béjaia

Le site de nidification du Grand Corbeau est situé à l'intérieur de l'université de Bejaia, au deuxième étage d'un bâtiment situé au sud du campus de Targa Ouzemmour. Le nid est bâti à l'intérieur d'une imposte de fenêtre du laboratoire de recherche N°12, à une hauteur d'une quinzaine de mètres (Fig. 2).

Nous avons suivi le comportement de nidification du début du mois de mars 2008 jusqu'à la fin de la saison de reproduction (mai) 2010. Nous avons utilisé une paire de jumelles 8x40 pour l'observation des oiseaux et un appareil photo de marque Canon (Power Shot A480, 3.3 x Optical zoom) pour la prise des photos.

Réultats

Le même nid a été utilisé durant trois années successives, de 2008 à 2010 mais le personnel de l'université nous a signalé que le Grand Corbeau avait niché sur l'ancienne bibliothèque du campus de Targa Ouzemmour, à 100 m du site actuel, trois ans auparavant, sans succès (élimination du nid par le personnel).

2008

Le couple de Grand Corbeaux est présent sur le campus depuis janvier au moins. Il passe la majorité de son temps sur le bloc d'enseignement N°6, juste en façe de l'emplaçement de leur futur nid. Les parades nuptiales démarrent dès janvier.

La construction du nid commence au début du mois du mars et les 4 œufs de la ponte éclosent entre le 18 et le 22 avril. Dès lors, les apports de nourriture se font à un rythme soutenu mais 20 jours plus tard un jeune est trouvé mort sous le nid. Durant la nuit, la femelle s'installe dans le nid et le mâle dans l'imposte gauche (Fig. 2).

Figure 2. Emplacement du nid

Le 26 mai, deux jeunes quittent le nid et se perchent dans un eucalyptus proche. L'un des parents suit les deux jeunes dans tous leurs déplacements, l'autre assure l'apport de nourriture et la surveillance du jeune restant.

Le lendemain 27 mai aux environs de 14 heures, le troisième jeune quitte le nid et rejoint ses frères. Tous trois restent avec leurs parents jusqu'au début du mois de janvier 2009 ; à partir de cette date, seuls les deux parents sont observés.

2009

Le 5 mars, un adule élimine d'anciennes brindilles et les remplace par des nouvelles (Fig. 3).

Figure 3. Un adulte éliminant une ancienne branche du nid

Comme l'année précédente, quatre œufs sont pondus et éclosent entre les 10 et 15 avril. A partir de ce moment, les parents défendent activement le nid.

Le 22 mai vers les 10 heures, trois jeunes quittent le nid et se posent sur un bâtiment proche. Le quatrième jeune s'envole le 24 mai mais se fracture une patte. Quelques jours plus tard, les parents accompagnent trois jeunes ; le dernier a dû périr. Les cinq oiseaux sont observés jusqu'en décembre.

2010

Le même comportement de remplacement de garniture du nid est observé en début de saison de reproduction.

Encore une fois, quatre œufs sont pondus, qui éclosent vers mi-avril. Deux jeunes quittent le nid le 25 mai, les deux autres deux jours plus tard. Les six oiseaux sont notés ensemble sur le site jusqu'en décembre.

Discussion

Trois années de suite, le Grand Corbeau s'est reproduit avec succès sur un bâtiment de l'université de Béjaia, en utilisant le même nid. Il s'agit, à notre connaissance, des premiers cas de reproduction connus en milieu urbain en Algérie.

Cette implantation récente est peut-être due à la dégradation de l'habitat naturel de cette espèce, en particulier à la suite des incendies de forêts annuels. Mais il est également très probable que l'augmentation des décharges publiques non contrôlées aux alentours de la ville, qui a provoqué une forte augmentation de la population de Goélands leucophées *Larus cachinnans*, ait joué un rôle déterminant dans l'installation du Grand Corbeau à Béjaia. Aujourd'hui, Grand Corbeau et Goélands y entrent en compétition…

Références

Cramp, S. & Perrins, C.M. (Eds.) 1994. *The Birds of the Western Palearctic*. Vol. VIII. Oxford University Press, Oxford.

Isenmann, P. & Moali, A. 2000. *Oiseaux d'Algérie* / *Birds of Algeria*. S.E.O.F. (Société d'Etudes Ornithologiques de France), Paris. 336 pp.

Isenmann, P. ; Gaultier, T. ; El Hili, A. ; Azafzaf, H. ; Dlensi, H. & Smart, M. 2005. *Oiseaux de Tunisie* / *Birds of Tunisia*. S.E.O.F. (Société d'Etudes Ornithologiques de France), Paris. 432 pp.

Thévenot, M. ; Vernon, J.D.R. & Bergier, P. 2003. *The Birds of Morocco*. British Ornithologist Union Checklist Series: 20. 594 pp.

Remerciements

Nous remercions le Pr. Iguer-Ouada Mmokrane, directeur du laboratoire de biologie animale à l'U.A.M.B. pour sa collaboration à ce travail, ainsi le Dr. Ayad Hanine, maître de conférences à l'U.A.M.B et Mr. Farid Belbachir, Institut zoologique de Londres. Nous remercions également Patrick Bergier, Go-South, pour ses aimables conseils et orientations depuis le début de ce travail.

The diet of the Maghrebian mouse-eared bat *Myotis punicus* (Mammalia, Chiroptera) in Kabylia, Northern Algeria

Régime alimentaire du Murin du Maghreb Myotis punicus *(Mammalia, Chiroptera) en Kabylie, nord de l'Algérie*

M. AHMIM and A. MOALI

Laboratoiry of ecologie and environnment
Faculty of nature sciences and life
Abderrahmane MIRA University, Bejaia, Algeria

E.mail: forestecolo@gmail.com
Aissa.moali@gmail.com

Abstract

This paper describes the composition of the diet of the Maghrebian mouse-eared bat species Myotis punicus *in the North of Algeria. The Maghrebian mouse-eared bat,* Myotis punicus *Felten, 1977 is classified by the IUCN Red List as a species of missing data and it is a specie whose knowledge of hunting habitat and diet are virtually unknown in Algeria. In our contribution we have studied the diet of the specie In the area located in the region of Kabylia Babors, in wilayates (districts) of Bejaia and Jijel in Algeria between the months of march 2007 and January 2008. The protocol used consisted of a sampling of guano in the different sites used by the species and the identification of remains of insects under microscope For analysis, samples of guano have been soaked at least one hour in 70% alcohol before being dissected using forceps under a binocular magnification 400× and the determination was made with a help of the identification key by Shiel* et al. *(1997). The results suggests that* Myotis punicus *in the studied sites of Algeria consumed prey belonging to three groups of arthropods: insects (frequence 96.06%),chilopods (2.82%) and spiders (1.12%).*

Résumé

Cet article décrit la composition du régime alimentaire du murin du Maghreb Myotis punicus *dans le nord de l'Algérie. En effet le Murin du Maghreb* Myotis punicus *est classé dans la liste rouge par l'*IUCN *comme une espèce manquant de données, alors qu'elles sont nécessaires pour sa classification dans la liste des espèces menacées.* Myotis punicus *est une espèce dont les connaissances des habitats de chasse et du régime alimentaire sont pratiquement inconnues en Algérie où à l'heure actuelle il n'est principalement connu que dans le nord du pays. Notre présente contribution consiste en l'analyse du régime alimentaire de cette espèce, La zone retenue pour l'étude est située dans la région de la Kabylie des babors plus exactement, dans les wilayates de Bejaia et de Jijel. Le protocole mis en place a consisté en un prélèvement d'échantillons de guano, ainsi, durant toute la période d'étude, 102 échantillons ont été récoltés au cours de 43 sorties, chaque échantillon étant représenté par 10 grains de guano donc 1 020 d'entre eux ont été analysés. Kervyn (1998) stipule qu'un échantillon annuel de 100 excréments est suffisant pour identifier les proies consommées. Les sorties ont été réalisées entre le mois de mars 2007 et le mois de janvier 2008, au rythme d'une sortie tous les 15 jours. Pour l'analyse, les échantillons de guano récoltés ont été trempés au moins une heure dans de l'alcool à 70 % avant d'être disséqués à l'aide de pinces sous une loupe binoculaire 10 X 40 et la détermination a été faite grâce à la clé de détermination de Shiel* et al. *(1997). Les résultats montrent que* Myotis punicus *consomme dans la zone étudiée en Algérie des proies appartenant à trois groupes d'arthropodes : insecta (fréquence 96,06 %), chilopoda (2,82 %) et Araneida (1,12 %).*

Keywords: *Myotis punicus*, diet, guano, North Algeria, preys.

Mots clés : Murin du Maghreb, *Myotis punicus*, Algérie du Nord, régime alimentaire, proies.

Figure 1 – Specimen of *Myotis punicus* from Algeria.

Introduction

Knowledge of lifestyle and biology of bats is a preliminary step essential to assess environmental contributions particularly important for reproduction of plant species, reforestation and the fight against pests.

The Maghrebian mouse-eared bat, *Myotis punicus* Felten, 1977, is a species whose knowledge of hunting habitat and diet are virtually unknown in Algeria, and its present is mainly known in the north of the country. *Myotis punicus* is distributed in the Mediterranean part of North Africa (Morocco, Algeria, Tunisia and Libya), and several West-Mediterranean islands: Malta, Corsica (France) and Sardinia (Italy) (Arlettaz *et al.* 1997). (Castella *et al.* 2000) and we have No results or data about its diet in italia (Agnelli *et al.* 2004).

Myotis punicus is classified by the IUCN Red List (Aulagnier *et al.* 2008) as a species of missing data.

This species is especially cave-dweller and only a limited number of colony roosts is known (Aulagnier *et al.* 2008). It was determined that its numbers are declining in Corsica, and probably it could be that this is the same case in Sardinia (Arlettaz *et al*. 1997).

In Malta, the work of monitoring of the species showed a decline estimated at 50% or more in three generations. There are about = 10,000 individuals, found in colonies (300-500 individuals) and in Corsica, there are approximately 4,000 individuals in four colonies. The total size of the population in Corsica, Sardinia and Malta is estimated between 7,000 and 9,000 individuals.

This specie is listed as “Near Threatened” (almost Meets Criteria Under A4cd VU) Temple (2009).

Materials and Methods

Study area and habitat mapping

The area selected for the study is located in the region of Kabylia Babors, in wilayates of Bejaia and Jijel.

The protocol used consisted of a sampling of guano in the different sites used by the species and the identification of remains of insects under microscope. The grains of guano of *Myotis punicus* are greater than the other about other species of bat represented by *Rhinolophidae* and *Miniopterus schreibersi*. These are expressed as percentage of frequency to allow comparison with other studies in other countries.

Our study area is located on the biogeographical east of the Great Kabylie (Kabylie of Djurdjura), which is a natural region of northeastern Algeria. It is a mountainous region characterized by a series of coastal links with an average elevation of 1000 m, whose highlights are the Jebel Babor (2004 m) and Jebel Tababor (1969 m). The topography of the region very rugged, with slopes often exceeding 25%, provides general guidance Southwest Northeast (Bellatreche 1994).

Ten sites were explored: the cave Taâssast, 4 caves Boukhiama, Fort Lemercier, Château de la Comtesse, Aokas cave, the cave of the elephants in Bejaia and the Cave of Boublatane in Jijel.

***Figure 2 – Guano of* Myotis punicus.**

Collect and analysis of the samples of guano

The trips were conducted between the months of March 2007 and January 2008, at a rate of one event every 15 days or about every week for each list according to the weather.

Thus, throughout the study period, 102 samples were collected after 43 outputs, each sample is represented by 10 pellets of guano, so 1020 pellets were analyzed in total. Kervyn (1998) stated that a sample of 100 annual dung is sufficient to identify the prey consumed only to identify but not specify its

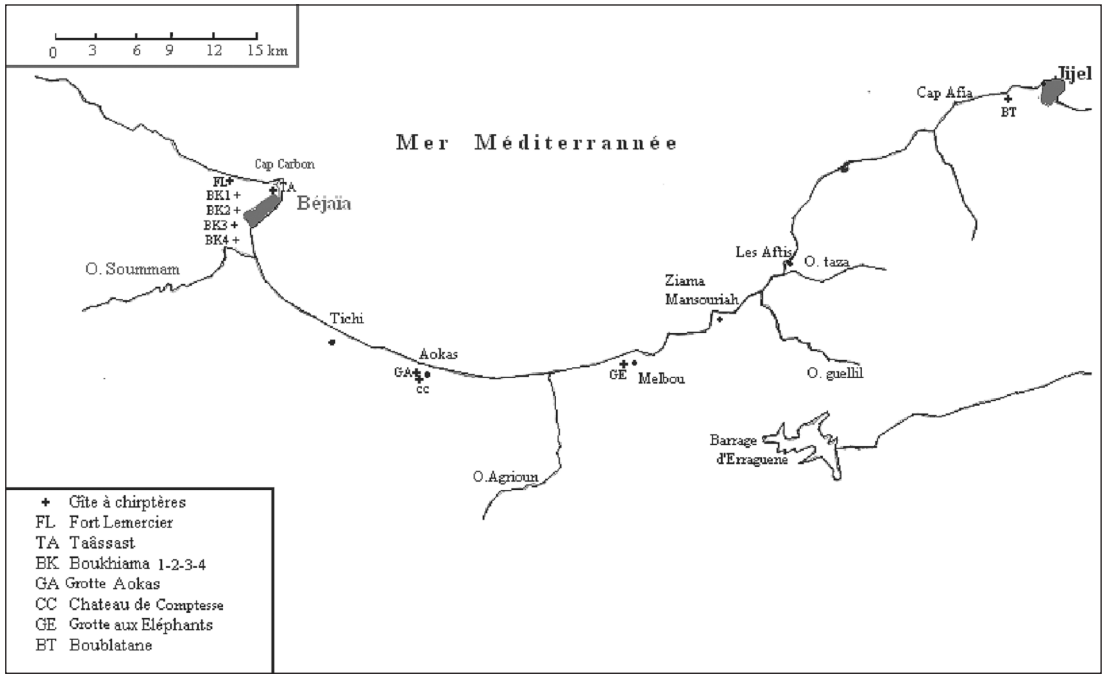


Figure 3 – Location of deposits sampled.

Table 1 – Release Calendar and number of samples collected.

Month	March	Avr	Mai	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Total
Number of Trips	03	03	07	04	04	03	05	04	05	01	04	**43**
Number of samples	28	03	10	03	03	04	13	10	16	06	06	**102**

composition and the annual changes of composition.

For analysis, samples of guano have been soaked at least one hour in 70% alcohol before being dissected using forceps under a binocular magnification 400× and the determination was made with a help of the identification key by Shiel *et al.* (1997).

Several methods of expressing results are used by the authors but their definition is not always harmonized throughoy publications. I referred the diet composition in accordance with Vaughan (1997); the results are expressed as percentage frequency of occurrence, *i.e.* the number of taxa equals the number of samples containing divided by the total number of occurrences, multiplied by 100.

Table 2 – Frequencies (in %) of the prey parts found in the guano of Myotis punicus*.*

	Order	*Effectif (prey parts)*	*Percentage*
Insecta	*Ephemeroptera*	*07*	*3.95*
	Dermaptera	*03*	*1.69*
	Hemiptera	*18*	*10.16*
	Neuroptera	*03*	*1.69*
	Coleoptera	*06*	*3.38*
	Siphonaptera	*02*	*1.12*
	Diptera	*82*	*46.32*
	Lepidoptera	*36*	*20.33*
	Trichoptera	*13*	*7.34*
Chilopoda	*spp.*	*05*	*2.82*
Arachnida	*Araneida*	*02*	*1.12*
		177	

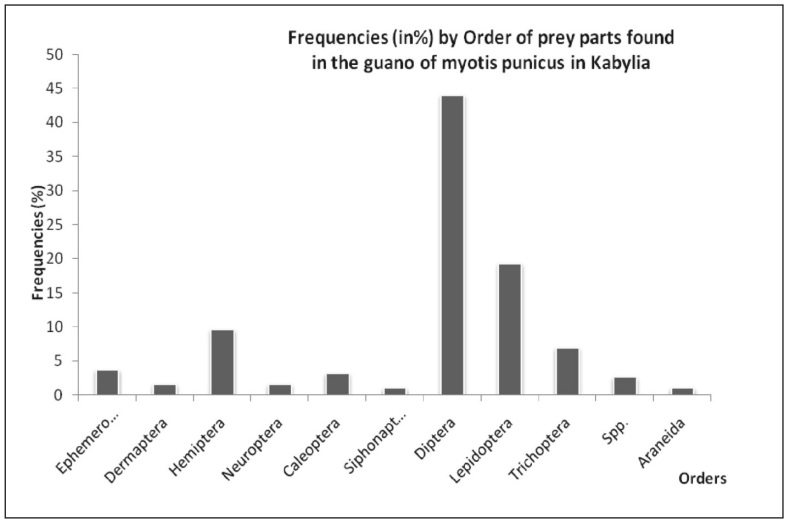


Figure 4 – Histogramme of the frequencies (in %) by Order of prey parts found in the guano of Myotis punicus *in Kabylia.*

Results

As shown in the table, *Myotis punicus* in the studied sites of Algeria consumed prey belonging to three groups of arthropods: insects (frequence 96.06%), chilopods (2.82%) and spiders (1.12%).

Diet composition of the Maghrebian mouse-eared bat *Myotis punicus*

The most consumed insect prey were dipterans with a frequency approaching a half of consumed taxa (46.32%). This percentage is composed mainly by Culicidae (15.59%), of Chironomidae/Ceratopogonidae (9.68%) and Tipulidae (6.45%).

The order Lepidoptera had also a good proportion in the diet of *M. punicus*; the butterflies created 20.33%. Hemipterans occupied 9.68%.

Discussion

The food needs of bats are important. They must accumulate fats to the period of hibernation. The daily ration of bats is equivalent to a quarter or a third of their own weight. It stresses the importance bats in the fight against insect pests for human.

The composition of diet is still very poorly known and we can study only analyzing the remains of food, since all species are protected and it is impossible to examine the contents of their stomachs. Bats catch butterflies, beetles, flies as well as dragonflies, crickets, grasshoppers and spiders. The wings and legs of butterflies and other insects are not eaten,

Table 3 – Frequencies (in %) of prey parts found in the guano of* Myotis punicus *in Kabylia.

Class	Order	Suborder	Superfamily or family	Effectif *(prey parts)*	Frequency (%)
Insecta	*Ephemeroptera*			**07**	**3,76**
	Dermaptera			**03**	**1,61**
	Hemiptera	*Heteroptera*	*Corixidae.*	10	5,38
		Homoptera	*Cercopedae*	03	1,61
			Aphidoidea	05	2,69
			subtotal	**18**	**9,68**
	Neuroptera		*Hemerobiidae*	02	1,08
			Chrysopidae	01	0,54
			subtotal	**03**	**1,61**
	Coleoptera	*Adephaga*	*Carabidae*	03	1,61
		Polyphaga	*Scarabaeoidea*	02	1,08
			Scolytidae	01	0,54
			subtotal	**06**	**3,23**
	Siphonaptera			**02**	**1,08**
	Diptera	*Nematocera*	*Tipulidae*	12	6,45
			Anisopodidae.	06	3,23
			Psychodidae.	05	2,69
			Culicidae.	29	15,59
			Chironomidae /Ceratopogonidae	18	9,68
			subtotal	**70**	**37,63**
		Cyclorrhapha	*Syrphidae*	01	0,54
			Sphaeroceridae.	06	3,23
			Calliphoridae	02	1,08
			Scathophagidae	02	1,08
			Total	**11**	**5,91**
		Brachycera	*Rhagionidae*	01	0,54
			subtotal	**82**	**44,09**
	Lepidoptera			**36**	**19,35**
	Trichoptera			02	1,08
			Limnephilidae.	06	3,23
			Hydropsychidae	05	2,69
			subtotal	**13**	**6,99**
		subtotal		**179**	**96,24**
Chilopoda				**05**	**2,69**
Arachnida	*Araneida*			**02**	**1,08**

fall to the ground and hoard them thereby detect the presence of bats.

In Algeria 11 orders of 3 classes are presents in the diet of *Myotis Punicus*, Our study showed that *Myotis punicus* diet in the study area consisted predominantly of dipterans with a frequency approaching a half of consumed taxa (46.32%) represented by Culicidae (15.59%) Chironomidae/Ceratopogonidae (9.68%) and Tipulidae (6.45%), The order of Lepidoptera 20.33% and Hemiptera 9.68%.

Comparison with Corsican and Maltese diet of *Myotis punicus*

In the Maltese Islands, a study of the diet of *Myotis punicus*, carried out using faecal material frombelow feeding perches, showed that the main prey species were of three insect orders: Orthoptera, Coleoptera and Lepidoptera (Borg 1998).

In Corsica we found 5 orders of Insecta (Orthoptera, Coleoptera, Lepidoptera, Diptera, and hymenoptera), the class of Araneida is present too.

In Corsica, *Myotis punicus* is hunting in open habitats where it captures Orthoptera, Coleoptera and Lepidoptera (caterpillars). The diet of 71 female *Myotis* cf. *punicus* has been identified During the breeding season They feed mainly on Orthoptera (36%) of Coleoptera (33%), Lepidoptera (caterpillars) (23%) and Diptera, Hymenoptera, and Araneida (Beuneux 2004).

Table 4 – Comparison of the diet composition of the Maghrebian mouse-eared bat Maghreb (*Myotis punicus*) in Algeria, Malta, and Corsica (France).

		Country		
	Order	**Algeria**	**Malta) (Borg, 1998)**	**Corsica (France)** *(Beuneux, 2004)*
Insecta	*Ephemeroptera*	X		
	Dermaptera	X		
	Hemiptera	X		
	Neuroptera	X		
	Coleoptera	X	X	X
	Siphonaptera	X		
	Diptera	X		X
	Lepidoptera	X	X	X
	Trichoptera	X		
	Orthoptera		X	X
	Hymenoptera			X
	Homoptera	X		X
Chilopoda	*spp.*	X		
Arachnida	*Araneida*	X		X

A comparison of the composition of frequencies of prey parts in the three countries see that there is a similitude only in 2 orders (Coleoptera and Lepidoptera), in Malta we haven't data about frequencies of the preys.

Between Corsica and Algeria the similitude is in 5 orders: Coleoptera represented by 3.38% in Algeria and 33% in Corsica; Lepidoptera 20.33% in Algeria and 23% in Corsica, Diptera and Arachnida respectively 46.32%, 1.12% in Algeria, and no quotation for Corsica; and we found 36% of Orthoptera in Corsica but this order is not netted in Algeria, The analyse of the composition of the prey parts in the two countries see that there are similitudes in 5 orders of insect with one great similitude about Lepidoptera (20.33% in Algeria and 23% in Corsica).

The prey who is largely consommed in Corsica is represented by the Orthoptera (36%) and in Algeria by the Diptera (46.32%).

Between the two islands Corsica and Malta there are 3 orders in similitude (Orthoptera, Lepidoptera and Coleoptera) and between Malta and Algeria the comparison see that we have 2 orders in similitude (Lepidoptera and Coleoptera).

The absence of Orthoptera in the diet of *Myotis punicus* in Algeria suppose that there is a concurrence with the other species of bats eating insect > 10 mm (*Rhinolophus ferrumequinum*), and possible insects may be caught in flight, or taken from vegetation, the ground, or water surfaces in a foraging style referred to as gleaning. Echolocation is generally used to locate prey although some bats use passive listening', homing in on the sounds made by the prey themselves (Hutson *et al.* 2001).

As second hypothesis concerning the absence of Orthoptera in the diet of *Myotis punicus* in Algeria, we suppose that it is due at the night-activity of dipterans, and that arthropods hide under stones during the night when Chiroptera in activity.

And we also suppose that it is also du at the nearness of the sea where we find many Culeidae, Chironomidae and Cerapogonidae.

Conclusion

Included in the diet of *Myotis punicus*, three classes of arthropods: Insecta, Chilopoda and Arachnida, including the predominance is attributed to insects (96.24%). The taxa most abundant insects (Lepidoptera, Diptera (Chironomidae and Culicidae/Ceratopogonidae) and Corixidae) are parasites and harmful.

It has this effect we can say that bats play a very important role in the ecological balance in many ways, especially as regards the fight against harmful interference, and that analysis of the diet of these gives us very important information on the limitations of numbers of insects and their diversity.

According to Beuneux (2004) the Maghrebian bat seems to be an opportunist gleaning bat.

And *Myotis punicus* is protected by national legislation in its European range states. There are also international legal obligations for its protection through the Bonn Convention (Eurobats) and Bern Convention, in parts of its range where these apply. It is included in Annex IV of the EU Habitats and Species Directive, and some habitat protection may be provided through Natura 2000. There is an ongoing project for the conservation of this species. Appropriate conservation measures include fencing cave entrances (but not gating) and obtaining legal protection for the species. In North Africa further research into population trends, establishment and management of protected areas, education, and implementation of national-scale legislation are needed (Aulagnier *et al.* 2008).

References

Agnelli P., Martinoli A., Patriarca E., Russo D., Scaravelli D. & Genovesi P. (a cura di), 2004. *Guidelines for bat monitoring: methods for the study and conservation of bats in Italy.* Quad. Cons. Natura, 19, Min. Environment, National Wildlife Institute.

Arlettaz R., Ruedi M., Ibanez C., Palmeirim J. & Hausser J., 1997. A new perspective on the zoogeography of the sibling mouse-eared bat species *Myotis myotis* and *Myotis blythii*: morphological, genetical and ecological evidence. *J. Zool.*, Lond. 24 2: 45-62.

Aulagnier S., Juste J., Karata A., Palmeirim J. & Paunovi M., 2008. Myotis punicus. *In*: IUCN 2009. IUCN Red List of Threatened Species. Version 2009. 2.

Bellatreche M., 1994. *Écologie et biogéographie de l'avifaune nicheuse forestière de la Kabylie des Babors (Algérie)*, Thèse. doct. Université de Bourgogne (France), 143 p.

Beuneux G., 2004. Morphometrics and ecology of *Myotis* cf. *punicus* (Chiroptera,Vespertilionidae) in Corsica. *Mammalia* 68 (4): 269-273.

Borg J.J., 1998. The Lesser Mouse-eared Bat *Myotis* blythi *punicus* Felten, 1977 in Malta. Notes on status, morphometrics, movements, and diet (Chiroptera, Vespertilionidae). *Naturalista Siciliano* 22 (3-4): 365-374, 1998.

Castella V., Ruedi M., Excoffier L., Ibanez C., Arlettaz R. & Hausser J., 2000. Is the Gibraltar Strait a barrier to gene flow for the bat *Myotis myotis* (Chiroptera: Vespertilionidae), *Mol. Ecol.* 9: 1761-1772.

Hutson A.M., Mickleburgh S.P. & Racey P.A. (comp.), 2001. *Microchiropteran bats: global status survey and conservation action plan*. IUCN/SSC Chiroptera Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK, X + 258 p.

Kervyn T., 1998. Méthodes de détermination du régime alimentaire des Chiroptères insectivores. *Arvicola 1998 6 Actes « Amiens 97 »* : 53-56.

Shiel C. *et al.*, 1997. *Identification of Arthropod Fragments in Bat Droppings* – occasionnal publication nº 17, The Mammals society, 53 p.

Vaughan N., 1997. The diets of British bats (Chiroptera). *Mammal. Rev*. 27(2): 77-94.

OPEN ACCESS Freely available online

PLoS one

Acetonic Extract of *Buxus sempervirens* Induces Cell Cycle Arrest, Apoptosis and Autophagy in Breast Cancer Cells

Ouardia Ait-Mohamed[1]*, Valentine Battisti[2], Véronique Joliot[2], Lauriane Fritsch[2], Julien Pontis[2], Souhila Medjkane[2], Catherine Redeuilh[3], Aazdine Lamouri[3], Christine Fahy[3], Mohamed Rholam[3], Djebbar Atmani[1], Slimane Ait-Si-Ali[2]*

1 Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la vie, Université de Béjaia, Béjaia, Algeria, **2** Laboratoire Epigénétique et Destin Cellulaire, UMR7216, Centre National de la Recherche Scientifique (CNRS), Université Paris Diderot Sorbonne Paris Cité, Paris, France, **3** Laboratoire ITODYS, UMR7086 CNRS, Université Paris Diderot Sorbonne Paris Cité, Paris, France

Abstract

Plants are an invaluable source of potential new anti-cancer drugs. Here, we investigated the cytotoxic activity of the acetonic extract of *Buxus sempervirens* on five breast cancer cell lines, MCF7, MCF10CA1a and T47D, three aggressive triple positive breast cancer cell lines, and BT-20 and MDA-MB-435, which are triple negative breast cancer cell lines. As a control, MCF10A, a spontaneously immortalized but non-tumoral cell line has been used. The acetonic extract of *Buxus sempervirens* showed cytotoxic activity towards all the five studied breast cancer cell lines with an IC_{50} ranging from 7.74 μg/ml to 12.5 μg/ml. Most importantly, the plant extract was less toxic towards MCF10A with an IC_{50} of 19.24 μg/ml. Fluorescence-activated cell sorting (FACS) analysis showed that the plant extract induced cell death and cell cycle arrest in G0/G1 phase in MCF7, T47D, MCF10CA1a and BT-20 cell lines, concomitant to cyclin D1 downregulation. Application of MCF7 and MCF10CA1a respective IC_{50} did not show such effects on the control cell line MCF10A. Propidium iodide/Annexin V double staining revealed a pre-apoptotic cell population with extract-treated MCF10CA1a, T47D and BT-20 cells. Transmission electron microscopy analyses indicated the occurrence of autophagy in MCF7 and MCF10CA1a cell lines. Immunofluorescence and Western blot assays confirmed the processing of microtubule-associated protein LC3 in the treated cancer cells. Moreover, we have demonstrated the upregulation of Beclin-1 in these cell lines and downregulation of Survivin and p21. Also, Caspase-3 detection in treated BT-20 and T47D confirmed the occurrence of apoptosis in these cells. Our findings indicate that *Buxus sempervirens* extract exhibit promising anti-cancer activity by triggering both autophagic cell death and apoptosis, suggesting that this plant may contain potential anti-cancer agents for single or combinatory cancer therapy against breast cancer.

Citation: Ait-Mohamed O, Battisti V, Joliot V, Fritsch L, Pontis J, et al. (2011) Acetonic Extract of *Buxus sempervirens* Induces Cell Cycle Arrest, Apoptosis and Autophagy in Breast Cancer Cells. PLoS ONE 6(9): e24537. doi:10.1371/journal.pone.0024537

Editor: Pranela Rameshwar, University of Medicine and Dentistry of New Jersey, United States of America

Received May 31, 2011; **Accepted** August 12, 2011; **Published** September 15, 2011

Funding: This work was supported by the Agence Nationale de la Recherche (ANR); the Association Française contre les Myopathies (AFM); the Fondation Bettencourt-Schueller; the Programme franco-algérien de formation supérieure en France (PROFAS) via the Centre des Oeuvres Universitaires et Scolaires (CROUS); the Centre national de la recherche scientifique (CNRS); and Université Paris Diderot. Dr. Ait-Mohamed was the recipient of a fellowship from the Programme franco-algérien de formation supérieure en France PROFAS managed by the CROUS. Dr. Battisti and Dr. Pontis are recipients of fellowships from the Ministère de l'enseignement supérieur et de la recherche. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ouardia.ait-mohamed@univ-paris-diderot.fr (OA); slimane.aitsiali@univ-paris-diderot.fr (SA)

Introduction

Breast cancer, a major worldwide health issue, is considered as the most common malignancy and the most common cause of cancer-related death in Western countries [1]. Standard cancer therapy generally combines surgery, multi-therapeutic agents and ionizing radiation [2]. These anticancer agents induce cell cycle arrest and/or cell death by apoptotic or non-apoptotic mechanisms including necrosis, senescence, autophagy and mitotic catastrophe [3,4].

Major issues concerning conventional anticancer chemotherapy are the occurrence of side effects induced by the non-specific targeting of both normal and cancer cells [5,6], and the emergence of drug-resistant cancer cells [7]. Based on this, there has been growing interest in the use of naturally occurring molecules with chemo-preventive and chemotherapeutic properties in cancer treatment [8–12]. Natural products will thus continue to play major role as active substances, model molecules for the discovery and validation of drug targets [13,14]. Among natural sources, plants have played an important role as a source of effective anticancer agents [15–17]. Four examples are well known: Taxol® from *Taxus brevifolia* L., vinca alkaloids from *Catharanthus roseus* G. Don, camptothecin from *Camptotheca acuminata*, Decne and podophyllotoxin from *Podophyllum peltatum* L. [18,19].

In folk medicine, *Buxus sempervirens* L. is used to treat rheumatism, arthritis, bile duct infections, diarrhea, fever and skin ulceration. Studies highlighted the unique feature of the genus *Buxus* regarding the presence of steroidal alkaloids (more than 200)

[20–23]. The latter are known for exhibiting promising biological activities including anti-acetylcholine esterase [24–27], cytotoxic [28] and immunosuppressive activities [29]. Nevertheless, to our knowledge, no anticancer activity of *Buxus sempervirens* L. extracts has been yet described.

Based on folk medicine, we investigated here the cytotoxic effect of the acetonic extract of *Buxus sempervirens* L. against five breast cancer cell lines: MCF7, MCF10CA1a, T47D, BT-20 and MDA-MB-435 or the spontaneously immortalized cell line MCF10A as a control. Our results showed that the *Buxus* extract has specific cytotoxic effects toward cancer cell lines by mainly inducing a decrease in cyclin D1. Interestingly, the extract induced autophagic cell death and apoptosis in breast cancer cells tested and a caspase 3-independent apoptosis cell death in the aggressive MCF10CA1a cells.

Results

Buxus acetonic extracts exhibit cytotoxic properties and induce phenotype modifications in breast cancer cells

In order to evaluate the cytotoxicity of the acetonic extract of *Buxus*, an MTT assay was monitored on five breast cancer cell lines. The MCF7, MCF10CA1a and T47D, which are aggressive triple positive breast cancer cells, and BT-20 and MDA-MB-435 that are triple negative breast cancer cells. The extract exhibited cytotoxic activity toward all cancer cell lines tested, displaying reduced IC_{50} (<20 μg/ml) (Figure 1A). Moreover, the IC_{50} obtained against the control cell line MCF10A was higher (IC_{50} = 19.24 μg/ml, Figure 1A). These results suggest a specific cytotoxic effect mainly against breast cancer cell lines.

In order to give a better understanding of the mechanisms of cytotoxicity in cancer cells, we decided to carry on experiments on aggressive triple positive cancer cells: MCF7, MCF10CA1a, T47D and the triple negative breast cancer cell line BT-20.

First, major phenotypic changes were noticed when cancer cell lines were incubated in the presence of *Buxus* extract. Hence, interestingly, the cancer cell lines treated with the same extract (corresponding IC_{50} during 72 h) displayed different apoptotic cell shapes regarding the apoptotic volume decrease (AVD) (Figure 1B and 1C). To further test this, cytoskeleton staining (anti-α-tubulin) was applied. Treated MCF7, T47D and BT-20 cells exhibited a reduced round-shape cellular form before complete detachment from cell culture dish (Figure 1B, 1D and 1E), while MCF10CA1a cells showed a distinct and severe shrinkage (Figure 1C). These specific shapes are well known as the AVD due to massive efflux of K^+ and Cl^- through their specific channels, leading to water escape from the cytoplasm, the latter being considered as a major hallmark of apoptotic cells [30,31].

Finally, while DMSO-treated cells showed large nuclei with distinguishable nucleoli, we have noticed the transformation of nuclei into a unique pyknotic mass in dramatically-injured cells (Figure 1 B–E). On the other hand, normal MCF10A cells did not exhibit such dramatic phenotype changes. Together, our results suggest a cytotoxic activity of the *Buxus* extract regarding cancerous cells *via* apoptotic cell death.

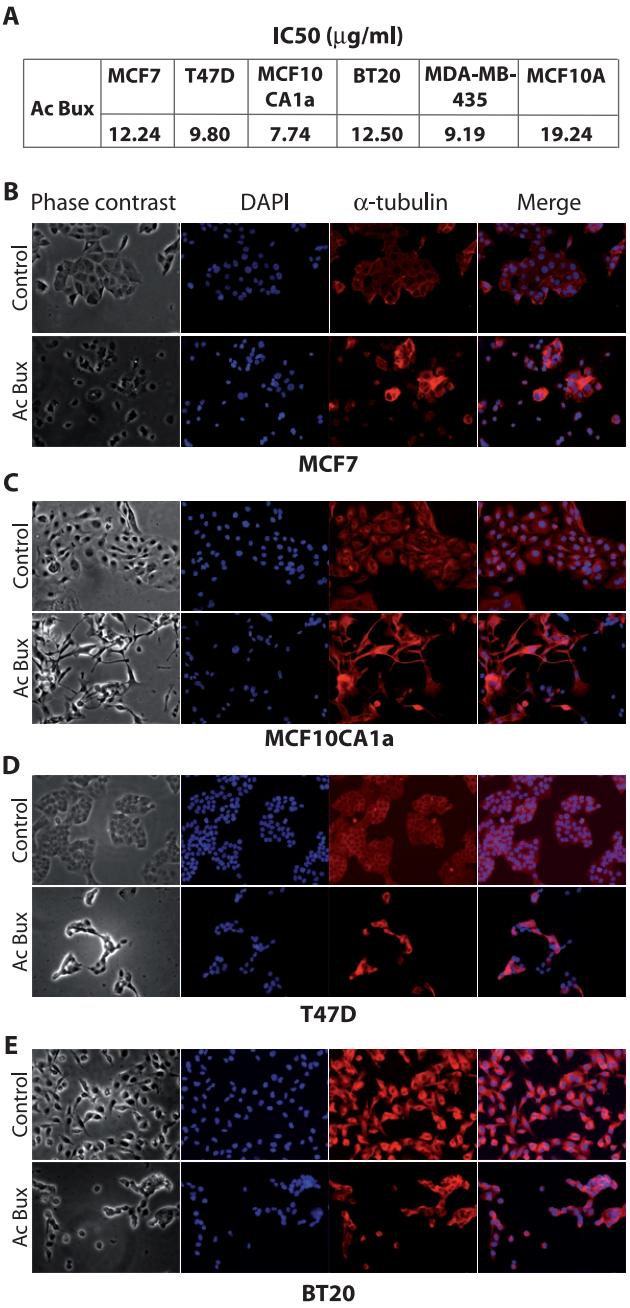


Figure 1. Cytotoxic effects of the acetonic extract of *Buxus sempervirens* L. towards breast cancer MCF7 and MCF10CA1a cells. A. IC_{50} determined by the dose-response curves obtained by the MTT assay. **B. C. D.** and **E.** Different cell shapes exhibited by MCF7, MCF10CA1a, T47D, MDA-MB-435 and BT-20, respectively, treated with *Buxus* extract at their respective IC_{50} during 72 h. Left panel: phase contrast images; Right panel: anti-α-tubulin fluorescence staining. Control cells are treated with vehicle DMSO (magnification ×200). Ac Bux: acetonic *Buxus* extract.
doi:10.1371/journal.pone.0024537.g001

Acetonic extract of *Buxus* induces cell cycle arrest

We studied the effect of the *Buxus* acetonic extract on the cell cycle of the studied breast cell lines. After 24 h incubation with the extract, stability is generally noticed in all cell cycle sub-populations of the control cell line MCF10A cells, with a slight increase in sub-G1 population observed with both concentrations applied (Figure 2C). We have also noticed a little decrease in the S-phase sub-population (Figure 2C). Interestingly, the IC_{50} were capable of triggering cell death of both cancerous cell lines. Thus, after 24 h of treatment, the sub-G1 sub-population sharply increased from 2.82% to 30.30% and from 7.31% to 20.64% for MCF10CA1a and MCF7, respectively (Figure 2A, Figure S1, S2). Concomitantly, there is a decrease in G0/G1 and S-phase sub-populations, mainly for MCF10CA1a cells from 69.59% to

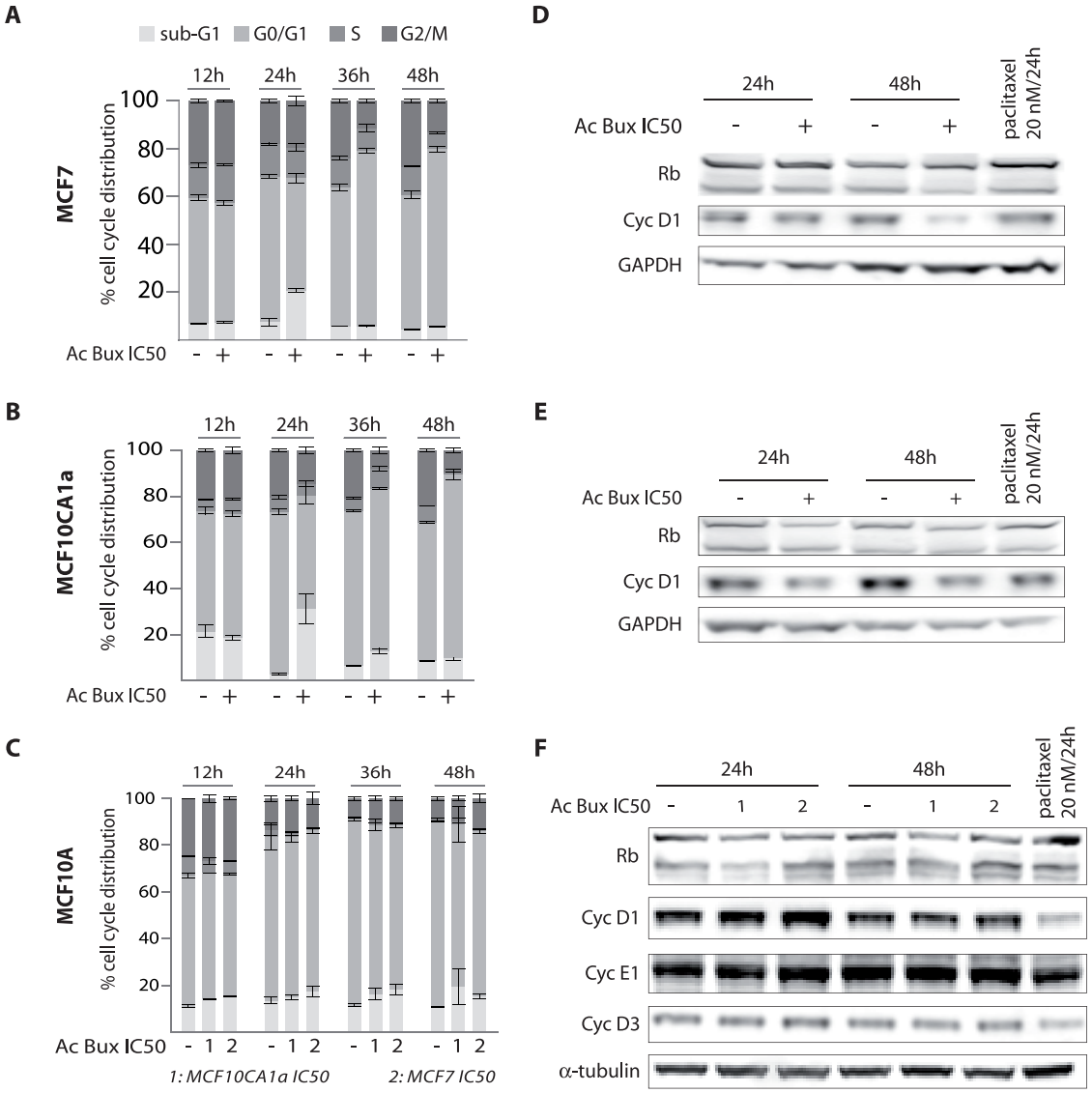


Figure 2. The acetonic extract of *Buxus* induces cell cycle arrest in MCF7 and MCF10CA1a breast cancer cell lines. A. MCF7 cells were incubated for increasing period intervals (12 h, 24 h, 36 h and 48 h) with their IC_{50} concentrations. The results represent means ± SEM of three experiments. **B.** MCF10CA1a cells were incubated for increasing period intervals (12 h, 24 h, 36 h and 48 h) with their IC_{50} concentration. The results represent means ± SEM of three experiments. **C.** MCF10A cells were incubated for the same period intervals (12 h, 24 h, 36 h and 48 h) with the IC_{50} of MCF7 and MCF10CA1a, respectively. The results represent means ± SEM of three independent experiments. **D.** Immunoblots of total cell extracts isolated from MCF7 treated or not with plant extract as indicated and probed with an anti-cyclin D1 antibody. GAPDH was used as a loading control. **E.** Immunoblots of total cell extracts isolated from MCF10CA1a treated or not with plant extract as indicated and probed with an anti-cyclin D1 antibody. GAPDH was used as a loading control. **F.** Immunoblots of total cell extracts isolated from MCF10A treated or not with plant extract (IC50s of MCF7 and MCF10CA1a concentrations) as indicated and probed with an anti-cyclin D1 antibody. α-tubulin was used as a loading control. Ac Bux: acetonic *Buxus* extract.
doi:10.1371/journal.pone.0024537.g002

48.05% and from 6.30% to 4.80%, respectively (Figure 2B). At 48 h, there is a significant increase in G0/G1 sub-population to the detriment of S and G2/M sub-populations (Figure 2A and 2B).

Finally, we have noticed in all cancer cell lines tested that a maximum of sub-G1 cell population is reached 24 h post-treatment, followed by a reduction (Figure 2A and 2B for MCF7 and MCF10CA1a, respectively). Concerning T47D and BT-20 cells, despite the observation of numerous floating dead cells, no major changes are illustrated in Sub-G1 sub-populations (Figure S3A and S3D). This could be due to the loss of the severely-damaged cells during washing steps. It is indeed established that the content of DNA remaining in apoptotic cells for cytometric analysis vary markedly depending on the extent of DNA degradation and cell washing steps [32]. Concerning MCF7 and MCF10CA1a, striking results were also noticed regarding the concentrations used: with high concentrations (2 times the IC_{50}), there is an increase in sub-G1 population, while with low concentrations there is a decrease in S and G2/M phases (Figure S1A and S2A).

Concerning cell cycle markers, all cancer cells tested treated with IC_{50} during 24 h and 48 h showed a noticeable decrease in cyclin D1 expression (Figure 2D and 2E, and Figure S3 B–C and E–F). No major changes in the expression of Rb were noticed in treated cells, we have noticed a slight decrease in

hypo-phosphorylated Rb protein levels 48 h after treatment (Figure 2D and 2E). Nonetheless, the IC_{50} of MCF7 and MCF10CA1a applied to MCF10A showed neither of the above effects (Figure 2C and 2F). These results indicate that the failure of tested breast cancer cells to enter S phase is due to a decrease in cyclin D1 induced by the *Buxus* acetonic extract.

Buxus acetonic extract induces autophagy in breast cancer cells

We have next investigated the role of *Buxus* acetonic extract in cell death. To this end, cells were collected after 24 h and 48 h treatment with respective IC_{50}, double-stained with PI and Annexin V-FITC and analyzed by FACS (Figure 3 and Figure S4). The kinetic of cell interaction with Annexin V revealed that the extract acts very fast (not shown). Interestingly, there is a discrepancy in the behavior of the breast cancer cell lines. Indeed, while with MCF10CA1a, T47D and BT-20 we revealed a pre-apoptotic sub-population (PI^-/Annexin V^+) (13.10% versus 25.57% after 24 h and 48 h of treatment, respectively for MCF10CA1a as an example), that latter shifted to a late apoptotic and/or a necrotic sub-population (PI^+/Annexin V^- quadrant) (Figure 3B, Figure S4 A–B). However, with MCF7 cell line, we noticed that the cell population shifted directly to PI^+ quadrants (dead cells) without transition by the PI^-/AnnexinV^+ (Figure 3A), even with reduced time contact kinetics (one hour intervals, data not shown). These findings suggested that the process of death induced by *Buxus* acetonic extract differs in the cancer cell lines; MCF10CA1a, BT-20 and T47D cells die *via* apoptosis pathway, while MCF7 cell death seemed to rely mainly on autophagy.

As previously seen with PI staining, reduced cell death is observed with MCF10A, even after 48 h of treatment, confirming the specific effect on cancerous cell lines. Paradoxically, a more lethal action is noticed after 24 h of incubation compared to 48 h (Figure 3C).

According to pictures obtained with transmission electronic microscopy, untreated MCF7 cells displayed normal characteristics with, however, the presence of some auto-lysosomes/auto-phagosomes in cell cytoplasm (Figure 4A), suggesting that even in normal growth conditions, MCF7 cells proceed to some controlled autophagy. Nevertheless, treated MCF7 cells with the *Buxus* acetonic extract (IC_{50} during 72 h) showed abundant auto-lysosomes/auto-phagosomes dispersed in the cytoplasm (Figure 4A). Hence, in the presence of the plant extract, the phenomenon is dramatically increased, leading to cell death without any damage to mitochondria and cytoplasmic membrane. These observations suggested that MCF7 death is due to autophagy rather than apoptosis. This is in agreement with previous reports showing that MCF7 cells do not undergo apoptosis after treatment with numerous apoptosis stimuli, including Tamoxifen [33], or injection of supra-physiological amounts of cytochrome C [34].

Concerning MCF10CA1a cells, pictures taken after IC_{50} treatment during 72 h, provided several hallmarks of apoptosis and autophagy (Figure 5A). We noticed the presence of initial autophagic vacuoles and degradative autophagic vacuoles, peri-nuclear localization of mitochondria, and most importantly, some of them were damaged.

To carry on our investigation concerning autophagy we studied a main autophagy marker, the Microtubule associated Light Chain 3 or LC3 protein. LC3 is the mammalian homolog of the yeast Apg8p protein, essential for amino acid starvation-induced autophagy [35,36]. LC3 is present in two forms in cells: LC3-I is the cytoplasmic form, which is processed into a lipidic LC3-II form, associated with the auto-phagosome membrane [35,36]. Therefore, we compared the LC3 distribution in *Buxus* acetonic extract-treated and untreated cells (Figures 4B–C, 5B–5C, Figure S5). In DMSO-treated cells, we noticed a homogeneous cytoplasmic distribution of unprocessed LC3-I, while in plant extract-treated cells (IC_{50}/72 h), many foci are depicted, corresponding to lipidic transformed LC3-II, mainly around nuclei (Figures 4C for MCF7, 5C for MCF10CA1a, Figure S5 A for T47D and C for BT-20). This specific signal corresponds to the auto-phagosome trans-membrane processed version of LC3. These results are in agreement with images taken with transmission electron microscopy (Figures 4A and 4B for MCF7 and MCF10CA1a respectively), where we noticed accumulation of late auto-phagosomes mainly around cell nuclei. In the case of MCF10CA1a cells, the foci pattern of LC3-II was difficult to confirm since there was very little cytoplasm around nuclei (Figure 5C).

Concerning immunoblots, the presence of LC3-II in untreated (24 h) MCF7 cells, demonstrated the occurrence of controlled-autophagy in normal cells, as already seen with transmission electron microscopy (Figure 4A). For MCF10CA1a aggressive cells, we found a decrease in LC3-II in *Buxus* acetonic extract-treated cells (Figure 5C). This is probably because LC3-II is present both on inner and outer auto-phagosome membranes, with the former being degraded inside auto-lysosomes, whereas LC3 on the outer membrane is deconjugated by Atg4 (Autophagy related gene 4) and returns to the cytosol [35]. Finally, concerning the control cell line MCF10A, a faint LC3-II signal is detected when the cells were treated with the IC_{50} of MCF7 (Figure S6). Immunoblots of total cell extracts from treated and non-treated T47D and BT-20 confirmed also autophagy processing since we have noticed the processed form of LC3 (LC3 II, 24 h and 48 h after treatment) (Figure S5 B and D for T47D and BT-20, respectively).

Acetonic *Buxus* extract induces caspase 3-independent apoptosis in MCF10C1a

In order to get more insights on the pattern of cell death, mainly in MCF10CA1a, we studied the activation of several additional markers related to apoptosis by immunoblot (Figure 6A). Pro-caspase 3 is undetectable in MCF-7 cells due to a 47-bp deletion within exon 3 of the procaspase-3 gene that alters the reading frame of the message, resulting in an unstable truncated polypeptide [34,37]. According to that, activated caspase 3 was assessed in MCF10CA1a (Figure 6A), as well as in the control cell line MCF10A (Figure S6). Surprisingly, active caspase 3 was absent after treatment with the plant extract, even with reduced incubation times (Figure 6A). This result is in contradiction with our previous finding concerning Annexin V staining; the aggressive cell line MCF10CA1a displayed PI^-/Annexin V^+ pattern after plant treatment, illustrating an apoptotic cell death concomitant to autophagy. Taken together, these results indicate that MCF10CA1a death can be related only to autophagy, triggered by metabolic stress created by damaged mitochondria that caused an energy-deprivation state, or the autophagy is coupled to an apoptosis cell death independent of caspase 3 activation, since we noticed occurrence of DNA damages related apoptosis (presence of cleaved PARP and γH2AX, Figure 6A).

As the cells displayed a G1-phase arrest, we were interested in testing levels of p21, a potent cell cycle inhibitor through inactivation of G1-phase cyclin/CDK complexes. Surprisingly, we have found a decrease in p21 levels in cancer cell lines tested (Figures 4B and 5B, Figure S5 B and D). In addition, the cells showed reduced levels of Survivin after plant extract treatment. In the control cell line MCF10A, Survivin was detected at 24 h but no effect on its levels is noticed after plant extract treatment. At

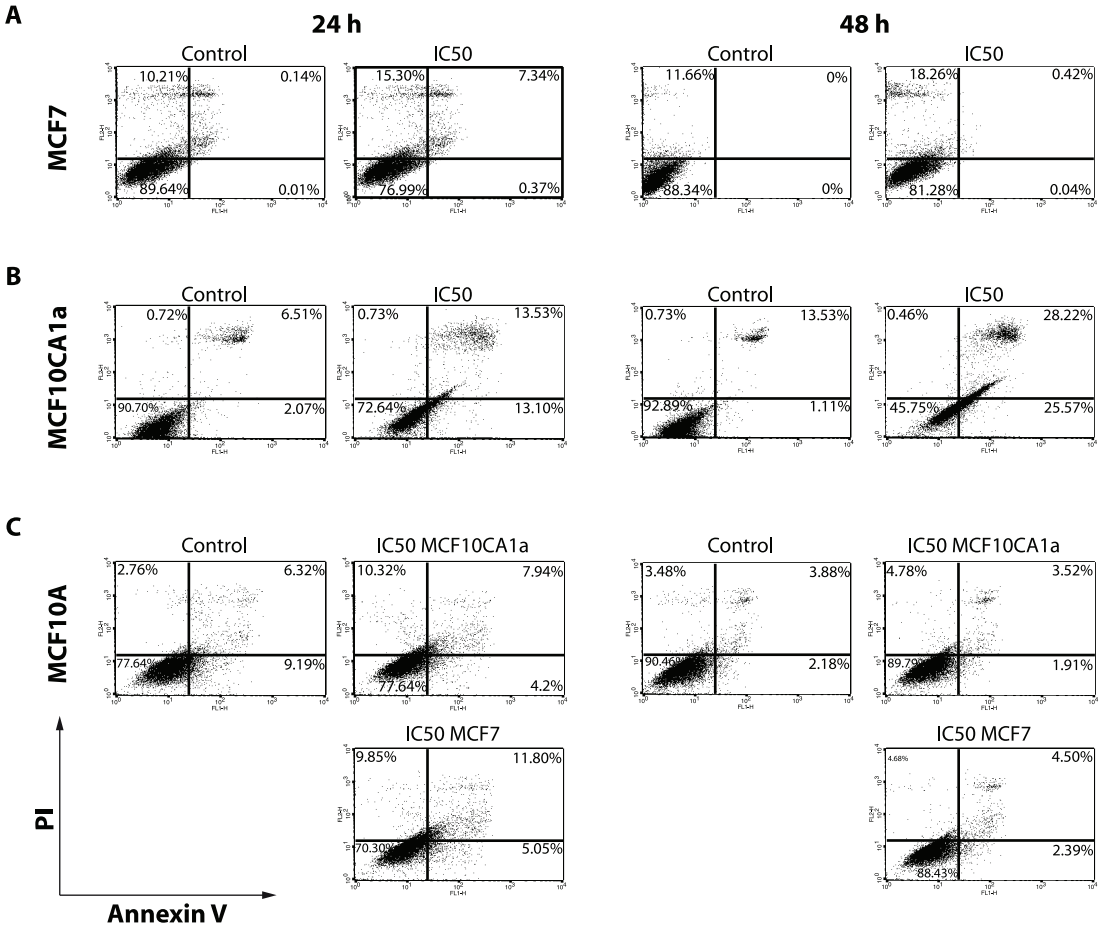


Figure 3. *Buxus* extract induces autophagy in cancer cells as evidenced by PI/Annexin V double staining and FACS analysis. A–B. PI/Annexin V double staining of untreated and treated MCF7 (A) and MCF10CA1a (B) cells with IC_{50} concentration for 24 h and 48 h. **C.** FACS analysis with PI/Annexin V double staining of MCF10A cell line (control cell line) treated with MCF7 and MCF10CA1a IC_{50} respective *Buxus* extract concentrations for 24 h and 48 h.
doi:10.1371/journal.pone.0024537.g003

48 h, the level of Survivin is undetectable along with Cyclin A2 (Figure S6), this can be explained by the fact that the cell line did not undergo mitosis and can hence explain the disappearance of Survivin.

It is known that the up-regulation of Survivin expression in cancer cells is independent of the cell cycle, suggesting an increase of its anti-apoptotic role compared to normal cells, in which its mitotic regulation functions may be predominant.

Beclin 1 is a 60-kDa protein that plays a critical role in the formation of auto-phagosomes in mammalian cells [38,39]. 40% of human breast carcinoma cell lines exhibit deletions of one or more alleles of *beclin 1* gene [40]. This decreased expression of Beclin 1 suggests that specific molecular alterations in autophagy pathways may contribute to tumorigenesis [41]. As illustrated in Figure 4, 6B and 6C, an increase in Beclin 1 levels was noticed in treated MCF7, T47D and BT-20, respectively, demonstrating that the plant extract triggers autophagic cell death.

Acetonic *Buxus* extract induces apoptosis in T47D and BT-20

Since we have noticed the presence of pre-apoptotic subpopulations in Annexin V-FITC stained cells, we decided to check the occurrence of apoptosis in these cell lines. As illustrated in Figure 6B and 6C for T47D and BT-20, respectively, after 3 h of treatment, there is occurrence of apoptosis since there is expression of certain apoptosis markers : caspase 3, γH2AX. In parallel, autophagy occurs in these cells, since there is a concomitant overexpression of Beclin-1 (Figure 6B and 6C).

Discussion

In this study, we report cytotoxic effects of a plant extract – acetonic extract of *Buxus sempervirens* L. – on several breast cancer cell lines. Cytotoxic activities concerning *Buxus* species are scarce; although an interesting cytotoxic activity is reported for triterpenoid alkaloids isolated from *Buxus microphylla* L. against HepG2 [28]. According to our results, in breast cancer cell lines, the *Buxus* acetonic extract induced cell cycle arrest in G0/G1 phase and triggered cell death by increased sub-G1 cell population. The observed effects could be mediated by two sub-classes of cytotoxic molecules, a first class could act fast and require high concentration to induce cell death, and a second class plays a role in cell cycle arrest by preventing the G1-to-S transition. Alternatively, all these effects could be attributed to a single molecule. This conclusion arises from previous similar results described in the literature with Resveratrol [42]. Indeed, this phytoalexin stilben isolated from grapes, wine and nuts, induces cell cycle arrest at low concentrations and cell death through

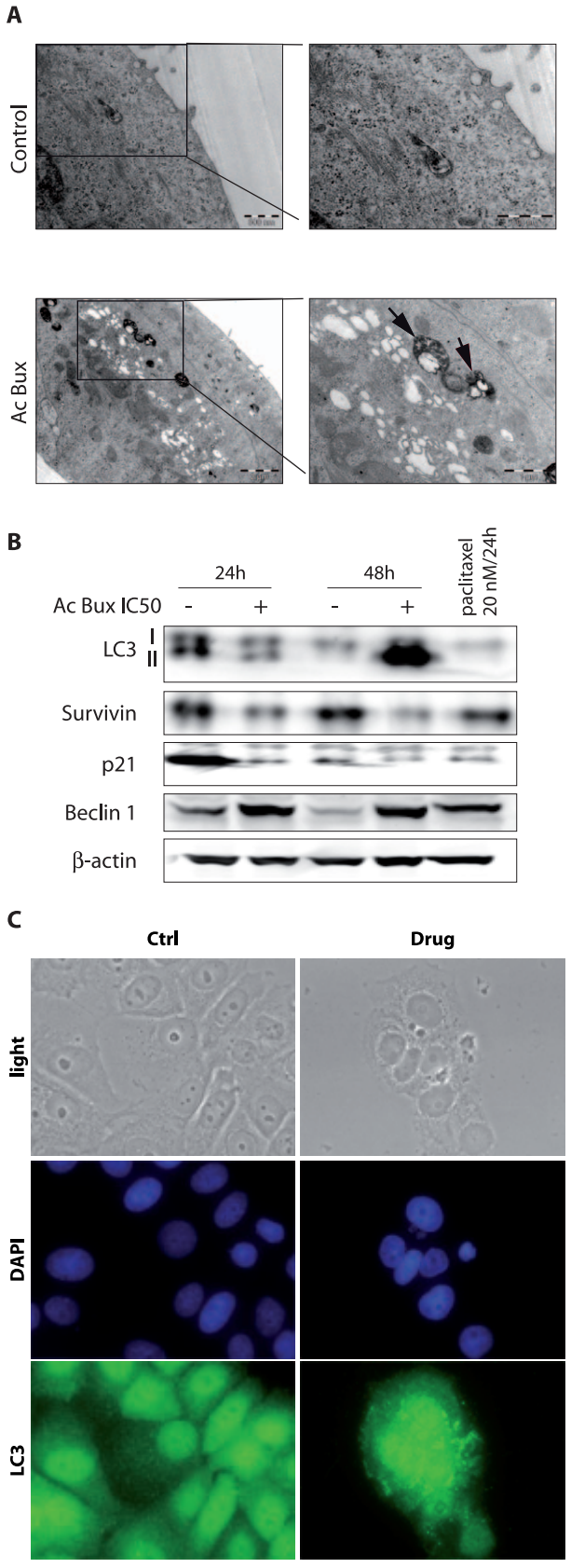


Figure 4. Acetonic extract of *Buxus* induces autophagy in MCF7 cell line. A. Transmission electron microscopy pictures of untreated and *Buxus* extract-treated MCF7 cells with IC_{50} concentration for 72 h. Black arrows show degradative autophagic vesicles. White arrows show lucent electron vesicles. **B.** Immunoblots of total cell extracts isolated from MCF7 (treated and untreated, as indicated) probed with different antibodies demonstrating the occurrence of autophagy. β-actin has been used as a loading control. **C.** Immunofluorescence targeting LC3 obtained with untreated and *Buxus* extract-treated MCF7 cells (IC_{50}, 72 h). Magnification ×400. Ac Bux: acetonic *Buxus* extract.
doi:10.1371/journal.pone.0024537.g004

auto-phagocytosis process in ovarian cancer cells at high concentrations [42]. This is a very striking finding, since our preliminary results revealed the absence of Resveratrol in *Buxus* extracts. Also, it is worthy to notice that the plant was collected in an area characterized by unfavorable growth conditions (mountainous and semi-arid region) which are known to trigger the production of phytoalexin substances.

Our investigation concerning cell cycle arrest revealed also a highly sought characteristic. The *Buxus* acetonic extract is able to block cell cycle in G0/G1 through the decrease in cyclin D1. Cyclin D1 belongs to the family of three closely related D-type cyclins, D1, D2 and D3, which are redundant in all proliferating cell types. D-cyclins together drive cell-cycle progression by activating their cyclin-dependent kinase partners, CDK4 and CDK6, which leads to phosphorylation of the retinoblastoma protein (Rb), and in turn to the advance through the G1 phase of the cell cycle [43,44]. Cyclin D1 is over-expressed in most breast tumor cell lines through over-expression and/or amplification at its genomic locus, 11q13. This feature has been shown to play a key role in tumorigenesis and confers bad prognosis in breast cancer [45–47]. Moreover, the cell cycle arrest observed cannot be the result of CDK inhibitors activation as shown by decreased levels of p21 and p27 (data not shown). Rather, the effect relies on a direct decrease in cyclin D1, strongly suggesting that inhibition of cyclin D1 by *Buxus* extract could be a good tool to improve prognosis in breast cancer.

Another interesting feature concerns the concomitant occurrence of the two programmed cell deaths, apoptosis and autophagy, in several breast cancer cells including triple positive and triple negative ones, since our results have shown markers related to both of them. Transmission electron microscopy analyses showed marked differences in localization and shapes of mitochondria in *Buxus* extract-treated MCF10CA1a cells. The cellular distribution of mitochondria is deeply affected during apoptosis. Mitochondria are normally dispersed throughout the entire cell; however, during apoptosis triggered by tumor necrosis factor (TNF), there is a peri-nuclear clustering of mitochondria is caused by an impaired activity of the molecular motor kinesin [48]. Also, the loss of integrity of the mitochondria outer membrane is a very important hallmark of apoptosis. Referred as MOMP (Mitochondrial Outer Membrane Permeabilization), it leads to the release of proteins normally found in the space between the inner and outer mitochondrial membranes, such as cytochrome C and AIF (Apoptosis Inducing Factor) [49]. It is well established that the release of these molecules initiates apoptosis. Cytochrome C binds to APAF-1 (apoptotic protease activating factor–1). In the presence of ATP, APAF-1 is allowed to oligomerization and forms the "apoptosome" which, in turn, activates Caspase 9 by dimerization. The active Caspase 9 activates executor caspases (Caspase 3 and 7) and this orchestrates apoptosis through the cleavage of key substrates within the cell [50]. Also, AIF has a direct effect on isolated nuclei, triggering chromatin condensation as well as large-scale chromatin fragmentation [51].

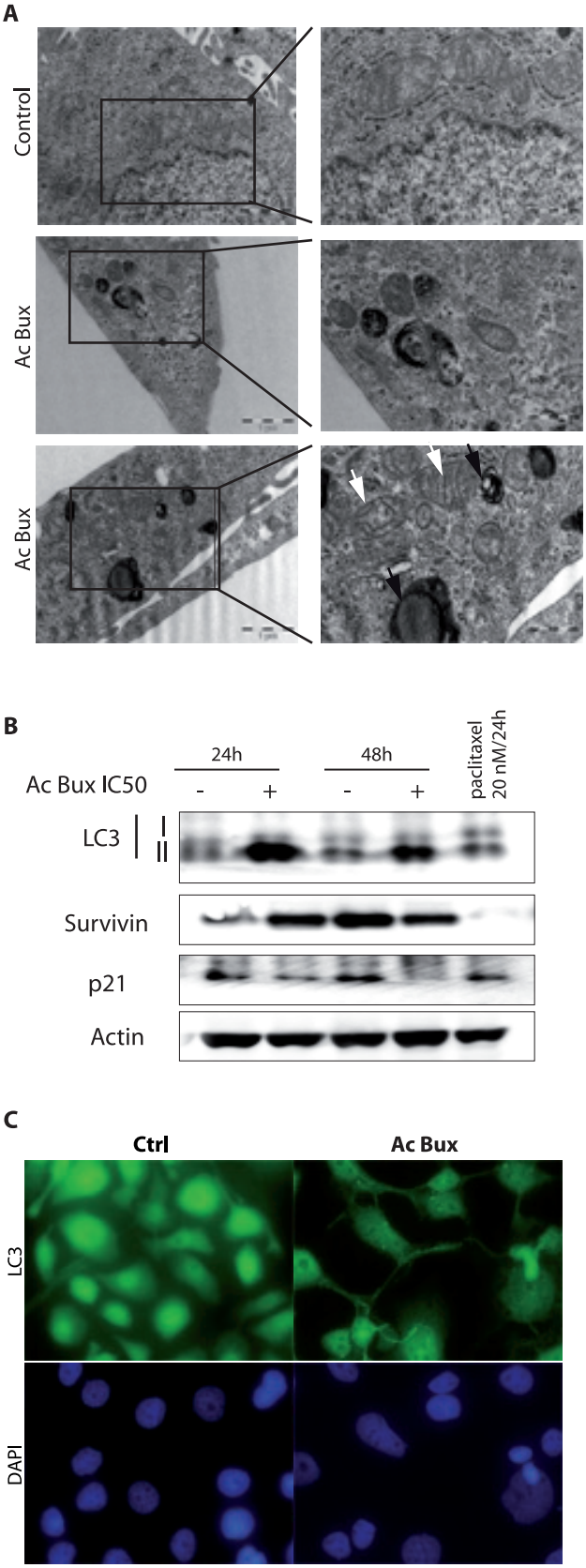


Figure 5. Acetonic extract of *Buxus* induces autophagy in MCF10CA1a cell line. A. Transmission electron microscopy pictures of untreated and *Buxus* extract-treated MCF10CA1a cells (IC_{50}, 72 h). Black arrows show degradative autophagic vesicles. White arrows show damaged mitochondria. **B.** Immunoblots of total cell extracts isolated from MCF10CA1a (treated and untreated, as indicated) probed with different antibodies demonstrating the occurrence of autophagy. β-actin has been used as a loading control. **C.** Immunofluorescence targeting LC3 obtained with untreated and treated MCF10CA1a cells (IC_{50}, 72 h). Magnification ×400. Ac Bux: acetonic *Buxus* extract.
doi:10.1371/journal.pone.0024537.g005

There is more and more evidence that autophagy is a mechanism of cell survival following plethora of extra-cellular and intra-cellular stimuli. Numerous studies have demonstrated that proceeding to autophagy allows cancer cells to escape cell death [52–55]. Nevertheless, the autophagy is highly contextual, it can exert both cyto-protective and death-promoting effects. Indeed, the effect of autophagy may vary dependent on the type of cancer, individual characteristics of cancer cells, microenvironments, and therapeutic treatment [52]. Nonetheless, it is clearly assumed that induction of autophagy to high levels leads to autophagic cell death [56,57].

Interestingly, the *Buxus* extract induced a decrease in p21 levels, which could be related to its involvement as an anti-apoptotic protein. This is exemplified by preventing apoptosis by protecting the N-terminal moiety of Caspase 3 preventing its activating proteolysis [58]. Lately, p21 has been reported to play a crucial role in autophagy [59]; although, the entire mechanism is not fully understood. Wild-type MEF (Mouse Embryonic Fibroblasts) undergo apoptosis upon C2-ceramide treatment, and $p21^{-/-}$ MEF undergo autophagy rather than apoptosis upon the same death stimulus. p21 triggers apoptosis by inhibiting the autophagic pathway through the suppression of the stability of autophagy-related proteins in MEF [59]. Hence, decreased levels of p21 observed in the cells treated with the plant extract can trigger cell death by autophagy.

By decreasing levels of p21, the *Buxus* extract seems to contain molecules that inhibit cytosolic p21 and trigger cell death. It has been already shown that targeting p21 (with an anti-sense oligodeoxynucleotide) attenuated the growth of Met-1 tumors in nude mice [60]. Finally, our data demonstrated that the *Buxus* extract also decreases levels of Survivin, a 16.5 kDa protein that belongs to the IAP family (Inhibitor of Apoptosis proteins) [61], which plays a key role in mitotic spindle formation [62]. However, two general considerations make Survivin an attractive therapeutic target in cancer: it is selectively expressed in tumor cells and it is required for their viability [63,64]. In cancer cells, Survivin correlates with unfavorable prognosis, resistance to therapy, and accelerated rates of recurrences [65].

In light of our results, we can conclude that *Buxus* sempervirens extract targets many proteins widespread in cancer cells cytoplasm, leading to cell cycle arrest and autophagy. There is however a crosstalk between apoptosis and autophagy, which determines cell fate, but the molecular mechanism is not fully understood. Previous data suggested that the removal or functional inhibition of essential proteins from the apoptotic machinery can switch a cellular stress response from the apoptotic default pathway to a state of massively increased autophagy. However, apoptosis develops only when autophagy is inhibited [66]. In our case, mechanisms of the concomitant occurrence of autophagy and apoptosis are unclear. A possible explanation for the autophagy observed in MCF10CA1a cells can be the presence of Ha-Ras. This aggressive cell line was obtained by transfecting MCF10A with this oncogene. It has been lately shown that the presence of this signature leads to the occurrence of autophagy [67].

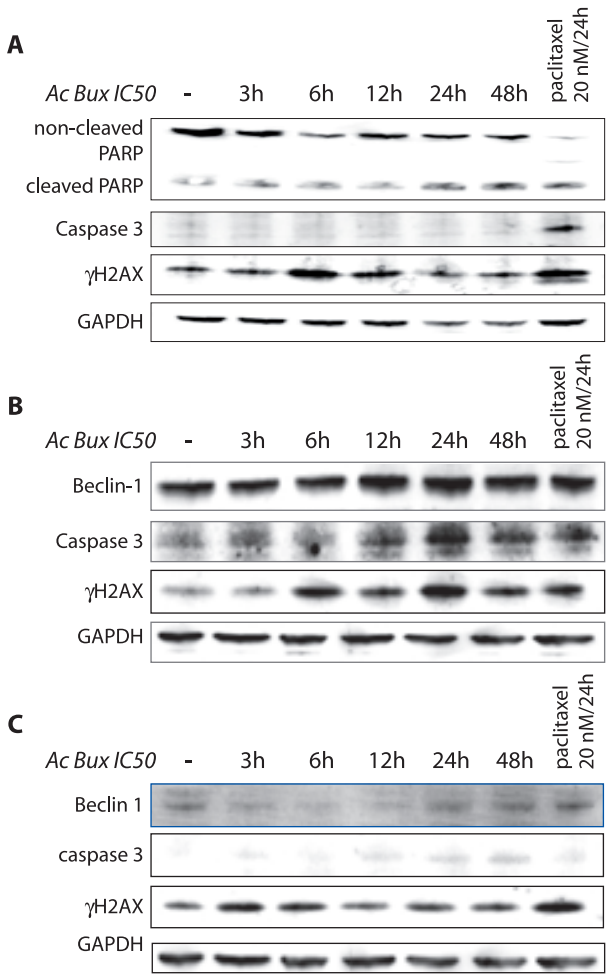


Figure 6. *Buxus* extract induces apoptosis in MCF10CA1a, T47D and BT-20 breast cancer cell lines. A. Immunoblots of total extracts from MCF10CA1a, revealing the presence of the cleaved from of PARP and γH2AX, hall marks of apoptosis, at the same time, the blot reveals the absence of active caspase 3, demonstrating the occurrence of apoptosis without caspase 3 activation. **B.** and **C.** Immunoblots of total extracts from T47D and BT-20, respectively, revealing the presence of the cleaved from of caspase 3 and γH2AX, hallmarks of apoptosis, demonstrating the occurrence of apoptosis and an up-regulation of Beclin-1, proving the occurrence of autophagy at the same time. GAPDH was used as a loading control. Ac Bux: acetonic *Buxus* extract.
doi:10.1371/journal.pone.0024537.g006

Conclusion

Nowadays, it is accepted that the major problem with conventional chemotherapy lies in the doses used: low doses have no effect on cancer cells and too high doses induce deleterious side effects. Thus, the presence of a "sensitizer" that can force cells to undergo apoptosis even with mild DNA-damaging agents would greatly enhance the efficacy and limit side effects of conventional chemotherapy drugs [68]. Hence, targeting p21 and Survivin can be a good adjuvant therapy to improve cell death in accompaniment to other conventional drugs [69,70]. *Buxus* extract probably contains molecules that inhibit p21 and Survivin and thus can be used in addition to commonly used drugs to trigger cell death.

Another important feature concerning *Buxus* extract is its capacity to target the cell cycle which is very promising in cancer chemotherapy. Agents that induce cell cycle arrest are increasingly used in combination with traditional cytotoxic drugs to overcome cell cycle–mediated drug resistance and to improve cytotoxic efficacy. Among them, Flavopiridol has been shown to directly inhibit many CDK proteins [71].

Taken together, our data suggest that *Buxus sempervirens* extract can induce cell death not only via apoptosis, but also by autophagy. This is very promising, since it indicates that the *Buxus* extract may contain molecules that can be potentially used in apoptosis-resistant cells. Also, it exhibited increased toxicity towards cancer cell lines, including triple negative breast cancer cells. Moreover, it induced cell cycle arrest, depletion of cell energy, leading to cell death. Finally, *Buxus* deserves further investigation to understand the potential use of its molecules in therapeutic application for cancer treatment.

Materials and Methods

Plant extract preparation

Buxus sempervirens L. (Buxaceae) was collected from remote places around the province of Béjaia (with the kind permission of the Parc National de Dujurdjura authorities, Northeastern region of Algeria) in March 2008. Plant parts (leaves and flowers) used in this study were chosen on the basis of their use in Algerian ancestral medicine.

Powdered material (2 g) was macerated in pure acetone (200 ml) during 24 h, at room temperature with light stirring (50 rpm), and then filtered using 0.22 µm filters (Millipore). The flow-through material was evaporated to dryness under reduced pressure and the solid extract was reconstituted in DMSO solvent (100 µg/µl stock solution) before storage at -20°C.

Cell culture

MCF7 cells (HTB-22, ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM), 4.5 g/l of glucose, supplemented with 5% fetal calf serum, 100 U/mL of penicillin (PAA), and 100 µg/mL of streptomycin (PAA). MCF10A cells (CRL-10317, ATCC) were cultured in DMEM/F-12 medium (PAA, Carlsbad, CA) supplemented with 10 µg/mL of human insulin (Sigma, St. Louis, MO), 20 ng/mL of epidermal growth factor (Sigma, St. Louis, MO), 0.5 µg/mL of hydrocortisone (Sigma, St. Louis, MO), 5% horse serum (Invitrogen), 100 U/mL of penicillin (PAA) and 100 µg/mL of streptomycin (PAA). T47D (HTB-133, ATCC), a generous gift from Dr Yegor Vassetzky were grown in DMEM, 4.5 g/l of glucose, supplemented with 10% horse serum, 100 U/mL of penicillin (PAA), and 100 µg/mL of streptomycin (PAA). BT-20 (HTB-20, ATCC) and MDA-MB-435 cells (HTB-129, ATCC) were cultured in DMEM, 4.5 g/l of glucose, supplemented with 10% fetal calf serum, 100 U/mL of penicillin (PAA), and 100 µg/mL of streptomycin (PAA). MCF10CA1a cells [72] were cultured in DMEM/F-12 medium supplemented with 5% fetal calf serum (PAA), 100 U/mL of penicillin (PAA) and 100 µg/mL of streptomycin (PAA). All cited cells were cultured at 37°C in a humidified atmosphere and 5% CO_2.

Viability assay

Cell proliferation was determined using the Cell Titer Glo assay (Promega). Cells were seeded at a density of 3×10^3 cells per well in 96-well plates and maintained 24 h for attachment and then treated with two-fold serial dilutions of the plant extract. After 72 h incubation, 20 µL of MTT reagent were added. The plates were incubated during 2 h and absorbance determined at 560 nm in Glomax Multi-detection System (Promega). Percentages of cell survival were calculated as follows: % cell survival = (absorbance of treated cells/ absorbance of cells with vehicle solvent)×100. The

half inhibitory concentration (IC_{50}) was calculated from the dose–response curve obtained by plotting the percentage of cell survival *versus* the concentration of plant extract used. All assays were performed three times in duplicate. During all experiments, DMSO dilutions of *Buxus* acetonic extract were adjusted in the culture media at a final concentration of 0.2% (v/v).

FACS analysis, PI and PI/Annexin V staining

In order to determine the effect of plant extract on the cell cycle, FACS analysis was carried out. For propidium iodide (PI) staining, cells were seeded in 6-well plates at a density of 10^4 cells/ml. After 24 h of attachment, cancer cells were treated with indicated plant extract concentrations for different time intervals. Floating and attached cells were harvested, washed in PBS, fixed in ice-cold ethanol (70% v/v) and stored at −20°C. For analysis, cells were washed in PBS and suspended in PI (25 mg/ml) in PBS with RNase A (200 µg/ml).

For PI/Annexin V double staining, treated cells were harvested and suspended in binding buffer (HEPES pH 7.4, $CaCl_2$ 2.5 mM, NaCl 140 mM). Aliquots of cells were incubated for 15 mn with Annexin V FITC and PI (5 µg/mL) (Invitrogen).

During all FACS analyses, 10^5 events for each sample were analyzed. Flow cytometry analyses were carried out on a FACScalibur system (BD Biosciences) followed by analysis using CellQuest Pro software (BD Biosciences).

Ultra-structural study by transmission electronic microscopy

Treated (IC_{50}/72 h) and control cells were fixed in buffered (0.1 M) sodium cacodylate, pH 7.4 and 2.5% glutaraldehyde solution for 2 h. After washing, the cells were post-fixed in 1% OsO_4 solution for 1 h at room temperature, rinsed and dehydrated in an ethanol gradient (70% to 100%, 10 min for each bath). Absolute ethanol was replaced by 2,3 epoxy propylether and further by propylene oxide. Cells were infiltrated by epoxy resin (R1165, Agar scientific) mixed to propylene oxide (50%-50%) overnight, followed by three baths with pure epoxy resin. Samples were polymerized at 60°C during 18 h. Ultra-thin sections (80 nm) cut with an ultra-microtome (Leica UC6) were stained with uranyl acetate (20 min) and Reynolds lead citrate (2 min). Sections were observed at 80 kV, in a TEM Phillips Tecnai equipped with an Olympus Keenview CCD camera.

Immunofluorescence

Cells were grown on Permanox slides during 24 h before *Buxus* acetonic extract treatment (IC_{50}, 72 h). They were fixed with a paraformaldehyde solution (4%) and permeabilized with 0.1% Triton X-100 in PBS, before incubation with appropriate antibodies: α-tubulin (1/5000, Sigma), LC3 (1/200, Sigma) overnight at 4°C. After extensive washing, slides were incubated 1 h at room temperature with red fluorescent Alexa Fluor 568 dye-labeled anti-mouse IgG for α-tubulin and green-fluorescent Alexa Fluor 488 dye-labeled anti-rabbit for LC3. Coverslips were mounted in DAPI (4′,6-diamidinole-2-phenolindole) (Sigma Aldrich). Finally, cells were observed with a Leica DMI 6000 B microscope and images were treated with MetaMorph software.

Western blot

Cell extracts were prepared in RIPA (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 1 mM EDTA containing protease inhibitor mixture (Roche Applied Science). After sonication on a Bioruptor (Diagenode) at high frequency during 7.5 mn (1 mn of pause *versus* 15 sec of sonication), the soluble protein fraction was collected after centrifugation at 13 500 g during 10 mn. Protein concentration was determined BCA kit according to manufacturer's instructions (Pierce, Rockford, IL). 30 µg of proteins were subjected to SDS-PAGE in 4 to 12% gradient gels and separated proteins were transferred to nitrocellulose membrane (Invitrogen). Incubation with different antibodies was monitored overnight at 4°C. Membranes were incubated with the appropriate secondary antibody coupled to HRP (Horseradish Peroxidase), revealed using West Dura kit (Pierce, Rockford, USA) and ChemiSmart 5000 system (Vilber Lourmat).

Antibodies

The antibodies against p21 (C-19, sc-397), cyclin A2 (C-19, sc-596), cyclin D1 (DCS-6, sc-20044), cyclin D3 (C-16, sc-182), cyclin E1 (E-4, sc-25303), Rb (C-15, sc-50) were from Santa Cruz Technologies. Anti-cleaved caspase 3 (Asp 175 9661) was purchased from Cell Signaling Technologies. Anti-PARP (33–3100) was from Zymed Inc, anti-LC3 (L8918). Antibodies against Beclin-1 (B6061), β-actin (T9026), α-tubulin (A5441) and GAPDH (G8795), normal mouse and normal rabbit IgG were from Sigma Aldrich. Anti-Survivin (Ab469) was from Abcam.

Statement of Ethics

Experimental research reported in the manuscript must have been performed with the approval of the ethic committee of our Department following the French and European rules. No research on humans has been carried out.

Supporting Information

Figure S1 Dose effect of the acetonic extract of *Buxus* on MCF7 cells. A. FACS analysis of treated MCF7 with increasing concentrations of *Buxus* extract. The results are the mean ± SEM of three experiments. The results demonstrate a dose effect with increased sub-G1 subpopulation upon plant extract treatment. B. Immunoblot analyses of total extract of treated MCF7 cells treated with increasing concentrations of *Buxus* showing the multiple targets of the extract at high concentrations. GAPDH was used as a loading control.
(EPS)

Figure S2 Dose effect of the acetonic extract of *Buxus* on MCF10CA1a cells. A. FACS analysis of treated MCF10CA1a with increasing concentrations of *Buxus* extract. The results represent means ± SEM of three experiments. The results demonstrate a dose effect with increased Sub-G1 subpopulation upon plant extract treatment. B. Immunoblot of total extract of treated MCF10CA1a cells treated with increasing concentrations of *Buxus* showing the multiple targets of the extract at high concentrations. GAPDH was used as a loading control.
(EPS)

Figure S3 Treatment of T47D (an aggressive triple positive breast cancer cell line) and BT-20 (a triple negative brast cancer cell line) cells with Bux acetonic extracts resulted in the accumulation of cells in G0/G1 phase in a dose- and time-dependent fashion. A. and D. T47D and BT-20, respectively, were treated in increasing concentrations of the plant extract (IC50/2, IC50 and 2× IC50) during 24 h and 48 h and resulted in an accumulation of cells in G0/G1 phase as demonstrated by FACS analyses. The results represent means ± SEM of three experiments. **B and E.** Western blots analysis of untreated and treated T47D and BT-20 cells, respectively, showing a decrease of cyclin D1 after their respective

IC50 treatment with the plant extract during 24 h and 48 h. **C and F.** Western blot analysis of total cell extracts from untreated and treated T47D and BT-20 cells, respectively, with increasing concentrations of Ac Bux (IC50/2, IC50 and 2× IC50) during 48 h, illustrating a dose effect of Ac Bux on the several targeted proteins probed. Ac Bux: acetonic Buxus extract.
(EPS)

Figure S4 Treatment of T47D and BT-20 cells with the plant extract resulted in the accumulation of apoptotic/necrotic cells. A. T47D cells were treated during 24 h and 48 h with the plant extract (IC_{50}) and resulted in the accumulation of apoptotic/necrotic cells as illustrated with the Annexin V-FITC stained cells analyzed by FACS. **B.** Annexin V-FITC stained BT-20 cells showing that after 24 h of plant extract treatment (IC_{50}), there is an emergence of pre-apoptotic cells that shift to apoptotic/necrotic cell population after 48 h of treatment.
(EPS)

Figure S5 *Buxus* extract treatment in T47D and BT-20 cells resulted in autophagy. A and C. Immunofluorescence targeting LC3 obtained with untreated and treated T47D and BT-20 cells, respectively, (IC_{50}, 72 h), showing the punctuated staining of the processed form LC3 II. Magnification ×400. Ac Bux: acetonic *Buxus* extract. **B and D.** Immunoblots of total cell extracts of untreated and treated T47D and BT-20 cells, respectively, (IC_{50} during 24 h and 48 h) demonstrating the occurrence of the processed form of LC3 and the decrease of p21 levels.
(EPS)

Figure S6 Immunoblot analysis of total cell extracts isolated from MCF10A demonstrating the absence of the processed LC3II and caspase 3 in treated cells. Only a small band related to LC3II is present when cells were treated with MCF7-IC50. α-tubulin was used as a loading control.
(EPS)

Acknowledgments

We warmly thank P. Arimondo for technical help. The authors thank E. Heard, G. Velasco, P.A. Defossez, C. Francastel and Yegor Vassetzky for sharing reagents. We thank Alexis Canette from the imaging facility ImagoSeine at the Jacques Monod Institute (Paris, France) for precious help in having transmission electronic microscopy pictures and pieces of advice in interpretation.

Author Contributions

Conceived and designed the experiments: OA AD SA. Performed the experiments: OA BV FL PJ JV MS. Analyzed the data: AO SA RC LA RM FC MS. Contributed reagents/materials/analysis tools: OA SA LA RM. Wrote the paper: OA SA.

References

1. Russo IH, Russo J (1998) Role of Hormones in Mammary Cancer Initiation and Progression. Journal of Mammary Gland Biology and Neoplasia 3: 49–61.
2. Morgan G, Ward R, Barton M (2004) The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies. Clinical Oncology 16: 549–560.
3. Okada H, Mak TW (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 4: 592–603.
4. Brown JM, Attardi LD (2005) The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 5: 231–237.
5. Gurung RL, Lim SN, Khaw AK, Soon JFF, Shenoy K, et al. (2010) Thymoquinone Induces Telomere Shortening, DNA Damage and Apoptosis in Human Glioblastoma Cells. PLoS ONE 5: e12124.
6. Johnston S New targets for therapy in breast cancer - Farnesyltransferase inhibitors.
7. Gibbs JB (2000) Mechanism-Based Target Identification and Drug Discovery in Cancer Research. Science 287: 1969–1973.
8. Mann J (2002) Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer 2: 143–148.
9. Surh YJ (2003) Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3: 768–780.
10. Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4: 206–220.
11. Newman DJ, Cragg GM (2007) Natural Products as Sources of New Drugs over the Last 25 Years⊥. Journal of Natural Products 70: 461–477.
12. Clardy J, Walsh C (2004) Lessons from natural molecules. Nature 432: 829–837.
13. Cragg GM, Grothaus PG, Newman DJ (2009) Impact of Natural Products on Developing New Anti-Cancer Agents†. Chemical Reviews 109: 3012–3043.
14. Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants. Life Sciences 78: 431–441.
15. Butler MS (2004) The Role of Natural Product Chemistry in Drug Discovery†. Journal of Natural Products 67: 2141–2153.
16. Li JWH, Vederas JC (2009) Drug Discovery and Natural Products: End of an Era or an Endless Frontier? Science 325: 161–165.
17. Paterson I, Anderson EA (2005) The Renaissance of Natural Products as Drug Candidates. Science 310: 451–453.
18. Pezzuto JM (1997) Plant-derived anticancer agents. Biochemical Pharmacology 53: 121–133.
19. Cragg GM, Newman DJ (2009) Nature: a vital source of leads for anticancer drug development. Phytochemistry Reviews 8: 313–331.
20. Atta ur R, Naz S, Noor-e-ain F, Ali RA, Choudhary MI, et al. (1992) Alkaloids from Buxus species. Phytochemistry 31: 2933–2935.
21. Fourneau C, Hocquemiller R, Guédon D, Cavé A (1997) Spirofornabuxine, a novel type of Buxus alkaloid. Tetrahedron Letters 38: 2965–2968.
22. Loru F, Duval D, Aumelas A, Akeb F, Guédon D, et al. (2000) Four steroidal alkaloids from the leaves of Buxus sempervirens. Phytochemistry 54: 951–957.
23. Nakano T, Hasegawa M (1964) Buxus alkaloids. Part III. The structure of "Alkaloid-L" and its correlation with cyclomicrophylline-A. Tetrahedron Letters 5: 3679–3682.
24. Khalid A, Azim MK, Parveen S, Atta-ur R, Choudhary MI (2005) Structural basis of acetylcholinesterase inhibition by triterpenoidal alkaloids. Biochemical and Biophysical Research Communications 331: 1528–1532.
25. Babar ZU, Ata A, Meshkatalsadat MH (2006) New bioactive steroidal alkaloids from Buxus hyrcana. Steroids 71: 1045–1051.
26. Ata A, Iverson CD, Kalhari KS, Akhter S, Betteridge J, et al. (2010) Triterpenoidal alkaloids from Buxus hyrcana and their enzyme inhibitory, anti-fungal and anti-leishmanial activities. Phytochemistry 71: 1780–1786.
27. Matochko WL, James A, Lam CW, Kozera DJ, Ata A, et al. (2010) Triterpenoidal alkaloids from Buxus natalensis and their acetylcholinesterase inhibitory activity. J Nat Prod 73: 1858–1862.
28. Yan YX, Hu XD, Chen JC, Sun Y, Zhang XM, et al. (2009) Cytotoxic Triterpenoid Alkaloids from Buxus microphylla. Journal of Natural Products 72: 308–311.
29. Mesaik MA, Halim SA, Ul-Haq Z, Choudhary MI, Shahnaz S, et al. (2010) Immunosuppressive Activity of Buxidin and Buxenone from Buxus hyrcana. Chemical Biology & Drug Design 75: 310–317.
30. Hernandez-Enriquez B, Arellano RO, Moran J (2010) Role for ionic fluxes on cell death and apoptotic volume decrease in cultured cerebellar granule neurons. Neuroscience 167: 298–311.
31. Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y (2000) Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. Proceedings of the National Academy of Sciences of the United States of America 97: 9487–9492.
32. Darzynkiewicz Z, Huang X, Okafuji M, King MA, Zbigniew Darzynkiewicz MR (2004) Cytometric Methods to Detect Apoptosis. Cytometry, 4th Edition: New Developments: Academic Press. pp 307–341.
33. Bursch W, Ellinger A, Kienzl H, Török L, Pandey S, et al. (1996) Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis 17: 1595–1607.
34. Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, et al. (1997) Cell-specific Induction of Apoptosis by Microinjection of Cytochrome c. Journal of Biological Chemistry 272: 30299–30305.
35. Mizushima N, Yoshimori T (2007) How to interpret LC3 immunoblotting. Autophagy 3: 542–545.
36. Tanida I, Ueno T, Kominami E (2008) LC3 and Autophagy. Methods in Molecular Biology (Clifton, NJ) 445: 77–88.
37. Kottke TJ, Blajeski AL, Meng XW, Svingen PA, Ruchaud S, et al. (2002) Lack of Correlation between Caspase Activation and Caspase Activity Assays in Paclitaxel-treated MCF-7 Breast Cancer Cells. Journal of Biological Chemistry 277: 804–815.
38. Cao Y, Klionsky DJ (2007) Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. Cell Res 17: 839–849.
39. Maiuri MC, Le Toumelin G, Criollo A, Rain J-C, Gautier F, et al. (2007) Functional and physical interaction between Bcl-XL and a BH3-like domain in Beclin-1. EMBO J 26: 2527–2539.

40. Aita VM, Liang XH, Murty VVVS, Pincus DL, Yu W, et al. (1999) Cloning and Genomic Organization of Beclin 1, a Candidate Tumor Suppressor Gene on Chromosome 17q21. Genomics 59: 59–65.
41. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, et al. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402: 672–676.
42. Opipari AW, Tan L, Boitano AE, Sorenson DR, Aurora A, et al. (2004) Resveratrol-induced Autophagocytosis in Ovarian Cancer Cells. Cancer Research 64: 696–703.
43. Sherr CJ (1996) Cancer Cell Cycles. Science 274: 1672–1677.
44. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes & Development 13: 1501–1512.
45. Zukerberg LR, Yang WI, Gadd M, Thor AD, Koerner FC, et al. (1995) Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc 8: 560–567.
46. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, et al. (1994) Amplification and Overexpression of Cyclin D1 in Breast Cancer Detected by Immunohistochemical Staining. Cancer Research 54: 1812–1817.
47. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, et al. (2000) β-Catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. Proceedings of the National Academy of Sciences of the United States of America 97: 4262–4266.
48. De Vos K, Goossens V, Boone E, Vercammen D, Vancompernolle K, et al. (1998) The 55-kDa Tumor Necrosis Factor Receptor Induces Clustering of Mitochondria through Its Membrane-proximal Region. Journal of Biological Chemistry 273: 9673–9680.
49. Green DR, Kroemer G (2004) The Pathophysiology of Mitochondrial Cell Death. Science 305: 626–629.
50. Spierings D, McStay G, Saleh M, Bender C, Chipuk J, et al. (2005) Connected to Death: The (Unexpurgated) Mitochondrial Pathway of Apoptosis. Science 310: 66–67.
51. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397: 441–446.
52. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A (2008) Blocked Autophagy Sensitizes Resistant Carcinoma Cells to Radiation Therapy. Cancer Research 68: 1485–1494.
53. White E, DiPaola RS (2009) The Double-Edged Sword of Autophagy Modulation in Cancer. Clinical Cancer Research 15: 5308–5316.
54. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA (2009) Autophagy Facilitates the Development of Breast Cancer Resistance to the Anti-HER2 Monoclonal Antibody Trastuzumab. PLoS ONE 4: e6251.
55. Milani M, Rzymski T, Mellor HR, Pike L, Bottini A, et al. (2009) The Role of ATF4 Stabilization and Autophagy in Resistance of Breast Cancer Cells Treated with Bortezomib. Cancer Research 69: 4415–4423.
56. Levine B (2007) Cell biology: Autophagy and cancer. Nature 446: 745–747.
57. Chen N, Debnath J (2010) Autophagy and tumorigenesis. FEBS Letters 584: 1427–1435.
58. Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, et al. (2005) The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation. Cell 120: 747–759.
59. Fujiwara K, Daido S, Yamamoto A, Kobayashi R, Yokoyama T, et al. (2008) Pivotal Role of the Cyclin-dependent Kinase Inhibitor p21WAF1/CIP1 in Apoptosis and Autophagy. Journal of Biological Chemistry 283: 388–397.
60. Fan Y, Borowsky AD, Weiss RH (2003) An Antisense Oligodeoxynucleotide to p21Waf1/Cip1 Causes Apoptosis in Human Breast Cancer Cells1. Molecular Cancer Therapeutics 2: 773–782.
61. Altieri DC (2003) Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3: 46–54.
62. Fengzhi L, Grazia A, Emily YC, Janet P, Simona T, et al. (1998) Control of apoptosis and mitotic spindle checkpoint by survivin Nature Publishing Group.
63. Altieri DC (2001) The molecular basis and potential role of survivin in cancer diagnosis and therapy. Trends in Molecular Medicine 7: 542–547.
64. Fukuda S, Pelus LM (2006) Survivin, a cancer target with an emerging role in normal adult tissues. Molecular Cancer Therapeutics 5: 1087–1098.
65. Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC (2003) Suppression of Survivin Phosphorylation on Thr34 by Flavopiridol Enhances Tumor Cell Apoptosis. Cancer Research 63: 230–235.
66. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nature Reviews Molecular Cell Biology 8: 741–752.
67. Elgendy M, Sheridan C, Brumatti G, Martin SJ (2011) Oncogenic Ras-Induced Expression of Noxa and Beclin-1 Promotes Autophagic Cell Death and Limits Clonogenic Survival Molecular Cell In Press, Corrected Proof.
68. Weiss RH (2003) p21Waf1/Cip1 as a therapeutic target in breast and other cancers. Cancer Cell 4: 425–429.
69. Tian H, Wittmack EK, Jorgensen TJ (2000) p21WAF1/CIP1 Antisense Therapy Radiosensitizes Human Colon Cancer by Converting Growth Arrest to Apoptosis. Cancer Research 60: 679–684.
70. Liu S, Bishop WR, Liu M (2003) Differential effects of cell cycle regulatory protein p21WAF1/Cip1 on apoptosis and sensitivity to cancer chemotherapy. Drug Resistance Updates 6: 183–195.
71. Schwartz GK, Shah MA (2005) Targeting the Cell Cycle: A New Approach to Cancer Therapy. Journal of Clinical Oncology 23: 9408–9421.
72. Strickland LB, Dawson PJ, Santner SJ, Miller FR (2000) Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. Breast Cancer Res Treat 64: 235–240.

Journal of Medicinal Plants Research Vol. 5(4), pp. 589-598, 18 February, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Antioxidant potential, cytotoxic activity and phenolic content of *Clematis flammula* leaf extracts

Dina Atmani[1], M. Begoña Ruiz-Larrea[2], José Ignacio Ruiz-Sanz[2], Leandro J. Lizcano[2], Fadil Bakkali[2] and Djebbar Atmani[1]*

[1]Laboratory of Applied Biochemistry, Faculty of Life and Nature Sciences, University of Bejaia 06000 Algeria.
[2]Department of Physiology, Medicine and Dentistry School, University of the Basque Country, Leioa, Spain.

Accepted 8 November, 2010

Five fractions of *Clematis flammula*, a plant widely used in the Mediterranean traditional medicine, were isolated from the leaves using a selective extraction procedure and their total antioxidant capacity was measured by both the ABTS and ORAC tests. Furthermore, their capacities to inhibit microsomal lipid peroxidation and to scavenge the hydroxyl radical were assessed. The cytotoxic potential of the crude ethanolic extract and the aqueous fraction obtained from chloroform was also evaluated on three human hepatoma cell lines CHL, PLC and HuH7. The results showed a stronger antioxidant capacity for the two aqueous phases obtained from ethyl acetate and chloroform concerning ABTS (7.9 and 10.5 mmoles Trolox eq/g of plant extract, respectively), ORAC (487 and 387 mmoles Trolox eq/g of plant extract, respectively) and hydroxyl radical scavenging activity (IC_{50} = 56.5 and 48.4 µg/mL, respectively), compared to their organic counterparts which, however, inhibited microsomal lipid peroxidation more efficiently (IC_{50} = 390.7 and 523.5 µg/mL, respectively). The ethanol crude extract exhibited a fairly good cytotoxic potential on the two cell lines CHL and PLC (IC_{50} = 58.5 and 47.3 µg/mL, respectively), in contrast to the aqueous phase obtained from chloroform (IC_{50} = 457.7 and 304.9 µg/mL, respectively). A positive correlation was also found between the phenol content and the different activities. These results provide experimental support for the therapeutic virtues of *Clematis flammula* leaf extracts.

Key words: *Clematis flammula*, chloroform extract, antioxidant, caffeic acid, lipid peroxidation.

INTRODUCTION

Excess reactive oxygen species (ROS) produced as a result of a redox disequilibrium inside the cell are believed to be largely responsible for the development of many diseases mainly chronic inflammations, neurodegenerative disorders, heart disease and cancer (Halliwell and Gutteridge, 1990). Plants constitute a natural source of a diverse array of antioxidant molecules that have the potential to eliminate or neutralize the deleterious ROS (Shahidi and Naczk, 2004). A recent surge in scientific research was directed to validate the use of medicinal plants and attempt to identify novel molecules of natural origin. Most of these turned out to be phenols, a class of secondary metabolites, with strong antioxidant activities (Rice-Evans et al., 1997), including strong ROS scavenging capacities (Atmani et al., 2009) and inhibition of free radical producing enzymes (Berboucha et al., 2009). In many cases, their antioxidant properties have outperformed synthetic antioxidants such as butylated hygroxyanisole (BHA) which is suspected of being toxic and even carcinogenic (Wichi, 1988). Moreover, polyphenolic compounds are believed to have chemopreventive and suppressive activities against cancer cells by inhibiting many metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle (Newman et al., 2002). The leaves of *C. flammula*, commonly found in the Mediterranean region, are used in Spain (Rivera and De Castrom, 1993), Italy (Guarino et al., 2008) and Turkey (Yesilada and Kupeli, 2007) but mostly in the Kabylie region in

*Corresponding author. E-mail: djatmani@yahoo.com. Tel: 0021334214513. Fax: 0021334214762.

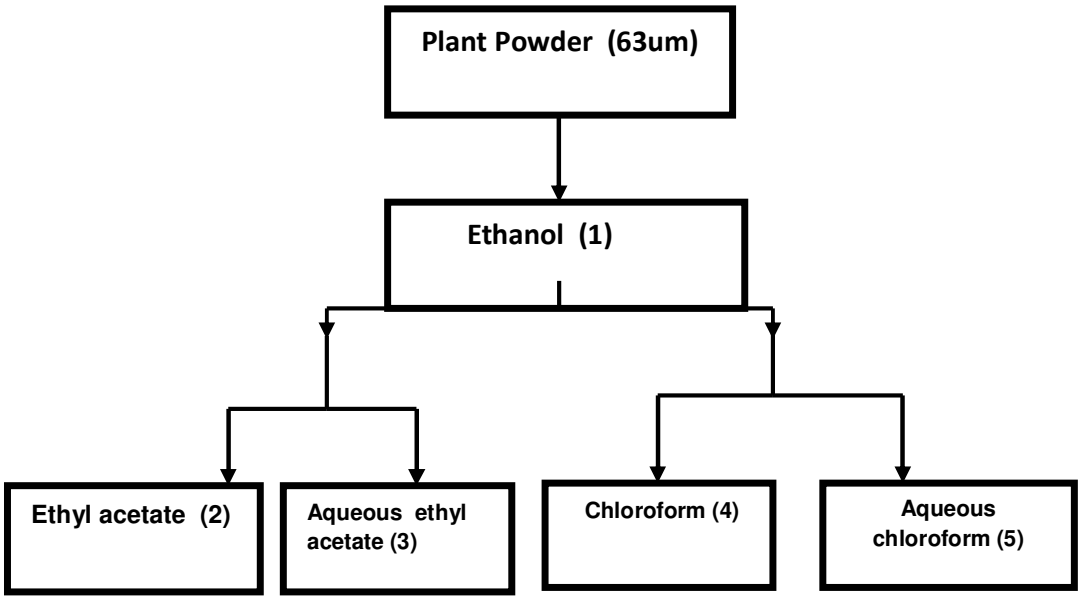


Figure 1. Extraction procedure from the leaves of *Clematis flammula*.

Algeria (Beloued, 1998) for medicinal purposes. According to epidemiological studies, infusions prepared from dried leaves are used to treat many ailments specifically arthritis, superficial burns and even cancer. Moreover, whole leaves are used as an insect repellent against deterioration of stored wheat. Recently, we reported the antioxidant potential of *C. flammula* leaf extracts using diphenyl picryl hydrazil (DPPH) and hydrogen peroxide (H_2O_2) scavenging activity tests and inhibition of linoleic acid peroxidation (Atmani et al., 2009). However, except for a preliminary study (Yesilada et al., 1997), the anti-cancer activity of this plant has not been tested. Therefore, our main objectives were to evaluate and compare (a) total antioxidant capacity of *C. flammula* fractions by using ABTS and ORAC assays, (b) their inhibition of microsomal lipid peroxidation and hydroxyl scavenging activities and (c) their anti-cancer activity against three different hepatoma cell lines by using the MTT assay. Furthermore, the quantification of phenols has been determined in order to establish a correlation between the content in phenols and the various activities tested.

MATERIALS AND METHODS

Preparation of plant extracts

The leaves of *C. flammula* (previously identified by Dr. M.S. Benabdelmoumene from the department of botany, where a specimen has been deposited) were collected in June in the forest of Tizi Neftah, Province of Amizour, Department of Bejaia in North-eastern Algeria. The whole plant was dried in an aerated but shaded area and the leaves were ground by an electrical grinder to obtain a powder of 60 µm of diameter. The ethanol extract was obtained by allowing 100 g of powder to macerate in ethanol for 24 h with continuous stirring. After decantation, the powder was separated from the corresponding ethanol extract which was left to dry at room temperature. Half of this crude extract was put in a mixture of chloroform and water (1:3:1, w/v/v) the other half in a mixture of ethyl acetate and water (1:3:1, w/v/v). Twenty four hours later, the organic phases were separated from the aqueous phases and dried gently to produce two aqueous and two organic fractions, as shown in Figure 1. The obtained crude ethanol extract (1) and the four subsequent fractions: ethyl acetate (2), aqueous of ethyl acetate (3), chloroform (4) and aqueous of chloroform (5) were tested separately.

ABTS test

The total antioxidant activity of the different fractions was measured by assessing their scavenging activity against the monocation radical $ABTS^{\cdot+}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) which is expressed as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC). The $ABTS^{\cdot+}$ radical solution was prepared as described by Re et al. (1999). One mL of this solution (adjusted to have an absorbance of 0.7 (+0.01 at 734 nm) was subsequently added to 10 µL of sample at different concentrations (50, 100, 200 and 400 µg/mL) at 30°C and for 12.5 min. The decolourization of $ABTS^{\cdot+}$ measured by a spectrophotometer (Uvikon 943) is a result of its reduction by hydrogen atoms given by the sample. TEAC was determined using the standard curve (R^2 = 0.99) of Trolox and expressed as mmoles Trolox equivalents per gram of fraction weight.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to the method described by Huang and his colleagues (Huang et al., 2002). Each diluted sample (25 µL) dissolved in 75 mM phosphate buffer solution (PBS) pH 7.4 was mixed with fluoroescein (8.16×10^{-2} mM)

in a 96-well polystyrene plate. This solution was then incubated in a preheated (37°C) microplate reader (Biotek Synergy HT) for 10 min. and the reaction was initiated by the addition of 25 µL (153 mM) AAPH (2, 2`-azobis (2-Amidinopropane hydrochloride). Fluorescence was recorded for 35 min. at the emission wavelength of 485 nm and the excitation wavelength of 530 nm. ORAC values were calculated by using the regression equation of a standard curve obtained by plotting Trolox concentrations against the area under the curve of the registered fluorescence along the time. These values are expressed as mmoles Trolox equivalents per gram of fraction weight.

Hydroxyl radical scavenging activity

The scavenging activity of *C. flammula* fractions and caffeic acid on hydroxyl radical was measured by the deoxyribose assay according to Halliwell et al. (1987) with some modifications. The reaction mixture containing 1750 µL of phosphate buffer (pH 7.4), 200 µL of sample, 50 uL of each of 2-deoxy-2-ribose (80 mM) dissolved in buffer, EDTA (4 mM), $FeCl_3$ (4 mM), H_2O_2 (20 mM) and ascorbic acid (4 mM), was vortexed and allowed to incubate for one hour at 37°C. One mL of 2% trichloroacetic acid (TCA) was then added followed by 1 mL of 1% thiobarbituric acid (TBA) and the tubes were allowed to stand in a boiling bath for 15 min. After cooling, the pink chromogen revealing the formation of thiobarbituric reactive substances (TBARS) was read at 532 nm (Specord 50). The percentage hydroxyl scavenging activity was calculated according to the following formula:

%OH scavenging= [(Ac-At)/Ac]x100

where Ac and At represent absorbance of the control and test solutions, respectively.

Inhibition of microsomal lipid peroxidation

The liver of male Sprague-Dawley rats (180 to 200 g) was cut into small pieces of about 2 g and then homogenised in 6 mL of 10 mM Tris-HCl buffer, 250 mM sucrose, 1 mM EDTA, pH 7.4 buffer solution (buffer A). The homogenate obtained was subsequently centrifuged at 1000 x g for 10 min at 4°C. The pellet was resuspended in 4 mL of buffer A and centrifuged twice at 1000 x g for 10 min at 4°C of which the resulting supernatants were centrifuged at 22,000 x g for 10 min at 4°C. Finally, the supernatant gathered was subjected to ultracentrifugation at 105000xg for 60 min at 4°C in a Centrikon T-2070 ultracentrifuge (Kontron). The pellet was immediately resuspended in 150 mM Tris-HCl pH 8.0 buffer and further centrifuged at 105,000 x g for 60 min at 4°C. The pellet obtained is the microsomal fraction which was immediately resuspended in 150 mM tris-HCl pH 7.4 and stored at -80°C until use. The microsomal proteins were quantified using the Bradford method (Bradford, 1976).

The inhibition of Fe^{2+}-ascorbate-induced microsomal lipid peroxidation by *C. flammula* fractions was measured according to the method of Fee and Teitelbaum (1972) that uses the formation of TBARS as an index of malondialdehyde (MDA) formation. The different fractions at concentrations ranging from 200 to 1000 µg/mL as well as the standard, caffeic acid (12.5 to 50 µg/mL) were dissolved in 150 mM Tris-HCl buffer, pH 7.4. 25 µL of the sample solution was then mixed with 725 µL of the same buffer followed by the addition of 50 µL of microsomes (8 mg/mL microsomal proteins) and the mixture was preincubated at 37°C for 10 min. Microsomal lipid peroxidation was started by the addition of a 100 µL of a solution containing a mixture of 25 µM $FeCl_3$ and 100 µM ascorbic acid dissolved in 10 mM KH_2PO_4. The mixture was incubated in a water bath for 10 min at 37°C and the reaction was stopped by the addition of 50 µL of 100% TCA. Ten minutes later, the tubes were centrifuged at 3,200 x g for 10 min at 4°C. An aliquot of 700 µL of the supernatant was withdrawn and 1.4 mL of 0.67% TBA solution prepared in 5% TCA was added. After heating at 100°C for 15 min, the intensity of the pink chromogen obtained was measured with a spectrophotometer at 535 nm (Uvikon 922).

Cytotoxic activity

Three human hepatoma cell lines (PLC, CHL, and HuH7) were used to assess the anti-cancer activity of the crude extract and fraction 5 which exhibited the best antioxidant activity as well as caffeic acid, used as a standard because of its confirmed anti-tumor activity (Tanaka et al., 1993), at concentrations ranging from 12.5 to 50 µg/mL. Cells were cultured in fresh Eagle Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin antibiotics. Cells were grown in a humidified incubator set at 5% CO_2 (Hera cell 150).

The dimethylthiazol diphenyltetrazolium bromide (MTT) colorimetric assay was carried out according to the method of Mosmann (1983) with some modifications. Cells were seeded into 96 well plates with a density of 2000 per well. At 50 to 60% confluency, they were treated with the plant extracts as well as caffeic acid for 48 h after which 100 µL MTT dye was added to each well. The microplates were further incubated for three hours at 37°C after which the supernatant was discarded and cells were then washed with 100 µL of PBS and 100 µL of a solution of dimethyl sulfoxide (DMSO) 10N NaOH (100:1) were added. Only metabolically active cells are capable to convert tetrazolium salt into a blue formazan product, the absorbance of which is measured at 570 nm using an ELISA microplate reader (Biotek Synergy HT). Results are expressed as percentage of control and derived from the mean of eight wells.

Quantification of total phenols

The amount of total phenols in the crude ethanol extract and in the different fractions was measured by using the Folin-Ciocalteu method (Kahkonen et al., 1999). Briefly, 200 µL of the sample solution was added to 1 mL of Folin-Ciocalteu and 0.8 mL of sodium carbonate (7.5%). After 30 min incubation in the dark, the absorbance was read at 765 nm. Phenol values obtained are expressed as catechin equivalent per gram of fraction weight.

Statistical analyses

Results are expressed as means of two different assays. Statistical analysis was done according to the Anova variance test using Statistica and $p<0.05$ was considered to be significant. Pearson's correlation was used to determine the relationship between covariates and Origin 7.5 to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Extraction yields

It has been reported that the efficiency of the extraction depends on many parameters, including the diameter of the powder and the volume and type of the solvents used (Escribano-Bailon et al., 2003). Furthermore, multi-solvent rather than single-solvent extractions were proven to be more efficient, leading to extracts with

Table 1. Extraction yields of *Clematis flammula* fractions.

Fraction	1	2	3	4	5
Percentage	20.3	3.5	6.7	10.2	25.4

Table 2. TEAC, ORAC and phenol content of *C. flammula* fractions: 1 (ethanol), 2 (ethyl acetate), 3 (aqueous of ethyl acetate), 4 (chloroform), and 5 (aqueous of chloroform) and caffeic acid.

Sample	TEAC mmoles trolox/g sample	ORAC mmoles trolox/g sample	Phenol content mg CE*/g sample
1	8.3 ± 0.3	33.05 ± 0.5	80.14 ± 20
2	4.12 ± 0.4	20.28 ± 0.2	232.18 ± 6
3	7.85 ± 0.5	487.3 ± 24	50.8 ± 2.5
4	4.85 ± 0.7	11.66 ± 0.3	210.36 ± 13
5	10.46 ± 0.5	387 ± 1.2	64 ± 2.5
Caffeic acid	120.15 ± 0.2	1710.9 ± 79.8	_

*Amounts of total phenols are expressed as catechin equivalent/g of sample

considerably higher antioxidant activity (Spigno et al., 2007). Table 1 clearly shows that the aqueous phase issued from chloroform (5) followed by the crude ethanol extract (1) exhibited the highest extraction yields. This result is in agreement with previous findings that consider ethanol as the most appropriate solvent to extract the maximum amount of phenols (Cowan, 1999).

Percent ethanol crude extract is expressed in g of plant powder. Percent of subsequent fractions is expressed in g of extract per g of the preceding fraction.

ABTS assay

The results summarized in Table 2 indicate that the aqueous fractions especially fraction 5 issued from chloroform extraction showed a significantly higher TEAC value ($p<0.5$) than that of the organic counterparts (2 and 4); however, the TEAC value of the crude ethanol extract (1) is comparable to that of the aqueous fraction 3 issued from ethyl acetate extraction ($p>0.5$). Moreover, caffeic acid, being a pure compound, was the most efficient in reducing $ABTS^{\cdot+}$, in agreement with previously reported data (Gulçin, 2006).

Even though the different fractions of *C. flammula* have previously been tested for their total antioxidant capacity using the DPPH test (Atmani et al., 2009), more than one method is necessary to evaluate the antioxidant capacity of a substance or plant extract since the underlying mechanisms can be different for each test (Frankel, 2001). In fact, we believe it is more relevant to evaluate the TEAC (total equivalent antioxidant capacity) by means of the ABTS cation radical scavenging assay because $ABTS^{\cdot+}$ trapping revealed to be more specific, being exclusively a hydrogen atom transfer (HAT) mechanism (Tang et al., 2007). DPPH, on the other hand, is a competition between HAT and sequential proton loss electron transfer (SPLET) mechanisms (Tang et al., 2007).

As the presence of the hydroxyl groups in a compound enhances its reaction with the ABTS cation radical and therefore the HAT mechanism (Re et al., 1999), it implies that the highly polar aqueous phase of chloroform (5) may contain a substantial amount of compounds with hydroxyl groups. Considering the high antioxidant potential obtained in previous experiments by the same fraction of *C. flammula* and other plants tested, *Pistacia lentiscus* and *Fraxinus angustifolia*, using the DPPH test (Atmani et al., 2009), it can be inferred that chloroform may be the most efficient solvent in the extraction procedure. In light of these results, it can be concluded that *C. flammula* fractions are considered to have a relatively high anti-radical activity regarding the ABTS assay when compared to other plant extracts tested in the same conditions (Maisuthisaku et al., 2006).

ORAC assay

The peroxyl radical ($ROO^{\cdot}$) is produced during lipid peroxidation *in vivo* and participates actively in its propagation (Porter, 1984). The ORAC assay measures the capacity of an antioxidant to protect fluorescein from the attack by this free radical, generated by thermal decomposition of AAPH. This assay is therefore, similarly to the ABTS test, widely accepted to estimate the antioxidant potential of a compound by comparison to a known reference, Trolox.

According to the results shown in Table 2, we notice a significantly higher antioxidant capacity for both aqueous

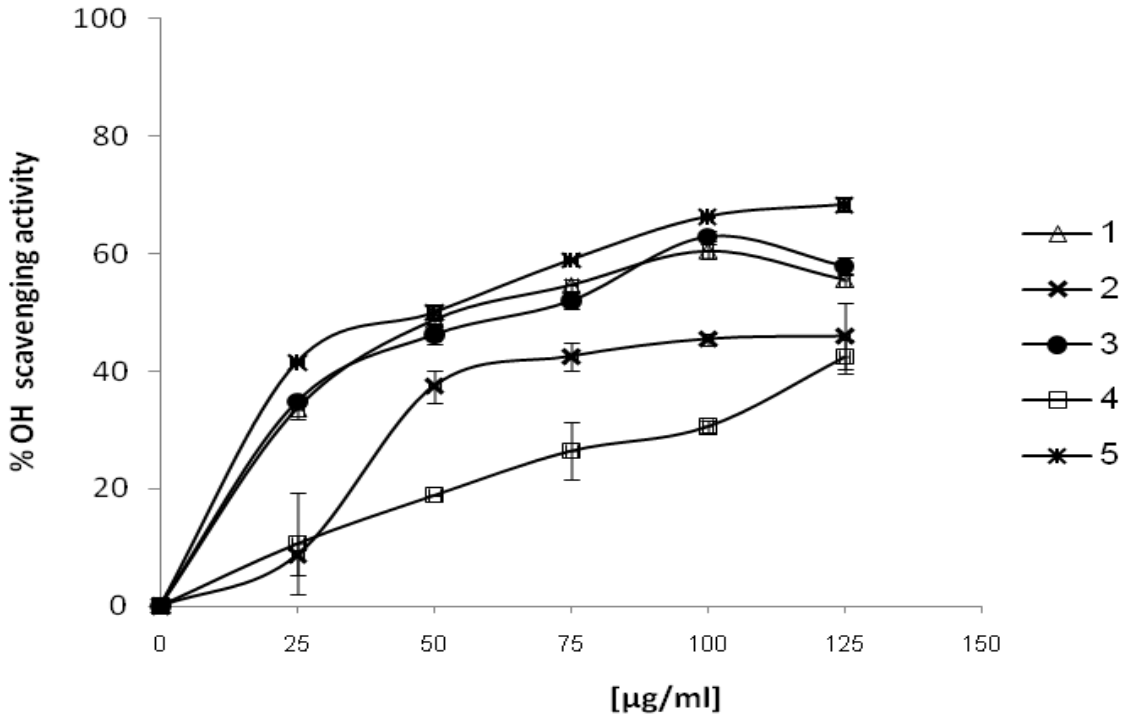


Figure 2. Percent hydroxyl radical scavenging activity of *C. flammula* fractions: 1 (ethanol), 2 (ethyl acetate), 3 (aqueous of ethyl acetate), 4 (chloroform), and 5 (aqueous of chloroform).

fractions 3 and 5, the most potent being fraction 3. On the other hand, their organic counterparts (1, 2 and 4) exhibited comparable results especially between ethanol and ethyl acetate ($p>0.5$) on one hand and ethyl acetate and chloroform ($p>0.5$) on the other. These results corroborate those of a structure-activity study that has been carried out on phenolic compounds regarding the ABTS, ORAC and DPPH assays where it was demonstrated that the higher the number of OH groups in a phenolic compound the higher its antioxidant capacity determined by the ABTS and DPPH assays (Villano et al., 2005). Nevertheless, the lack of a strong correlation between the ORAC and ABTS values reported by these authors and consolidated by our results ($r=0.61$) was explained by the fact that, in addition to the high polarity of a phenolic compound, the catechol moiety contributes greatly to the scavenging activity of peroxyl radicals which could account for the high ORAC value exhibited by caffeic acid (Table 2). This test is believed to be more specific than the ABTS method, considering the wider range of values found (Table 2), in agreement with the findings of the above mentioned authors (Villano et al., 2005).

Scavenging activity against OH radical

The formation of OH radical by the Fenton reaction is representative of the events that occur *in vivo* in iron-rich tissues like liver where it contributes to the initiation of lipid peroxidation (Porter, 1984). Besides, its high reactivity can lead to DNA mutagenesis and inactivation of various proteins (Villano et al., 2005). Therefore, the scavenging activity of this radical can be considered as one of the best indicators of the antioxidant potential of a compound. The results shown in Figure 2 indicate a dose-response increase in the capacity to quench hydroxyl radicals for all fractions, especially the highly polar ones (1, 3 and 5) with comparable IC_{50} values (54.4, 56.5 and 48.4 µg/mL, respectively) ($p>0.5$). On the other hand, the two organic fractions prevented equally the degradation of deoxyribose ($p>0.5$) (IC_{50} =126.7 and 128.7 µg/mL). Therefore, as appears from the present data and from previous investigations (Singh et al., 2007), polarity seems to play a central role in the hydroxyl radical scavenging efficiency of plant extracts, related to their high content in phenol compounds with hydroxyl groups (Yang et al., 2001). In contrast, caffeic acid exhibited a low efficiency against hydroxyl radical (IC_{50}=942.3 µg/mL), even showing a pro-oxidant potential at low concentrations. In fact, caffeic acid's metal chelating character renders it capable of redox cycling of the ferrous metal ion required for hydroxyl generation (Li et al., 2000). Similar results concerning the hydroxyl scavenging potential of caffeic acid were obtained by using electron spin resonance (Tran et al., 2000). Moreover, it has been established that the catechol moiety found in caffeic acid enhances considerably the

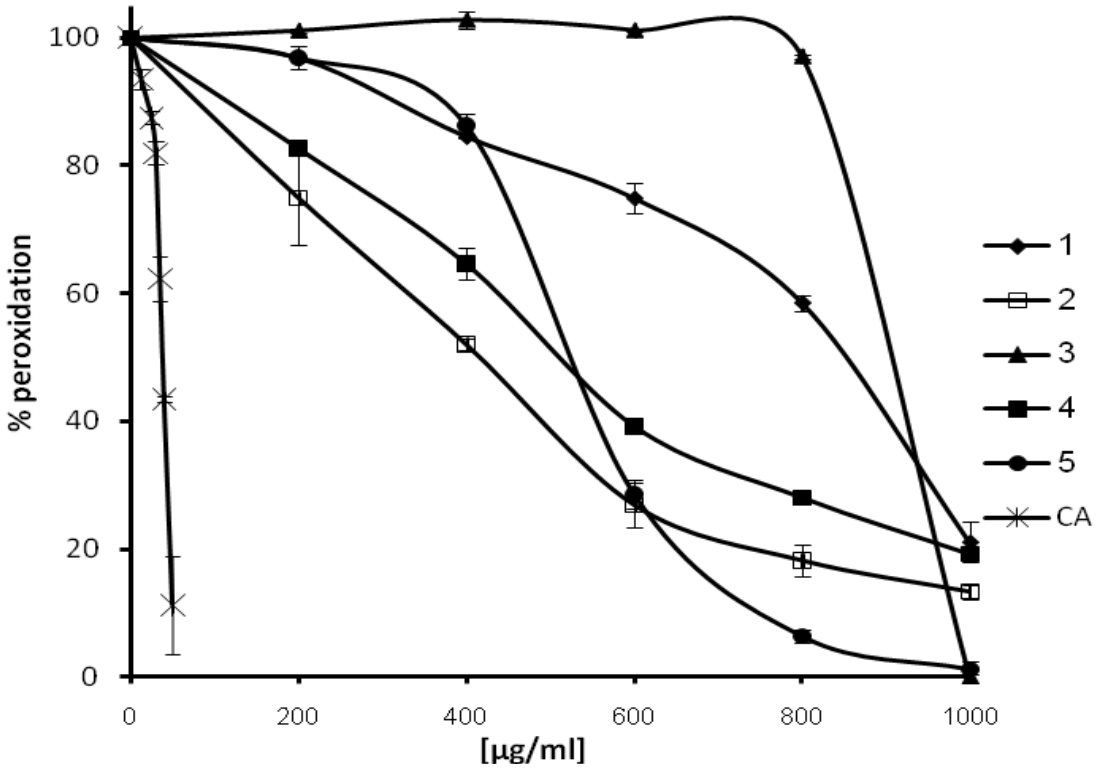


Figure 3. Inhibition of microsomal lipid peroxidation by *C. flammula* fractions 1 (ethanol), 2 (ethyl acetate), 3 (aqueous of ethyl acetate), 4 (chloroform), and 5 (aqueous of chloroform), compared to caffeic acid (CA).

formation of hydroxyl radicals during the Fenton reaction (Iwahashi et al., 1989). Later on, a kinetic study using the deoxyribose assay confirmed the pro-oxidant effect of caffeic acid beyond ten minutes of incubation (Cheng et al., 2003).

Inhibition of microsomal lipid peroxidation

A dose-dependent inhibition of microsomal lipid peroxidation is observed (Figure 3) for all the fractions as well as caffeic acid, the latter (IC_{50}=39 µg/mL) greatly outperforming all the *C. flammula* fractions. On the other hand, the IC_{50} values of the organic fractions 2 and 4 (390.7 and 523.5 µg/mL, respectively) indicate their higher capacity to prevent microsomal lipid peroxidation, as predicted by previous experiments using the ferrothiocyanate (FTC) method (Atmani et al., 2009). In fact, it seems that the higher lipophilic character of caffeic acid and the organic fractions 2 and 4 determines their lower IC_{50}. This is in agreement with previous reports that demonstrated that the hydrophobic nature of a phenolic compound enhances considerably its antioxidant capacity against microsomal lipid peroxidation (Wu et al., 2007). Furthermore, a protective and stabilizing role of the membrane assigned to such compounds was attributed to their high partition coefficients (Schroeter et al., 2000). However, all the *C. flammula* fractions have displayed a much lower efficiency against microsomal lipid peroxidation than that previously reported with the FTC method (Atmani et al., 2009). This can be explained by the complexity of the microsomal system which is considered to be a better bio-membrane model than a simple suspension of linoleic acid, thus allowing a more thorough understanding of the interactions between plant extracts and membrane surface. In fact, it has been suggested that antagonistic interactions between compounds in plant extracts with endogenous antioxidants such as Vitamin C and glutathione exist, thereby leading to the attenuation of their capacity to preserve the membrane from lipid peroxidation (Desmarchelier et al., 1997).

On the other hand, fraction 5 seems to overcome the microsomal membrane barrier since it possesses an IC_{50} (552.1 µg/mL) comparable to that of its organic counterpart (4) ($p>0.5$), with, nevertheless, a slower rate of efficiency (Figure 3). The iron-ascorbate system used in the present study induces lipid peroxidation by the formation of hydroxyl radicals which attack the microsomal membrane, producing peroxyl free radicals that propagate a chain reaction. Therefore, a complete reduction of lipid peroxidation displayed by both water-soluble fractions (3 and 5) could be accounted for by their high anti-radical potential especially concerning peroxyl

and hydroxyl radicals (Table 2 and Figure 2), suggesting that their constituents might be hydrophilic chain-terminating antioxidants. The involvement of other constituents present in fraction 5 such as secondary antioxidants with strong reducing power in the recycling of endogenous compounds such as vitamin E may be ruled out in light of the poor reducing power of the plant extracts reported earlier (Atmani et al., 2009).

MTT assay

The results in Figure 4a show that the crude ethanol extract exerts a pronounced dose-dependent effect on PLC (IC_{50}= 58.5 µg/mL), and CHL (IC_{50}= 47.3 µg/mL) cell lines, comparable to that of caffeic acid (IC_{50}= 37.1 µg/mL) ($p>0.5$), implying that it may constitute a source of anti-cancer metabolites. However, the crude extract was less efficient on HuH7 cell line, reaching only 67% viability at the highest concentration (IC_{50}=138.9 µg/mL). Caffeic acid, has demonstrated a significant effect at low concentrations (Figure 4c) as expected, especially on CHL cell line where it reduced viability to 20% at the highest concentration used (50 µg/mL). Again, the HuH7 cell line has shown more resistance to the action of the aqueous phase of chloroform extraction (5) and caffeic acid although less conspicuous than that observed for the ethanol fraction (1). However, the most interesting finding is that for both fractions as well as caffeic acid, an optimal concentration is reached, above which no dose-response to the high concentrations used was observed. In the case of fraction 5, higher concentrations had to be used to obtain a noticeable effect on all the cell lines (Figure 4b) (IC_{50}=304.9, 457.7 and 469.3 µg/mL on cell lines A, B and C, respectively), indicating a low cytotoxic potential and despite having higher antioxidant activities than its ethanol counterpart with respect to almost all the previous tests. In fact, it has been established that ROS scavenging can result in the dysfunction of enzymes involved in the activation of critical signaling pathways implicated in the initiation, promotion and progression of cancer (Simon et al., 1998).

Nevertheless, a compound with strong antioxidant potential can also be implicated in DNA protection and prevent apoptosis (Rajkumar et al., 2010), rendering the association between antioxidant and anti-cancer activities less evident. In fact, pro-oxidant compounds such as Salograviolide A isolated from *Centaurea ainetensis* cause growth inhibition and apoptosis in neoplastic epidermal cells, by fragmenting their DNA (Ghantous et al., 2008). Moreover, the hydrophilic character of a compound may hamper its interaction with the membrane where all the signal transduction pathways occur. In support of this assumption, it has been shown that caffeic acid phenyl ester has a higher efficiency against HepG2 hepatoma cell line, probably due to its higher lipophilic character (Chung et al., 2004). Indeed, a structure-activity study has led to the conclusion that cytotoxic activity of phenolic derivatives is directly related as much to their antioxidant potential as to their lipophilicity which favours their better incorporation into cells (Fiuza et al., 2004). Considering all the above mentioned studies and the fact that the ethanol crude extract exhibited very low inhibitory efficiency against lipoperoxidation (IC_{50} = 821 µg/mL), we can suppose that the anti-proliferative activity of this fraction cannot be due solely to its lipophilic constituents but to a synergy between both lipophilic and hydrophilic compounds, the latter shared by fraction 5 and exhibiting some cytotoxic activity.

Total phenol content

The results shown in Table 2 illustrate the enrichment in total phenol content of the organic fractions (2 and 4), compared to their aqueous counterparts (3 and 5) which could explain the significantly higher correlations found between the amount of phenols and the inhibition of microsomal lipid peroxidation (r^2 = 0.97) than that with TEAC (r^2 = 0.84) and hydroxyl (r^2 = 0.80) where the aqueous fractions (3 and 5) exhibited greater activities. In addition to that, higher antioxidant activity exhibited by fraction 5 in most tests used in the present study may be consistent with its high extraction yield. These results strongly indicate that the antioxidant potential of the various fractions of *C. flammula* is largely due to their high content in phenols in accordance with many investigations concerning the antioxidant potential of plant extracts (Lizcano et al., 2010). In contrast, regarding the ORAC assay, a much lower correlation was observed (r^2 = 0.57). This can lead to the assumption that individual phenolic compounds or antagonistic interactions between them are rather responsible for that activity. Regarding cytotoxic activity, a high correlation was found between the total phenol content and the anti-cancer activities against PLC and CHL cell lines (r^2 =0.7and 0.8 respectively), in contrast to the HuH7 cell line (r^2 =0.35).

Conclusion

The different polarities of the isolated fractions obtained by the selective extraction procedure adopted in this study seem to influence considerably their biofunctionality since the water-soluble fractions showed a predominant antioxidant potential greater than that of their organic counterparts. Higher activities exhibited by fraction 5 specifically plead in favour of considering it as a potential inexpensive source of antioxidant molecules despite displaying less cytotoxic activity, in contrast to the ethanol crude extract which contains both polar and non polar compounds that may act in synergy to achieve a high used chemotherapeutic agents revealed to be rather

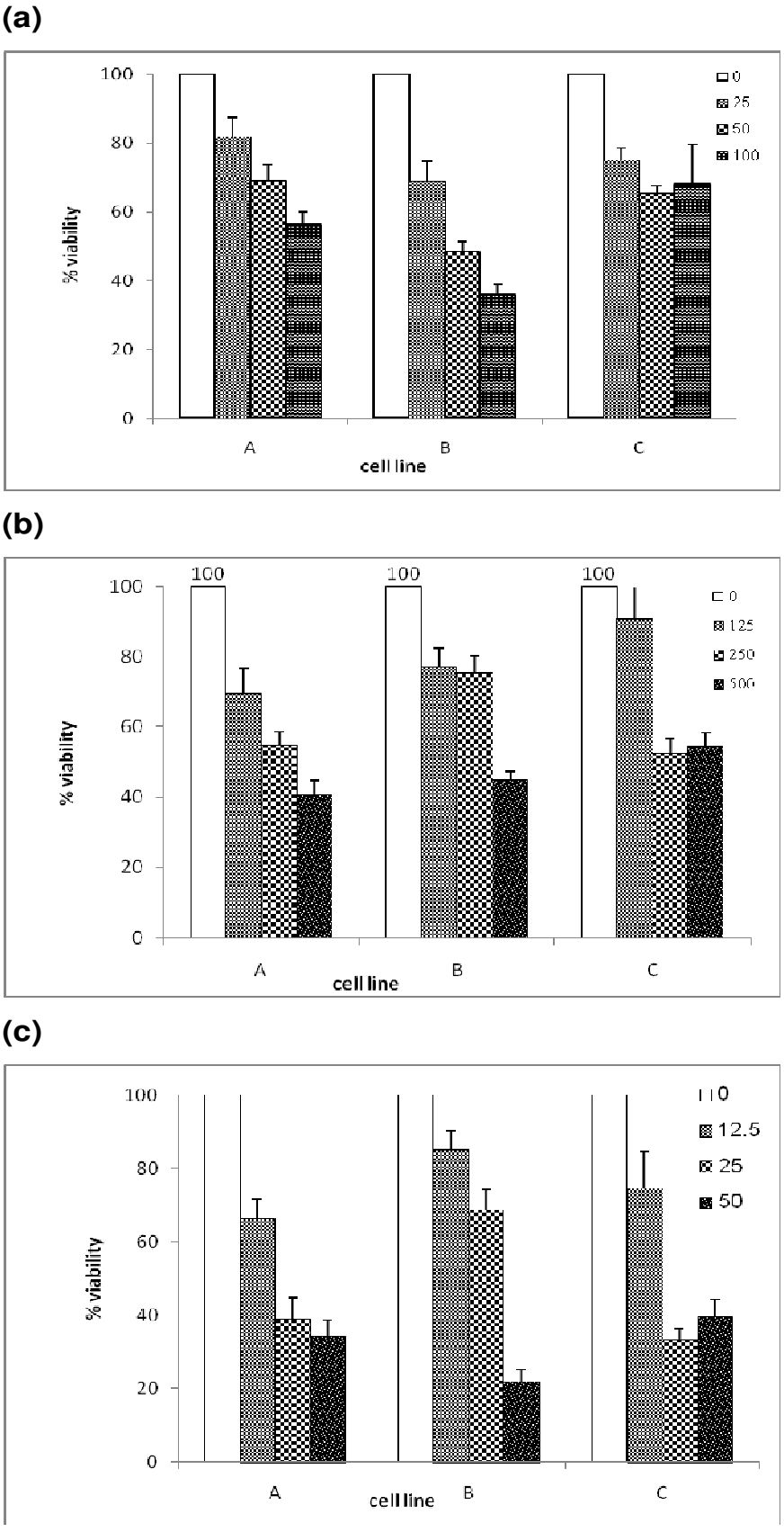


Figure 4. Cytotoxic activities of fractions a) 1 (ethanol), b) 5 (aqueous of chloroform) and c) caffeic acid on three hepatoma cell lines A= PLC B=CHL and C = HuH7.

anticancer potential. Elucidation of the molecular mechanisms involved and isolation of the bioactive molecules implicated may help the development of plant-derived anti-cancer drugs which can replace the clinically toxic. Therefore, further investigations need to be undertaken for full identification and characterization of the molecules responsible for antioxidant and anticancer activities of *C. flammula* leaf extracts.

ACKNOWLEDGEMENTS

We wish to thank the Algerian Ministry of High Education and Scientific Research for sponsoring this work. We also thank Dr. Lisardo Boscá, Instituto de Investigaciones Biomédicas Alberto, Consejo Superior de Investigaciones Cientificas, Madrid, Spain) for kindly donating the cell lines.

REFERENCES

Atmani D, Chaher N, Berboucha M, Ayouni K, Lounis H, Boudaoud H, Debbache N, Atmani D (2009). Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chem., 112: 303-309.

Atmani D, Atmani D, Chaher N, Boudaoud H, Debache N, Berboucha M (2009). Flavonoids in human health: from structure to biological activity. Curr. Nutr. Food Sci., 5, 225-237.

Beloued M: (1998). Plantes médicinales d'Algérie (Medicinal plants of Algeria). Office des Publications Universitaires (OPU), Algiers, Algeria, p. 277.

Berboucha M, Ayouni K, Atmani D, Atmani D, Benboubetra M (2010). Kinetic study on the inhibition of xanthine oxidase by extracts from two selected Algerian plants traditionally used for the treatment of inflammatory diseases. J. Med. Food, 13 (4): 1-9.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Cheng Z, Li Y, Chang W (2003). Kinetic deoxyribose degradation assay and its application in assessing the antioxidant activities of phenolic compounds in a Fenton- type reaction system. Anal. Chim. Acta., 1: 129-137.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microb. Rev. 12: 564-582.

Chung T, Moon S, Chang Y, Ko J, Lee Y, Cho G, Kim S, Kim J, Kim C (2004). Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. The FASEB J., 18: 1670-1681.

Desmarchelier C, Mongelli E, Coussio J, Ciccia G (1997). Inhibition of lipid peroxidation and iron (II)-dependent DNA damage by extracts of *Pothomorphe peltata* (L.) Miq. Braz. J. Med. Biol. Res., 30: 85-91.

Escribano-Bailon MT, Santos-Buelga C (2003). Polyphenols extract from food. In: "Methods in polyphenol analysis". Ed. Roy. Soc. Chem., 1-16.

Fee JA, Teitelbaum HD (1972). Evidence that superoxide dismutase plays a role in protecting red blood cells against peroxidative hemolysis. Biochem. Biophys. Res. Comm., 49: 150-158.

Fiuza SM, Gomes C, Teixeira LJ, da Cruz G Cordeiro MNDZ, Milhazes N Borges F, Marques MPM (2004). Phenolic acid derivatives with potential anticancer properties-a structure-activity relationship study. Part 1: Methyl, propyl and octyl esters of caffeic and gallic acids. Bioorg. Med. Chem., 12(13): 3581-3589.

Frankel EN (2001): Interfacial lipid oxidation and antioxidation. J. Oleo. Sci., 50: 387-391.

Ghantous A, Abou Tayyoun A, Abou Lteif G, Saliba NA, Gali-Muhtasib H, El-Sabban M, Darwiche N (2008). Purified Salograviolide A isolated from *Centaurea ainetensis* causes growth inhibition and apoptosis in neoplastic epidermal cells. International J. Oncol., 32: 841-849.

Guarino C, De Simone L, Santoro S (2008). Ethnobotanical study of the Sannio Area, Campania, Southern Italy. Ethnobotanical Res. App., 6: 255-317.

Gulçin I (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicol., 217: 213-220.

Halliwell B, Gutteridge JMC (1990): Role of free radicals and catalytic metal ions in human disease. An overview. Methods in Enzymol., pp. 1-85.

Halliwell B, Gutteridge JMC, Aruoma OI (1987). The Deoxyribose Method: A simple "Test-Tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem., 165: 215-219.

Huang D, Ou B, Hampsh-Woodill M, Flanagan JA, Prior R (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem., 50: 4437-4444.

Iwahashi H, Morishita H, Ishii T, Sugata R, Kido R (1989). Enhancement by catechols of hydroxyl radical formation in the presence of ferric ions and hydrogen peroxide. J. Biochem., 105(3): 429-434.

Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem., 47: 3954-3962.

Li C, Xie B (2000). Evaluation of the antioxidant and pro-oxidant effects of tea- catechin oxypolymers. J. of Agri. and Food Chem., 2000; 48: 6362-6366.

Lizcano LJ, Bakkali F, Ruiz-Larrea B, Ruiz-Sanz JI (2010). Antioxidant activity and polyphenol content of aqueous extracts from Colombia Amazonian plants with medicinal use. Food Chem., 119: 1566-1570.

Maisuthisaku P, Pongsawatmanit R, Gordon M (2006). Characterization of the phytochemicals and antioxidant properties of extracts from Teaw (*Cratoxylum formosum* Dyer). Food Chem., 100: 1409-1418.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Meth., 65: 55-63.

Newman DJ, Gragg GM, Holbeck S, Sausville EA (2002). Natural products as leads to cell cycle pathway targets in cancer chemotherapy. Cur. Cancer Drug Targets., 2: 279-308.

Porter NA (1984). Chemistry of lipid peroxidation. Methods in Enzymol., 105: 263-273.

Rajkumar V, Guha H, Kumar RA (2010). Antioxidant and anticancer potentials of Rheum emodi rhizome extracts. eCAM., 1-9.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Bio. Med., 26: 1231-1237.

Rice-Evans C, Miller NJ, Papanga G. (1997) Antioxidant properties of phenolic compounds. Trends Plant Sci. Rev., 2(4): 152-159.

Rivera N, De Castro O (1993). Ethnopharmacology of Murcia (SE Spain). Médicaments et aliments: L' approche ethnopharmacologique, pp. 215-239.

Schroeter H, Williams RJ, Matin R, Iversen L, Rice-Evans CA (2000). Phenolic antioxidants attenuate neuronal cell death following uptake of oxidized low density lipoprotein. Free Rad. Biol. Med., 29: 1222-1233.

Shahidi F, Naczk M (2004). Phenolics in food and neutraceuticals CRC Press. Boca Raton, FL.

Simon AR, Rai U, Fanburg BL, Cochran BH (1998). Activation of the JAK-STAT pathway by reactive oxygen species. Am. J. Physiol. Cell Physiol., 275: 1640-1652.

Singh R, Singh S, Kumar S, Arora S (2007). Evaluation of antioxidant potential of ethyl acetate extract/fractions of *Acacia auriculoformis* A. Cunn. Food Chem. Toxicol., 45:1216-1223.

Spigno G, Trqmelli L, De Faveri DM (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J. Food Eng., 81(1): 200-208.

Tanaka T, Kojima T, Kawamori T, Wang A, Suzui M, Okamoto K, Mori H (1993). Inhibition of 4-notroquinoline-induced rat tongue carcinogenesis by the naturally occurring plant phenolic acids caffeic,

ellagic, chlorogenic and ferulic acids. Carcinogenesis., 14: 1321-1325.
Tang Y, Liu Z (2007). Free-radical scavenging effect of carbazole derivatives on DPPH and ABTS radicals. J. Am. Oil Chem. Soc., 84: 1095-1100.
Tran TT, Cronin MT, Dearden JC, Morris H. (2000). Determination of anti-oxidant activity by electron spin resonance spectroscopy. Pest Manage. Sci., 56(9): 818-820.
Villano D, Fernandez Pachon MS, Troncoso AM, Garcia Parrilla MC (2005). Comparison of antioxidant activity of wine phenolic compounds and metabolites *in vitro*. Anal. Chimica Acta, 38(1-2): 391-398.
Wichi HP (1988). Enhanced tumour development by butylated hydroxyanisole (BHA) from the prospective of effect on forestomach and oesophagal squamous epithelium. Food Chem. Toxicol., 26: 717-723.
Wu W, Lu L, Long Y, Wang T, Liu L, Chen Q, Wang R (2007). Free radical scavenging and antioxidative activities of caffeic acid phenethyl ester (CAPE) and its related compounds in solution and membranes: a structure-related study. Food Chem., 105: 107-115.
Yang YH, Jung YY, Chung HY (2001). Peroxynitrite and hydroxyl radical scavenging activity of dihydroxybenzaldehydes. Kor. J. Gerontol., 11(3): 24-28.
Yesilada E, Üstün O, Sezik E, Takaishi Y, Ono Y, Honda G (1997). Inhibitory effects of Turkish folk remedies on inflammatory cytokines: interleukin n-1α, interleukin-1β and tumor necrosis factor α. J. Ethnopharmacol., 58: 59-73.
Yesilada E, Kupeli E (2007). *Clematis vitalba L.* aerial part exhibits potent anti-inflammatory aminoceptive and anti-pyretic effects. J. Ethnopharmacol., 110: 504-515.

ISSN : 0974 - 7508 Volume 7 Issue 2

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 7(2), 2011 [66-70]

Antibacterial activity of four species of Algerian algae

F.Bedjou, F.Benabdesselam-Maiza*, K.Saidani, N.Touati

Laboratory of Plant Biotechnology and Ethnobotany, Faculty of Life and Nature Sciences, A/Mira University of Béjaia, 06000, Béjaia, (ALGERIA)

E-mail: fadilamaiza@yahoo.fr

Received: 14th April, 2011 ; Accepted: 24th April, 2011

ABSTRACT

Antibiotic resistance in Bacteria is one of the emerging health related problem in the world nowadays. Plants and among them algae are valuable natural sources effective against infectious agents. Methanolic extracts of four marine algae of Algeria coast were investigated for antibacterial activity against six pathogenic bacteria (*Bacillus subtilis, Listeria innocua, Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*). Susceptibility assays using disc diffusion and broth microdilution test for the determination of minimum inhibitory concentration (MIC) were employed to asses the antibacterial activity of methanolic extracts of algae. All algae extracts showed antibacterial activity against four of the six pathogenic bacteria tested with MIC values ranged between 0.25-3mg/ml. Extract of *Rhodomela confervoïdes* exhibited the highest activity against *Bacillus subtilis* (24 mm). *Cystoseira tamariscifolia* exhibited the highest activity against *Listeria innocua* (19.67mm)

KEYWORDS

Algae;
Polyphenols;
Active metabolites;
Antibacterial activity.

INTRODUCTION

Marine organisms, as algae, are a rich source of biologically active metabolites[2,4]. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry[13]. The cell extracts, and active constituents of various algae, have been shown to have antibacterial activity *in vitro* against Gram positive and Gram negative bacteria[12]

In this investigation antibacterial activity of four marine algae of Algeria coast belonging to families such as Rhodophyceae (*Rhodomela confervoïdes*), Chlorophyceae (*Ulva lactuca*), Phaeophyceae (*Cystoseira tamariscifolia* and *Padina pavonica*) was studied against pathogenic bacteria (*Bacillus subtilis ATTCC 6633, Listeria innocua CLIP 74915, Staphylococcus aureus ATCC 6538, Escherichia coli NAR, Klebsiella pneumonia E47, Pseudomonas aeruginosa ATCC27853*.

Tuney and al.[12] and Bansamir and al.[1] have shown that cell extracts and active constituents of various algae have antibacterial activity against Gram positive and Gram negative bacteria

MATERIALS AND METHODS

Algae materials

Marine algae were hand collected from the submerged marine rocks of Bejaia coats in north Algeria

Full Paper

during March 2009. Seaweeds were identified by from botanic laboratory (University of Béjaia). Epiphytes and sediment were removed by washing first in sea water and then in fresh water. The algae were transported to the laboratory in polyethylen bags at ice temperature.

Preparation of methanolic extract of algae

The samples of algae were dried at room temperature, powdered, and sieved. The powder was dissolved with methanol (1/10 w:v) and kept at room temperature, under agitation for overnight. The solution was centrifuged at 2220g for 10 minutes (sigma). The supernatant, which contains polyphenol extract was recovered. The residue was dissolved twice in methanol (1/10 w:v). The supernatants were recovered and filtered through 4 layered cheese cloths and concentrated in kika labortechnik rotavapor. The dried sample was dissolved in methanol and stored at 4°C until further use[3]

Tested microorganisms

Tested microorganisms were obtained from applied microbiology laboratory (university of Bejaia). Six human pathogenic microorganisms, such as : *Bacillus subtilis* (ATCC 6633), *Listeria innocua* (CLIP 74915), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (N.A.R), *Klebsiella pneumonia* (E 47), *Pseudomonas aeruginosa* (ATCC 27853) were used in the present study for evaluation of the antimicrobial activity of algae extracts.

The pathogenic bacteria were cultured individually on selective broth at 37°C for 18h, before inoculation for assay[5]. 100µl of broth culture, which contain 10^8UFC/ml were used for inoculate Muller-Hinton agar medium[7,8]

Antibacterial assays

Antibacterial activity was evaluated by agar diffusion method[10]. Spots which contain 25µl of crude extract of algae were applied on Muller-Hinton agar medium, sowed with bacteria (10^8UFC)[6].

Inhibition zones, around spots, were evaluated after incubation during 24h at 37°C[6]. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the algae extract required for complete inhibition of growth of the bacteria being tested after incubation at 37°C for 18h or 24h. All the essays were carried out in triplicate. Methanol (50%) without algal extract was used as a negative control and in this case, no antibacterial activity was observed.

RESULTS

Antibacterial activity

The values of Diameters of microbial inhibition zones (mm) recorded with the different extracts are reported

TABLE 1 : Antibacterial activity of methanolic extracts of Algerian algae against pathogenic bacteria strains

Marine algae	Dilution (mg/25µl)	Microbial inhibition zone diameter (mm)					
		B. subtilis	*L. innocua*	*S. aureus*	*E. coli*	*K.pneumoniae*	*p.aeruginosa*
Cystoseira tamariscifolia	1,25	00,00±00,00^{a}	19,67±00,57^{jkl}	18,33±00,57^{jk}	18,00±00,00^{mn}	15,67±00,57^{fg}	18,00±00,00^{lmn}
	0,625	00,00±00,00^{a}	17,66±00,57^{gh}	15,67±00,57^{ef}	17,00±00,00^{jk}	17,00±00,00^{hij}	17,66±00,57^{kl}
	0,312	00,00±00,00^{a}	16,67±00,57^{f}	15,33±00,57^{de}	16,00±00,00^{h}	17,00±00,00^{hij}	16,33±00,57^{jk}
	0,156	00,00±00,00^{a}	17,00±00,00^{fg}	14,67±00,57^{d}	18,00±00,00^{mn}	19,33±00,57^{k}	15,67±00,57^{ghi}
Padina pavonica	1,25	19,33±00,57^{f}	13,00±00,00^{c}	13,33±00,57^{c}	12,33±00,57^{e}	00,00±00,00^{a}	12,33±00,57^{b}
	0,625	09,33±00,57^{b}	00,00±00,00^{a}	16,33±00,57^{fg}	16,33±00,57^{hi}	00,00±00,00^{a}	15,33±00,57f^{gh}
	0,312	00,00±00,00^{a}	00,00±00,00^{a}	16,66±00,57^{gh}	14,00±00,00^{f}	00,00±00,00^{a}	16,33±00,57^{ij}
	0,156	00,00±00,00^{a}	00,00±00,00^{a}	12,66±00,57^{c}	16,00±00,00^{h}	00,00±00,00^{a}	13,33±00,57^{b}
Rhodomela confervoides	1,25	22,67±00,57^{ig}	20,33±00,57^{l}	16,33±00,57^{fg}	18,33±00,57^{no}	17,33±00,57^{ij}	16,33±00,57^{ij}
	0,625	22,33±00,57^{i}	18,00±00,00^{h}	15,66±00,57^{ef}	17,33±00,57^{kl}	16,33±00,57^{gh}	14,67±00,57^{ef}
	0,312	24,00±00,00^{kl}	18,00±00,00^{h}	11,33±00,57^{b}	16,00±00,00^{h}	16,33±00,57^{gh}	16,33±00,57^{ij}
	0,156	23,33±00,57^{gk}	19,33±00,57^{ijk}	15,67±00,57^{ef}	18,67±00,57^{o}	12,33±00,57^{d}	15,67±00,57^{ghi}
Ulva lactuca	1,25	00,00±00,00^{a}	16,33±00,57^{f}	16,00±00,00^{efg}	14,33±00,57^{f}	00,00±00,00^{a}	15,00±00,00^{efg}
	0,625	00,00±00,00^{a}	16,33±00,57^{f}	13,00±00,00^{c}	00,00±00,00^{a}	00,00±00,00^{a}	00,00±00,00^{a}
	0,312	00,00±00,00^{a}	13,00±00,00^{c}	13,00±00,00^{c}	00,00±00,00^{a}	00,00±00,00^{a}	00,00±00,00^{a}
	0,156	00,00±00,00^{a}	13,00±00,00^{c}	00,00±00,00^{a}	00,00±00,00^{a}	00,00±00,00^{a}	00,00±00,00^{a}

Full Paper

on TABLE 1

Crude extracts of the four species of algae showed antibacterial activity (Figure 1). No activity was observed from four methanolic extracts : *Cystoseira tamariscifolia* against *Bacillus subtilis*, *Padina pavonica* against *Klebsiella pneumoniae*, *Ulva lactuca* against *Bacillus subtilis* and *Klebsiella pneumoniae*. The majority of algal extracts were active against four or five microorganisms.

All the extracts showed activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria innocua*.

The extract of *Rhodomela confervoïdes* exhibited the highest activity against *Bacillus subtilis* (24 mm). *Cystoseira tamariscifolia* exhibited the highest activity against *Listeria innocua* (19.67mm). all the extracts exhibited a lowest activity than the polyphenol standards TABLE 2

TABLE 2 : Antibacterial activity of some polyphenol standards

Standard polyphénol	Dilution (mg/25 µl)	Microbial inhibition zone diameter (mm)					
		B. subtilis	*L. innocua*	*S. aureus*	*E. coli*	*K.pneumoniae*	*p.aeruginosa*
Cafeic acid	1,25	25,00±00,00^{mn}	24,33±00,57^{n}	37,33±00,57^{r}	24,00±00,00^{q}	23,33±00,57^{l}	23,33±00,57^{r}
	0,625	22,33±00,57^{hi}	24,67±00,57^{n}	25,67±00,57^{p}	26,33±00,57^{r}	22,67±00,57^{l}	24,33±00,57^{s}
	0,312	23,33±00,57^{gk}	18,33±00,57^{hi}	19,33±00,57^{l}	17,33±00,57^{l}	19,00±00,00^{k}	18,33±00,57^{mn}
	0,156	17,67±00,57^{de}	19,00±00,00^{ij}	19,33±00,57^{l}	16,66±00,57^{ijk}	17,33±00,57^{ij}	18,00±00,00^{lmn}
Gallic acid	1,25	20,67±00,57^{g}	28,33±00,57^{o}	27,33±00,57^{q}	20,00±00,00^{p}	19,33±00,57^{k}	18,67±00,57^{no}
	0,625	19,33±00,57^{f}	22,67±00,57^{m}	24,33±00,57^{o}	18,33±00,57^{no}	19,33±00,57^{k}	21,33±00,57^{q}
	0,312	18,33±00,57^{e}	20,33±00,57^{l}	15,33±00,57^{de}	18,33±00,57^{no}	16,67±00,57^{hi}	19,33±00,57^{op}
	0,156	15,33±00,57^{c}	19,00±00,00^{i}	17,33±00,57^{hi}	16,33±00,57^{hi}	17,33±00,57^{ij}	16,00±00,00^{hij}
T tannic acid	1,25	25,33±00,57^{n}	20,33±00,57^{b}	27,33±00,57^{q}	24,00±00,00^{q}	14,33±00,57^{e}	26,33±00,57^{t}
	0,625	24,66±00,57^{lmn}	15,33±00,57^{e}	24,33±00,57^{o}	17,67±00,57^{lm}	15,33±00,57^{f}	27,33±00,57^{u}
	0,312	21,67±00,57^{h}	18,33±00,57^{hi}	15,33±00,57^{de}	18,00±00,00^{mn}	17,67±00,57^{j}	21,33±00,57
	0,156	19,67±00,57^{fg}	15,00±00,00^{de}	17,33±00,57^{hi}	17,00±00,00^{jk}	14,33±00,57^{e}	17,67±00,57^{lmq}
Catechin	1,25	20,33±00,57^{g}	18,33±00,57^{hi}	20,33±00,57^{m}	17,00±00,00^{jk}	17,33±00,57^{ij}	17,33±00,57^{kl}
	0,625	19,33±00,57^{f}	19,33±00,57^{jk}	18,33±00,57^{jk}	09,33±00,57^{d}	16,67±00,57^{hi}	15,33±00,57^{fgh}
	0,312	18,33±0,57^{e}	20,00±00,00^{kl}	18,00±00,00^{ij}	06,33±00,57^{c}	19,33±00,57^{k}	16,67±00,57^{jk}
	0,156	18,33±00,57^{e}	19,00±00,00^{ij}	19,00±00,00^{kl}	04,00±00,00^{b}	18,67±00,57^{k}	14,33±00,57^{e}
Quercetin	1,25	17,33±00,57^{d}	14,33±00,57^{d}	17,33±00,57^{hi}	17,33±00,57^{kl}	12,00±00,00^{d}	18,33±00,57^{mn}
	0,625	20,33±00,57^{g}	20,00±00,00^{k}	22,33±00,57^{n}	15,33±00,57^{g}	17,33±00,57^{ij}	20,00±00,00^{p}
	0,312	25,00±00,00^{mn}	16,33±00,57^{fl}	19,33±00,57^{l}	18,00±00,00^{mn}	11,00±00,00^{c}	23,33±00,57^{r}
	0,156	24,33±00,57^{lm}	18,33±00,57^{hi}	19,00±00,00^{kl}	16,00±00,00^{h}	14,00±00,00^{b}	23,33±00,57^{r}

NT: Not tested

*** Methanol used as a negative control has shown no bacterial activity.**

Determination of minimum inhibitory concentration (MIC)

TABLE 3 shows the MIC results of four algal extracts against six bacteria strains

The highest MIC (l < 3mg/ml) is observed for *E.coli*, *L.innocua* and *K.pneumoniae* with *Cystoseira tamariscifolia* and *Rhodomela confervoïdes* extracts. The lowest MIC (>0.25mg/ml) is obtained for *S.aureus* with *Rhodomela confervoïdes* extract.

Among the standards tested, gallic acid and tannic acid showed the lowest MIC against *L.innocua*, *S.aureus* and *P.aeruginosa* for the first one (0.1mg/ml), and against *B.subtilis* and *P.aeruginosa* for tannic acid (TABLE 3)

DISCUSSION

The main objective of this study was to evaluate the ability of algal extracts of Bejaia coast, to inhibit the growth of pathogenic bacteria, with the aim to use them in the future as alternative to common antibiotics

Full Paper

TABLE 3 : Determination of minimum inhibitory concentration of extract

Marine algae	MIC (mg/ml)					
	B.subtilis	*L.innocua*	*S.Aureus*	*E.Coli*	*K.pneumoniae*	*p.aeruginosa*
Cystoseira tamariscifolia	NT	>3	1,2	>3	>3	1,2
Padina pavonica	>3	>3	1,2	>3	NT	1,2
Rhodomela confervoides	0,5	>3	0,25	>3	>3	1,2
Ulva lactuca	NT/NT	>3	1,2	>3	NT	1,2

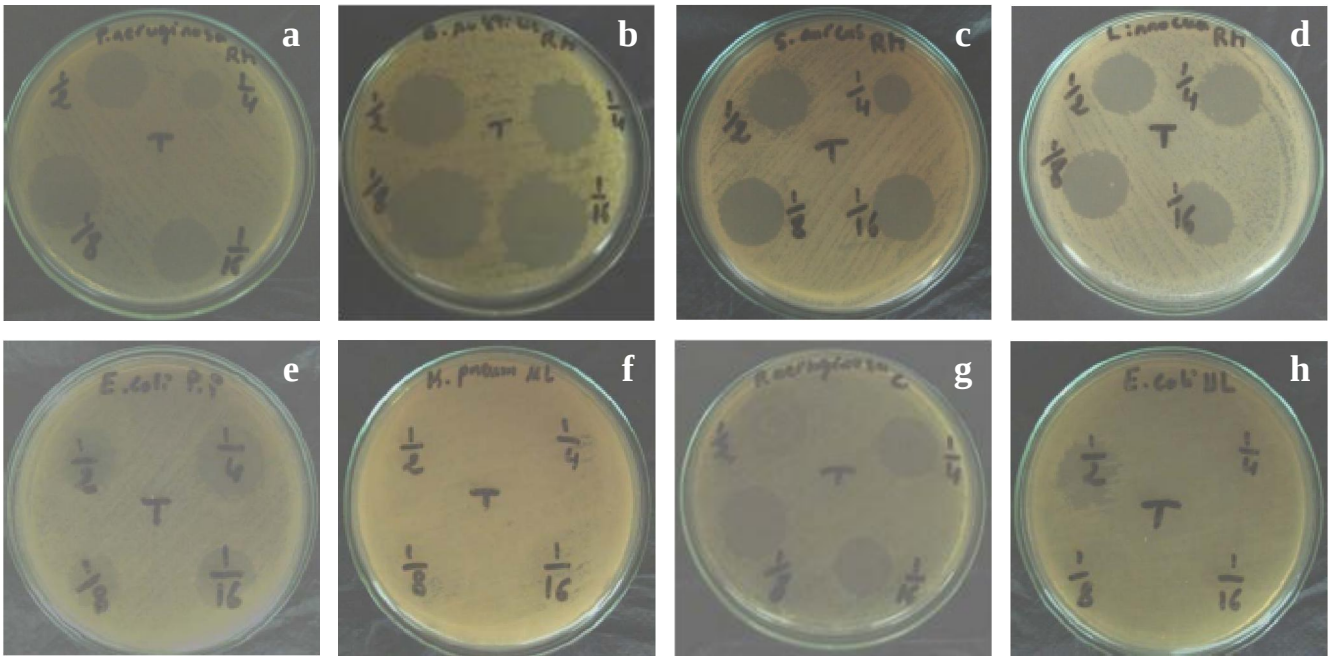


Figure 1 : Photos of some inhibition zones obtained with methanolic extracts: *Rhodomedela confervoides* vs *P. aeruginosa* (a,d), *B. subtilis* (b), *S.aureus* (c) *Ulva lactuca* vs *E. coli* (h), *K. pneumoniae* (f) *Cystoseira tamariscifolia* vs *P. aeruginosa* (g); *Padina pavonica* vs *E. coli* (e).

in human therapeutic.

Most active species were *Rhodomela confervoïdes* and *Cystoseira tamariscifolia* respectively against *B.subtilis* (inhibition zone˜22 at 23mm) and L.innocua (20mm) for *Rhodomela*, and against *L.innocua* (19.67mm) and *S.aureus* (18.33mm) for *Cystoseira*.

No activity was observed against *B.subtilis* with *Cystoseira tamariscifolia* and *Ulva lactuca* extracts. The same result was obtained with the extracts of *Padina pavonica* and *Ulva lactuca* against *K.pneumoniae*.

The high activity of seaweeds belonging to the Rhodophyceae agrees with the results of previous studies using other test microorganisms[7,8]. The result of the present study revealed that Gram positive organisms were generally more susceptible to the crude extracts of algae, which agrees with the results of others studies[11]

The more susceptibility of the Gram positive bacteria to algal extract is due to the differences in their cell wall structure and their composition[9]. In Gram negative bacteria, the presence of a thick murine layer in the cell wall prevents entry of the inhibitors.

The results obtained in this study suggest that algal extracts of Bejaia coast have a good antibacterial activity against pathogenic bacteria which makes them interesting for programs screening natural products. This ability is not restricted to one order or division within the macroalgae: all of them offer opportunities for producing new types of bioactive compounds.

REFERENCES

[1] A.Bansemir, M.Blume, S.Schröder, U.Lindequist; Aquaculture, **252**, 79-84 **(2006)**.

[2] M.A.Borowitzka, I.J.Borowitzka; Vitamins and Fine Fine Chemicals from Micro Algae. In Microalgal Biotechnology. Cambridge University Press, Great Britain, 179 **(1992)**.

Full Paper

[3] S.H.Cho, S.E.Kang, J.Cho, A.Kim, S.Park, Y.Hong, D.Ahn; Journal of Medicinal Food, **10(3)**, 479-485 **(2007)**.

[4] R.Ely, T.Suprya, C.G.Naik; J.Exp.Boil.and Ecol., **309**, 121-127 **(2004)**.

[5] M.Kandhasamy, K.D.Arunachalam; African Journal of Biotechnology, **7(12)**, 1958-1961 **(2008)**.

[6] N.U.Karabay-Yavasoglu, A.Sukatar, G.Ozdemir, Z.Horzum; Phytotherapy Research, **21**, 153-156 **(2007)**.

[7] K.B.Oh, J.H.Lee, S.H.Chung, J.Shin, H.J.Shin, H.K.Kim, H.S.Lee; Bioorganic and Medicinal Chemistry Letters, **18**, 104-108 **(2008)**.

[8] J.K.Patra, A.P.Patra, N.K.Mahapatra, H.N.Thatoi, S.Das, R.K.Sahu, G.C.Swain; Malaysian Journal of Microbiology, **5(2)**, 128-131 **(2009)**.

[9] E.A.Paz, R.N.Lacy, M.Bakhtian; The B-Lactum Antibiotics Penicillin and Cephalosporin in Perspective. Hodder Strong London, 324 **(1990)**.

[10] I.Suay, F.Arenal, F.J.Asensio, A.Basilio, M.A.Cabello, M.T.Diez, J.B.Garcia, A.Gonzalez del Val, J.Gorrochategui, P.Hernandez, F.Pelaez, M.F.Vicente; Antonie van Leeuwenhoek, **78**, 129-139 **(2000)**.

[11] E.Taskin, M.Ozturk, E.Taskin, O.Kurt; African Journal of Biotechnology, **6(24)**, 2746-2751 **(2007)**.

[12] I.Tüney, B.H.Çadirci, D.Ünal, A.Sukatar; Turk J.Biol., **30**, 171-175 **(2006)**.

[13] V.Venugopal; Marine Products for Healthcare Functional and Bioactive Nutraceutical Compounds from the Ocean. Ed. CRC Press Taylor & Francis Group. Boca Raton, London and New York, 552 **(2009)**.

THE CONTRIBUTION OF LEGUMES TO POST-FIRE REGENERATION OF *QUERCUS SUBER* AND *PINUS HALEPENSIS* FORESTS IN NORTHEASTERN ALGERIA

Farid BEKDOUCHE[1*], Mohamed SAHNOUNE[1], Fazia KROUCHI[2], Samira ACHOUR[1], Nora GUEMATI[1] & Arezki DERRIDJ[2]

RÉSUMÉ. — *Contribution des Légumineuses à la régénération après feu des forêts de* Quercus suber *et* Pinus halepensis *du Nord-Est Algérien*. — Le rôle des Légumineuses dans la régénération après feu a été largement étudié sur la rive nord de la Méditerranée, mais aucune donnée n'est disponible concernant la rive sud. Le but de cet article est d'analyser la dynamique des Légumineuses dans une subéraie et une pinède à Pin d'Alep du Nord-Est Algérien durant les premiers mois après un feu de faible intensité. Les aspects de la démographie des germinations des Légumineuses, leur contribution à la biomasse totale et la richesse spécifique sont considérés. Les deux communautés ne présentent pas de différences majeures. Toutes deux montrent des modèles démographiques similaires : les germinations de Légumineuses commencent à émerger très tôt (novembre) après le feu (octobre) et continuent à s'établir abondamment avec un premier pic en décembre et un deuxième en mars, avec un ralentissement significatif en janvier et février. La germination diminue à partir d'avril pour atteindre son minimum en mai et complètement s'arrêter en début juin. Ce modèle en deux pics plutôt qu'un seul, comme déjà rapporté, serait vraisemblablement dû au froid exceptionnel durant janvier et février 2005 (neige jusqu'au niveau de la mer) d'où un ralentissement de la germination et son décalage à mars. Huit mois après l'incendie (vers la fin juin, période où la végétation herbacée commence à sécher), la biomasse aérienne des Légumineuses affiche un maximum de 37,8 ± 1,83 g/m^2 dans la subéraie et 26,7 ± 1,63 g/m^2 dans la pinède. Sept mois après le feu, relativement aux autres familles de plantes, les Légumineuses sont floristiquement bien représentées avec 19,8 ± 1,11 % des espèces dans la subéraie et 24,3 ± 2,05 % dans la pinède, valeurs nettement supérieures à celles rapportées pour d'autres écosystèmes de type méditerranéen (3,8 - 13,3 %), probablement en raison de la localisation géographique (basse latitude) de notre aire d'étude et la pression de surpâturage qui favorise l'installation des Légumineuses.

SUMMARY. — The role of legumes in post-fire forest regeneration has been extensively studied in the northern Mediterranean basin but no published data are available concerning the southern part of the area. The aim of the present paper is to analyse the dynamics of the leguminous species in a *Q. suber* and a *P. halepensis* forests in Northeastern Algeria during the first months after a wildfire of low intensity. The aspects of seedling demography of legumes, their contribution to total biomass and species richness were considered. No major differences were detected between the two communities. They presented similar demographic patterns: legume seedlings began to emerge very early (November) after the fire (October) and continued to increase massively with a peak in December and another in March, and a significant slowdown in January and February; in April, they resumed to gradually decrease again to reach their minimum in May and completely stop in early June. This pattern with two peaks rather than only one in seedling demography was likely due to the exceptional coldness of January and February 2005 (snow down to sea level) whereby the germination slowdown and its delay to March. Eight months after the fire (towards the end of June, as the herbaceous vegetation began to dry), the above-ground biomass of legumes reached a maximum of 37.8 ± 1.83 g/m^2 in the *Q. suber* forest and 26.7 ± 1.63 g/m^2 in the *P. halepensis* one. Seven months after the

[1] University A. Mira of Bejaia, Laboratory of Ecology and Environment, Targa Ouzemmour, 06000 Bejaia, Algeria. E-mails: bekdouche_21@yahoo.fr; sahnounemohamed@yahoo.fr

[2] Department of Agronomy, Faculty of Agronomical and Biological Sciences, University M. Mammeri, PB 17, Tizi Ouzou, RP 15000, Algeria. E-mails: krfaz@yahoo.com; aderridj@yahoo.fr

* Corresponding author: Phone number: (213) 551.35.51.31; Fax: (213) 34.21.47.62

disturbance, in comparison with the other taxa, the legumes were floristically well represented with 19.8 ± 1.11 % and 24.3 ± 2.05 % of species in the Cork oak and the Allepo pine forests respectively. These richness values are far higher than those reported for other Mediterranean-type ecosystems (3.8 - 13.3 %) probably because of the geographic location (lower latitude) of our study area and the overgrazing pressure favouring installation of leguminous species.

In the Mediterranean basin and Mediterranean-type areas, vegetation is mainly shaped by the action of frequently recurring fires (Naveh, 1975; Trabaud & Lepart, 1980; Arianoutsou, 1998; Pausas *et al.*, 2008). It has been clearly demonstrated that the seed bank and the number of species are significantly reduced in the case of a long period without fire (Keeley *et al*., 2005). That's why post-fire seed germination is to be interpreted as a strategy of adaptation to fire-prone habitats of hard-coat-seeder taxa, such as *Leguminosae* and *Cistaceae* (Arianoutsou, 1998).

Legumes and *Cistaceae* recovery is accomplished by massive seedling emergence closely confined to the first post-fire years through their soil seed bank whose dormancy is broken by the impact of fire (Auld & O'Connell, 1991; Roy & Sonié, 1992; Thanos *et al*., 1992; Herranz *et al*., 1998, 1999; Ferrandis *et al.,* 1999; Baeza & Vallejo, 2006). This fast and abundant germination after fire is mainly provided by the soil seed bank (Arianoutsou, 1998), the contributions by dissemination being less significant (Luzuriaga *et al*., 2005).

While the role of *Cistaceae* is practically limited to soil protection against water erosion during the first months after fire, the leguminous species play multiple other roles represented in hampering the invasion by exogenous species and soil enrichment before an effective resumption of native woody plants (Arianoutsou & Margaris, 1981; Trabaud & Oustric, 1989; Buhk *et al*., 2007). Input of organic N from legumes can stimulate recovery of microbial activity and N cycling which can serve to facilitate community succession and promote ecosystem resilience (Johnson *et al.*, 2004; Chambers *et al*., 2007; Goergen & Chambers, 2009).

Because of all these important roles during the first months after fire, many studies have been dedicated to legumes in Mediterranean Europe (e.g. Papavassiliou & Arianoutsou, 1993; Arianoutsou & Thanos, 1996; Herranz *et al*., 1998; Kokkoris & Arianoutsou, 2000; Baeza *et al.*, 2006; De Luis *et al.*, 2006, 2008a, b). However, in the southern part of the area, no published data have hitherto been reported on the subject except on the effect of fire on flora and vegetation in general, without any specifications to legumes (Madoui *et al*., 2006; Ouelmouhoub & Benhouhou, 2007; Bekdouche *et al*., 2008).

In Algeria, the National Forest Service statistics show that, from 1963 through 2009, approximately 1.6 10^6 ha of vegetation were burnt, representing an annual average of 34 10^3 ha. *Q. suber* and *P. halepensis* forests were the most affected ecosystems especially in 1994 when about 9 10^4 ha of *Pinus* and 4 10^4 ha of *Quercus* forests were consumed by fires. To better manage the situations caused by the wildfires, every aspect of the phenomenon should be correctly understood. With the present contribution, we aim to assess the legumes dynamics during the first months (1st year) in a *Q. suber* and a *P. halepensis* forests of Northeastern Algeria after a wildfire of low intensity. The aspects analysed were (1) the seedling demography of legumes, (2) their above ground biomass and (3) the species richness. The presentation will consist on a comparison between the two communities and a confrontation of the results with those reported for other Mediterranean-type ecosystems through a review of the main relevant documentation on the topic.

MATERIALS AND METHODS

STUDY SITES

In October 2004, a wildfire devastated the *Q. suber* forest (QF) of Sidi Boudraham (36° 44' N; 5° 00' E) and the *P. halepensis* forest (PF) of Adrar Oufarnou (36° 46' N; 5° 00' E) located on the heights near the coastal city of Bejaia, *c.* 200 km east of Algiers. The previous fire in the site occurred in 2001. Given this short period of only three years,

the ecosystems would not have accumulated much fuel from which we deduce that the fire of October 2004 is to be considered of low (medium at the most) intensity. Three years old vegetation is rather young, low and loose with high rates of moisture which does not favour fire severity (Trabaud, 1980).

The dominant species are *Phillyrea latifolia, Arbutus unedo* and *Erica arborea* in QF, and *Phillyrea media* and *Erica multiflora* in PF. Average altitude of the two study sites is *c.* 380 m. According to the nearest weather station (Bejaïa), for the period 1970-2009, the average of maximum and minimum temperatures of the hottest month (August) and the coldest month (January) are 29.58 °C and 7.52 °C respectively; the annual average rainfall is 780 mm. Following Emberger (1971), the area is classified under the hot variant of the sub-humid bioclimatic stage. For the period of study (October 2004 - June 2005), the monthly precipitations and temperatures are included in Fig. 1.

Experimental design and monitoring

All the legume species were considered. To monitor the regeneration of these species, regular observations were made during the first seven months after the fire (i.e. from early November 2004 through late May 2005). For each of the two communities, counts of the different legume species seedlings were performed monthly on ten permanent 1 m^2 quadrats, regularly distributed on two equal rows. The two rows were c. 25 m apart and stretched over a 45 m long transect. Every month, the leguminous seedlings were carefully collected (destructive sampling). In both communities, the above-ground biomass was assessed on eight randomly chosen plots of 4 m^2 each. At intervals of two months (from early December 2004 through late June 2005), the above-ground biomass of a 1 m^2 quadrat was collected from each of the eight plots. The plant material was split into two categories (legumes and other non legume plant families), oven-dried at 105 °C for 24 hours and then weighed to the nearest 0.001 g. Finally, in order to determine the contribution of legumes to the richness in each forest type seven months after the fire, ten floristic relevés, made on 100 m^2 plots (Trabaud, 1980) randomly distributed over each study site, were analysed for their species composition, including all plant families (see results, Tab. I).

Species identification and nomenclature

The species were identified using the Flora of Quézel & Santa (1962-1963) and their nomenclature up-dated according to Dobignard (2010). Voucher specimens are deposited in the Herbarium of the laboratory of Ecology, Department of Biology of Organisms and Populations, University of Bejaïa (Algeria).

Statistical analysis

Repeated measures ANOVA and LSD tests were used for comparison between months within each community, and the Student t-test served for comparison between the two communities ($P = 0.05$). Prior to statistical analysis, data were tested for parametric assumptions with Kolmogorov-Smirnov test of normality and Levene's test of variance homogeneity. Outputs showed that there was no violation of parametric tests conditions, except for the biomass data witch had undergone a logarithmic transformation ($y = \log(x+1)$) to homogenize the variances. The statistical processing was performed using SPSS 10.0 for the inferences and MicroSoft Excel 2007 for the charts. The results were presented as means ± SD or means ± 2SE.

RESULTS

Emergence of legume seedlings

In November 2004, one month after the fire, the average numbers of legume seedlings were 3.5 ± 0.45 /m^2 in QF and 1.5 ± 0.40 /m^2 in PF (Fig. 1). The two communities presented similar demographic patterns. In both forests, the leguminous seedlings began to arise very early after the fire (early November) and continued to emerge until late May; a first peak was observed in December (29.2 ± 1.16 /m^2 in QF and 30.0 ± 0.94 /m^2 in PF) and another in March (21.2 ± 1.10 /m^2 in the QF and 19.2 ± 1.14 /m^2 in PF) with a significant slowdown in January and early February; in April, the germinations resumed to gradually decrease in number again to reach their minimum in May and completely stop in early June (Fig. 1).

Changes in the above-ground biomass of legumes

The above-ground biomass of legumes collected during the survey was very low during the first four months after the fire (Fig. 2). In April 2005, six months after the disturbance, the biomass values reached 11.17 ± 1.150 g/m^2 in QF and 8.580 ± 0.998 g/m^2 in PF. The maximal biomass was recorded in June 2005, eight months after the fire, with 37.84 ± 1.835 g/m^2 in the *Quercus* forest and 26.70 ± 1.634 g/m^2 in the *Pinus* one. The two communities presented similar biomass dynamics with values slightly higher in the *Quercus* forest, especially during the eighth month ($P < 0.05$). In late June, the herbaceous vegetation had completed its growth and began to dry.

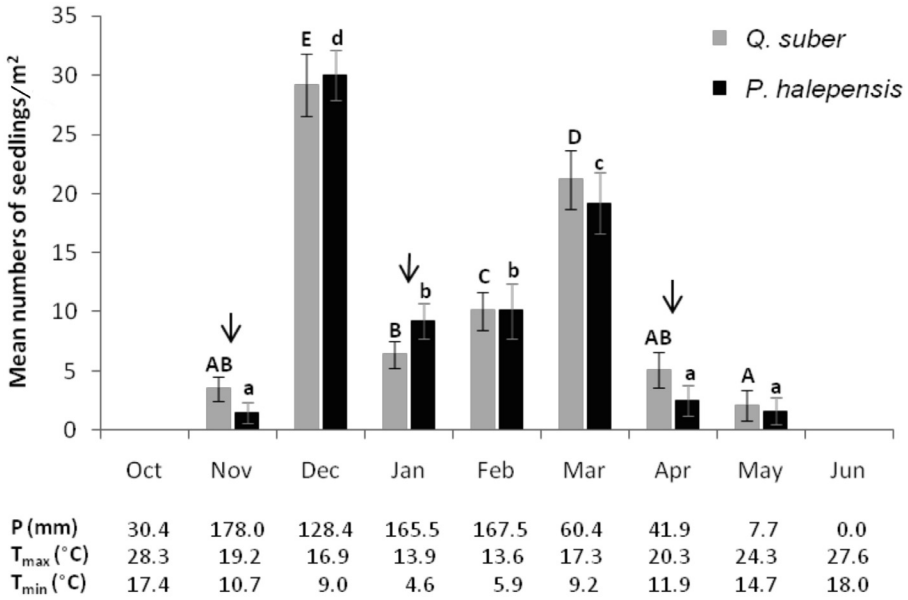


Figure 1. — Comparison of mean numbers ± 2SE of legume seedlings per m^2 after fire in a *Quercus suber* and a *Pinus halepensis* forests of Northeastern Algeria. Within each community, the values with different letters are significantly different (LSD test, $P < 0.05$). Between the two communities, only the values pointed at with arrows are significantly different (t-test, $P < 0.05$). The three lines facing the month axis are, respectively, mean precipitation values (mm), and maximal and minimal mean temperatures (°C) recorded from October 2004 through June 2005. Maximal and minimal mean temperatures of January and early February 2005 were the lowest ever registered. For the period 1978-2008 the second lowest maximal temperature was 14.5 °C (January 1981 and 1985) and the lowest minimal temperature was 5 °C (January 1983) (Data from The Meteorological Station of Bejaia, Algeria).

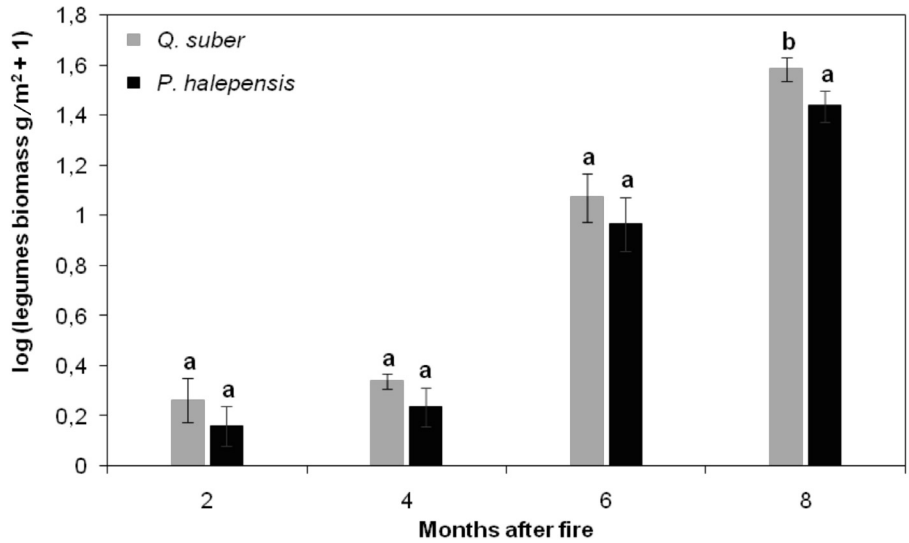


Figure 2. — After fire changes in mean ± 2SE above-ground biomass (g) of legumes in a *Quercus suber* and a *Pinus halepensis* forests of Northeastern Algeria. The two communities are significantly different at the eighth month only (t-test, $P < 0.05$).

The major difference between the two communities lies in their dominant species. In the *Quercus* forest, *Scorpiurus muricatus* dominated because of its extensive vegetative system. In the *Pinus* community, this role was performed by *Hedysarum coronarium*, similar to *S. muricatus* with its dense vegetative structure. Two other species, *Anthyllis tetraphylla* and *Ebenus pinnata*, showing high abundance, were associated with *Hedysarum coronarium*.

With regard to total biomass, the legumes constituted the most represented family during the first months after the fire in the two surveyed communities. Their maximal contribution to the total biomass was reached in November 2004 (66.8 ± 8.99 % in QF and 63.2 ± 2.96 % in PF) and their minimal relative biomass was recorded in January 2005 (13.7 ± 2.66 % in QF and 11.2 ± 1.34 % in PF) (Fig. 3).

The legume species, herbaceous in majority (only three out of thirty species were woody), dominated the two regenerating communities until woody shrubs of the other families recovered (Tab. I). The significant biomass of the remaining non legume species was essentially due to woody shoots. The weight of a shoot of *Arbutus unedo*, *Phillyrea media*, *Erica arborea* or *Pistacia lentiscus* can equal or even exceed numerous individuals of an herbaceous species. In addition, this woody biomass was strongly enhanced by the *Cistus* species that regenerated quickly and massively by seeds.

Legume species richness

The legumes were equally well represented (t-test, $P < 0.05$) in the two communities seven months after the fire, with 19.8 ± 1.11 % in the *Quercus* forest and 24.3 ± 2.05 % in the *Pinus* one. The number of legume species was 16 out of 81 in QF and 23 out of 95 in PF (Tab. I).

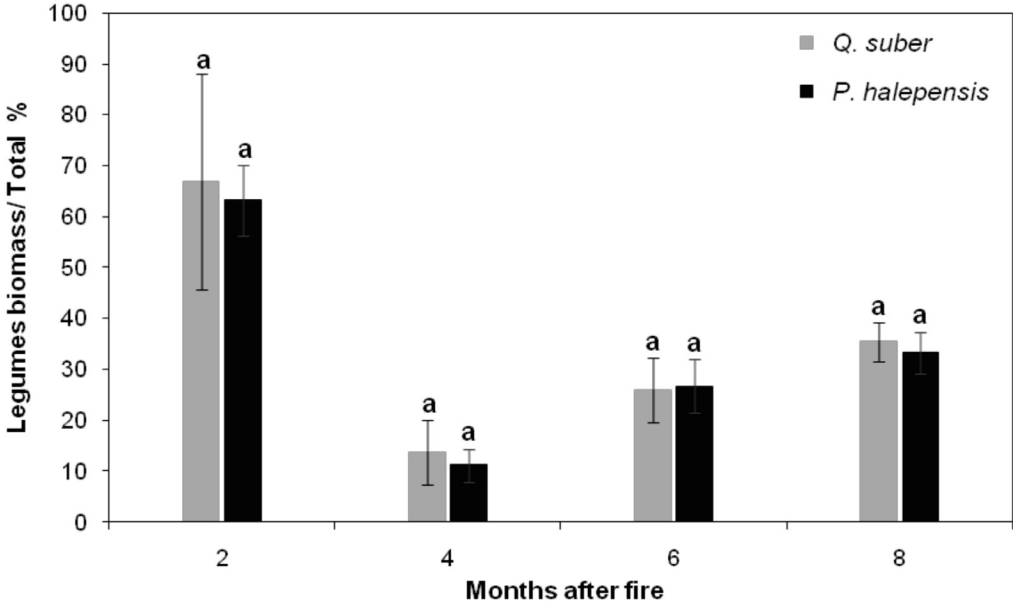


Figure 3. — Contribution of legumes to total above-ground biomass (%) over 8 months after fire (November 2004-June 2005) in a *Quercus suber* and a *Pinus halepensis* forests of Northeastern Algeria. The two communities are totally similar over the 8 months (t-test, $P < 0.05$).

TABLE I

Plant species recorded in a Quercus suber *and a* Pinus halepensis *forests of Northeastern Algeria seven months after fire. QF:* Quercus suber *forest; PF:* Pinus halepensis *forest; +: Presence; -: Absence; Fire Reg.: Post-fire regeneration strategy; R = Obligate resprouter; S = Obligate seeder; SR = Seeder-resprouter; MPh: Macrophanerophyte; NPh: Nanophanerophyte; PHl: Phanerophyte liana; Ch: Chamaephyte; H: Hemicryptophyte; G: Geophyte; Th: Therophyte.*

Species	Family	QF	PF	Fire reg.	Life form
Tripodion tetraphyllum (L.) Fourr.	Fabaceae	-	+	S	Th
Anthyllis vulneraria L.	Fabaceae	-	+	S	Th (H)
Astragalus hamosus L.	Fabaceae	-	+	S	Th
Astragalus monspessulanus L.	Fabaceae	-	+	S	H
Biserrula pelecinus L.	Fabaceae	+	-	S	Th
Calicotome spinosa (L.) Link	Fabaceae	+	+	R S	NPh
Ceratonia siliqua L.	Fabaceae	-	+	R	Ph
Coronilla juncea L.	Fabaceae	-	+	S	Ch
Ebenus pinnata Aiton	Fabaceae	-	+	S	Ch
Genista tricuspidata Desf.	Fabaceae	+	+	R S	NPh
Hedysarum coronarium L.	Fabaceae	-	+	S	Th
Hippocrepis multisiliquosa L.	Fabaceae	-	+	S	Th
Lotus corniculatus L.	Fabaceae	+	-	S	H
Lotus creticus L.	Fabaceae	-	+	S	Ch
Lotus edulis L.	Fabaceae	+	+	S	Th
Lotus ornithopodioides L.	Fabaceae	+	+	S	Th
Medicago orbicularis (L.) Bartal.	Fabaceae	+	-	S	Th
Onobrychis caput-galli (L.) Lam.	Fabaceae	-	+	S	Th
Ononis natrix L.	Fabaceae	+	+	S	Th
Ononis reclinata L.	Fabaceae	-	+	S	Th
Ononis sicula Guss.	Fabaceae	-	+	S	Th
Scorpiurus muricatus L.	Fabaceae	+	+	S	Th
Trifolium angustifolium L.	Fabaceae	+	-	S	Th
Trifolium bocconei Savi.	Fabaceae	+	-	S	Th
Trifolium campestre Schreb.	Fabaceae	+	+	S	Th
Trifolium glomeratum L.	Fabaceae	+	+	S	Th
Trifolium retusum L.	Fabaceae	+	+	S	Th
Trifolium stellatum L.	Fabaceae	-	+	S	Th
Trifolium tomentosum L.	Fabaceae	+	-	S	Th (H)
Vicia lutea L.	Fabaceae	+	-	S	Th
Anacyclus clavatus (Desf.) Pers.	Compositae	-	+	S	Th
Andryala integrifolia L.	Compositae	+	+	S	H
Bellis annua L.	Compositae	+	+	S	Th
Rhaponticoides africana (Lam.) M.V.Agab. & Gr.	Compositae	+	-	SR	H
Chrysanthemum coronarium L.	Compositae	+	+	S	Th
Filago pygmaea L.	Compositae	+	+	S	Th
Galactites elegans (All.) Soldano	Compositae	+	+	S	Th
Hypochaeris radicata subsp. radicata	Compositae	+	+	SR	H
Hypochaeris alliatae (Biv.) Gal	Compositae	-	+	S	Th
Phagnalon saxatile (L.) Cass.	Compositae	-	+	SR	Ch
Pulicaria odora (L.) Rchb.	Compositae	+	+	SR	H
Scolymus hispanicus L.	Compositae	-	+	SR	H
Sonchus oleraceus L.	Compositae	+	+	S	Th
Aira cupaniana Guss.	Poaceae	+	-	S	Th

Ampelodesmos mauritanicus (Poir.) Durand & Sch.	Poaceae	+	+	SR	H
Avena sterilis L.	Poaceae	+	-	S	Th
Brachypodium sylvaticum (Huds.) P. Beauv.	Poaceae	+	+	SR	H
Briza maxima L.	Poaceae	+	-	S	Th
Bromus hordeaceus L.	Poaceae	+	+	S	Th
Cynosurus elegans Desf.	Poaceae	+	+	S	Th
Dactylis glomerata L.	Poaceae	+	+	SR	H
Festuca paniculata (L.) Schinz & Thell.	Poaceae	+	+	SR	H
Hyparrhenia hirta (L.) Stapf	Poaceae	-	+	SR	H
Melica minuta L.	Poaceae	+	-	SR	H
Catapodium rigidum (L.) C.E.Hubb.	Poaceae	-	+	S	Th
Clinopodium vulgare L.	Lamiaceae	-	+	SR	H
Lavandula stoechas L.	Lamiaceae	+	+	S	Ch
Stachys ocymastrum (L.) Briq.	Lamiaceae	+	+	S	Th
Teucrium flavum L.	Lamiaceae	-	+	SR	Ch
Teucrium fruticans L.	Lamiaceae	-	+	SR	Ch
Thymus algeriensis Boiss. & Reut.	Lamiaceae	+	-	SR	Ch
Arbutus unedo L.	Ericaceae	+	-	R	NPh
Erica arborea L.	Ericaceae	+	+	SR	NPh
Erica multiflora L.	Ericaceae	-	+	SR	NPh
Erica scoparia L.	Ericaceae	+	+	SR	Ch
Galium lucidum All.	Rubiaceae	+	+	S	H
Galium scabrum L.	Rubiaceae	+	+	S	Th
Rubia peregrina L.	Rubiaceae	+	+	SR	NPh
Sherardia arvensis L.	Rubiaceae	+	-	S	Th
Bupleurum plantagineum Desf.	Umbelliferae	-	+	SR	NPh
Daucus carota L.	Umbelliferae	+	+	SR	Th (H)
Eryngium tricuspidatum L.	Umbelliferae	+	+	SR	H (G)
Torilis arvensis (Huds.) Link	Umbelliferae	-	+	S	Th
Cistus salviifolius L.	Cistaceae	+	+	SR	NPh
Cistus monspeliensis L.	Cistaceae	+	+	S	NPh
Fumana thymifolia (L.) Webb	Cistaceae	-	+	S	Ch
Gladiolus italicus Mill.	Iridaceae	+	+	SR	G
Moraea sisyrinchium (L.) Ker Gawl.	Iridaceae	+	-	SR	G
Iris juncea Poir.	Iridaceae	+	+	SR	G
Allium roseum L.	Alliaceae	-	+	SR	G
Allium nigrum L.	Alliaceae	+	+	SR	G
Cerastium glomeratum Thuill.	Caryophyllaceae	+	-	S	Th
Silene secundiflora Otth	Caryophyllaceae	+	+	S	Th
Convolvulus althaeoides L.	Convolvulaceae	-	+	R	H
Convolvulus tricolor L.	Convolvulaceae	-	+	S	Th
Borago officinalis L.	Boraginaceae	-	+	S	Th
Echium asperrimum Lam.	Boraginaceae	-	+	S	H
Biscutella didyma L.	Cruciferae	+	-	S	Th
Sinapis arvensis L.	Cruciferae	+	+	S	Th
Carex distachya Desf.	Cyperaceae	+	-	R	H
Carex halleriana Asso	Cyperaceae	+	+	R	H
Quercus suber L.	Fagaceae	+	-	R	MPh
Quercus coccifera L.	Fagaceae	+	+	R	NPh
Linum corymbiferum ssp. *corymbiferum*	Linaceae	+	+	S	Ch
Linum bienne Mill.	Linaceae	+	+	S	Ch

Olea europaea subsp. europaea	Oleaceae		+	R	MPh
Phillyrea latifolia L.	Oleaceae	+	+	R	NPh
Fumaria capreolata L.	Papaveraceae	+	+	S	Th
Papaver somniferum ssp. *setigerum* (DC.) Arcang.	Papaveraceae	-	+	S	Th
Anagallis arvensis L.	Primulaceae	+	+	S	Th
Cyclamen africanum Boiss. & Reut.	Primulaceae	+	+	R	G
Clematis flammula L.	Ranunculaceae	+	+	SR	Phl
Ranunculus macrophyllus Desf.	Ranunculaceae	+	-	SR	H
Crataegus monogyna Jacq.	Rosaceae	-	+	R	NPh
Rosa sempervirens L.	Rosaceae	+	+	R	Ph
Pistacia lentiscus L.	Anacardiaceae	+	+	R	NPh
Arisarum vulgare Targ. Tozz	Araceae	+	+	R	G
Asparagus acutifolius L.	Asparagaceae	+	+	SR	Phl
Simethis mattiazzii (Vand.) G. L.	Asphodelaceae	+	-	R	G
Campanula dichotoma L.	Campanulaceae	+	-	S	Th
Lonicera implexa Aiton	Caprifoliaceae	+	-	R	Phl
Euphorbia pterococca Brot.	Euphorbiaceae	+	+	S	Th
Geranium robertianum ssp. *purpureum* (Vil.) Nym.	Geraniaceae	+	-	SR	Th (H)
Lavatera arborea L.	Malvaceae	-	+	R	NPh
Myrtus communis L.	Myrtaceae	+	+	R	NPh
Pinus halepensis Mill.	Pinaceae	-	+	S	MPh
Rhamnus alaternus ssp. *alaternus*	Rhamnaceae	-	+	R	NPh
Smilax aspera L.	Smilacaceae	+	+	R	PHl
Solanum nigrum ssp. *nigrum*	Solanaceae	-	+	S	Th
Daphne gnidium L.	Thymelaeaceae	+	+	SR	NPh

DISCUSSION

Legumes seed germination after fire

Many of the dominant species in Mediterranean ecosystems after fire are resprouters (Lloret, 1998). Other species have no ability to survive severe damage to the adult and rely entirely on seed germination for regeneration (Ferrandis *et al*., 1999). Seed germination is the major mechanism adopted by legumes for their post-fire regeneration (Papavassiliou & Arianoutsou, 1993). Similarly, 90 % of the legumes inventoried during the present survey regenerated exclusively by seeds (Tab. I), resprouting was observed only for *Calicotome spinosa, Ceratonia siliqua* and *Genista tricuspidata*. Despite their perennial habit, herbaceous species such as *Anthyllis vulneraria, Astragalus monspessulanus, Coronilla juncea, Lotus creticus, Lotus corniculatus* and *Ononis natrix* regenerated only by seeds. Why one or the other strategy of post-fire regeneration is favoured is not yet clear (Férnandez-Santos *et al*., 2004). The germination of legumes of the burnt areas is induced by heat shock (Auld & O'Connell, 1991; Keeley & Bond, 1997; Hanley & Fenner, 1998; Herranz *et al*., 1998) or strong insolation of the stripped areas (Bazzaz, 1998). In the Sydney region, Auld & Denham (2006) have estimated that about 15 % of the fire-prone flora has dormancy broken by heat shock. Generally, temperatures ranging between 60-150 °C affect germination positively (De Luis *et al*., 2005b). For some tropical legumes, temperatures of 80-100 °C significantly increase seed germination (Williams *et al*., 2004). For some species, this germination is stimulated by the smoke generated during the combustion of vegetation (Crosti *et al*., 2006; Dayamba *et al*., 2008)). The effect of thermal shock and smoke on seed germination of some taxa of fire-prone vegetation have been shown to be additive and generate a lifting of dormancy of the soil seed bank (Roy & Sonié, 1992; Thanos *et al*., 1992; Doussi & Thanos, 1994; Pérez-Fernandez & Rodriguez-Echeverria, 2003; Thomas *et al*., 2003; Crosti *et al*., 2006; Scott *et al*., 2010). In the Sydney

region, approximately half of the fire-prone flora has seed dormancy broken by the interaction of heat and smoke (Auld & Denham, 2006). Smoke promotes germination increase up to a maximum and then a decrease is observed because high concentrations or duration of smoking become germination inhibitory (Light *et al*., 2002) or seed lethal (Keeley & Fotheringham, 1998). In the same way, heat intensity or duration stimulate seed germination increasingly until an optimum and then a decrease is observed because excessive thermal shocks cause seed mortality (Auld & O'Connell, 1991; Baeza & Vallejo, 2006). High temperatures affect seed viability and consequently germination rates (Lloret, 1998). In general, the responses to heat and smoke are variable. Keeley & Bond (1997) have noted that, in some cases, seed germination behaviour is phylogenetically inherited as in legume species where heat will commonly enhance germination. Crosti *et al*. (2006), in a comparison of several studies from different regions with Mediterranean-type vegetation, have concluded that there are both phylogenetic and biogeographical patterns in post-fire regeneration behaviour.

In our case, germination of legumes from the soil seed banks of the two investigated forests (QF and PF) began very early after the disturbance. The first germinations were observed after the first rains in November 2004 (Fig. 1). The seedling demography showed a bimodal pattern with a major peak in December and a second smaller one in March. This pattern with two peaks rather than only one as it has been already observed during the first season after fire (Arianoutsou, 1998; De Luis *et al*., 2008a, b) was likely due to the exceptional meteorological conditions (Fig. 1); January and February 2005 in our study area were colder than usual (snow down to sea level for a prolonged period), so germination was slowed down and delayed to March, hence the two peaks.

In a *Ulex parviflorus* dominated gorse shrubland of Eastern Spain observed over two consecutive years after a fire experimentally set in October 1996, De Luis *et al.* (2008a) recorded that seedling demography of *Leguminosae* and *Cistaceae* shows a monomodal pattern in each year with a first peak in the autumn just after the fire (1st year) and a second the following autumn (2nd year), without any peak in spring; but the *Cistaceae* dominated during the first year and the *Leguminosae* during the second year. These observations differ from ours not only by the absence of the spring peak explained above but also by the domination of *Cistaceae* before *Leguminosae* during the first year after fire.

Legume species dominated in our communities during the first year after fire because most of them are annual herbs (see Tab. I), thus they could quickly reconstruct their seed bank. Contrarily, the *Cistus* species are woody plants reaching maturity after two or three years (Roy & Sonié, 1992; Tavşanoğlu & Gurkan, 2005; Duguy & Vallejo, 2008), so, they could not sufficiently reconstitute their seed bank since the previous fire of 2001 (Three years time). Survival of *Cistus* species which reproduce only by seed may be seriously threatened by fires occurring before they have produced and accumulated seeds (Trabaud, 1980). Duguy & Vallejo (2008) have also suggested that frequent fire recurrences with repeated short intervals between fires might cause a reduction of woody species cover, probably due to a decrease of the seed bank replenishment, and increase of the presence of herbaceous species.

Furthermore, a low severity of fires stimulates mainly germination of seeds near the soil surface (De Luis *et al.*, 2008a), but the depth of burial depends essentially on the seed size (Bond *et al.*, 1999). The *Cistus* seeds being lighter than those of the woody legumes of the gorse shrubland studied by De Luis *et al.* (2008a) would occur at the superficial level, while those of the woody legumes would burrow deep in the soil. Our results seem to agree with this pattern too, because the seeds of herbaceous legumes, lighter than those of *Cistus* species (personal observation), would likely remain nearer to the soil surface which would favour their massive germination after a low intensity fire. As a rule, to accurately evaluate the effect of any factor on post-disturbance vegetation, the whole set of interacting factors must be considered, including pre-disturbance vegetation (determined by past disturbance regime), disturbance characteristics, spatial factors and post-disturbance conditions (Duguy & Vallejo, 2008).

Legumes biomass and species richness

Despite their relatively low contribution to the species richness (20-24 % in May 2005), the leguminous species participated with a significant proportion to the total biomass of the two

communities (up to 67 % in November 2004) in comparison with the whole of the other flora (especially *Cistaceae*, *Poaceae* and woody sprouts of *Erica arborea*, *E. multiflora*, *Phillyrea latifolia*, *Pistacia lentiscus*, *Myrtus communis* and *Arbutus unedo*; tree seedlings contributing very little to biomass). The relatively low number of legume species was compensated by their remarkable abundance and dominance.

Seven months after the disturbance, the number of legume species was significant for both communities (Tab. I), resulting from seed germination stimulated by the fire. This enhancement in legumes germination densities is likely due to a heat-induced rupture of the hard coat of their seeds (Doussi & Thanos, 1994). Similar observations have been made for *Cistaceae* species (Arianoutsou & Margaris, 1981; Trabaud & Oustric, 1989; Roy & Sonié, 1992; Ferrandis *et al*., 1999). The Mediterranean-type ecosystems throughout the globe show that legume richness after fire ranges from 3.8 % in the Chilean matorral (Rundel, 1981) to 13.3 % for the sclerophyll forests of South Australia (Specht, 1972). In a *Pinus halepensis* forest of Greece, legumes representing approximately 9 % of the flora of a mature stand have more than doubled immediately after a fire (Kazanis & Arianoutsou, 1996). Our results revealed that the legumes proportions were 19.8 ± 1.11 % and 24.3 ± 2.05 % of species in QF and PF respectively. These relatively higher richness values were probably due to the fact that our floristic samples were done during the earlier stages after the fire in the two communities shaped by the overgrazing pressure (Arianoutsou & Thanos, 1996) and situated in a climatic region (lower latitude) favouring the establishment of legumes (Garcillán *et al.*, 2003). Legumes are very present in Mediterranean-type ecosystems because of their morphological and physiological adaptations and their various dispersal modes (endozoochory, autochory, etc.) (Arianoutsou & Thanos, 1996). Their adaptive mechanisms are related to nitrogen fixation through development of nitrogen fixing root nodules in soils with limited nitrogen availability and formation of mycorrhizal associations that enhance phosphorus uptake (Goergen & Chambers, 2009). One of the particularities of the Mediterranean region is the high human pressure particularly through overgrazing which favour herbaceous legumes characterized by their potential adaptation to increase their seed germination after fire (Arianoutsou & Thanos, 1996).

Soon after the fire, the legumes achieved a high density and a rapid increase in biomass (Figs. 1 & 2). Because of these demographic strategies, they present great affinities with the initial stages of post-fire successions (Arianoutsou & Margaris, 1981).

Perturbations such as agricultural activity or fire provide space, nutrients and light for early successional species (mostly therophytes) which are low in the competitive hierarchy but have a strong colonization capacity in open spaces (Duguy & Vallejo, 2008). The legume species, with their massive germinations as a result of the conditions created by fire, lead the way to species of mature stages of the succession by improving the soil fertility which may favour ecosystem richness.

Roles of legumes in plant formations dynamics

Legumes play multiple roles in the dynamics of plant formations. Above all, they protect soil against water erosion during the first months after fire because the woody species in burnt communities recover more slowly by resprouting. With wildfire occurrence and strong rainfall, changes in soil properties have been reported as a major reason for accelerated erosion (Pardini *et al.*, 2004; De Luis *et al.*, 2005a). The loss of topsoil and decreased regeneration of vegetation may inhibit the formation of soil organic matter and the establishment of plant species, thereby favouring the intensification of the erosive processes (Andreu *et al*., 1996). The legumes share the function as soil protectors against water erosion with the *Cistus* species (Trabaud & Oustric, 1989).

The herbaceous species play a more important role in protecting soil against soil erosion because of their rapid establishment soon after fire through germination or resprouting (Beyers, 2009). This is why these species are preferred in burnt landscapes rehabilitation programmes. Perennial resprouter grasses are usually used because of their faster growth and higher rate of survival; they usually dominate the first stages of post-fire recovery in Mediterranean ecosystems (De Luis *et al.*, 2005a). However, successful establishment of seeded grasses displaces native

herbaceous vegetation, particularly annuals, and can reduce the survival rates of tree seedlings (Beyers, 2004). Seeding with herbaceous native species can provide additional benefits such as preventing invasion by undesirable plants. Several studies suggest that relatively high cover of perennial herbaceous species can increase the ecosystems resilience after fire and increase resistance to invasion by exotic species (Chambers *et al.*, 2007). The rapid and massive recovery of herbaceous legume species may better favour the installation of tree seedlings because they are not as competitive as the aggressive non-native perennial grass species (*Bromus inermis, Dactylis glomerata, Festuca ovina*) generally used in post-fire rehabilitation (Beyers, 2009).

The legumes are the only plants replacing quickly the nitrogen lost via volatilization during fires by inputs from nitrogen fixers after fire (Arianoutsou & Thanos, 1996; Boring *et al*., 2004; Johnson *et al*., 2004; Goergen & Chambers, 2009). Johnson *et al*. (2005) have estimated that post-fire nitrogen gain equals or even exceeds total nitrogen losses due to fire and salvage logging in a Sierran forest.

After fire, legumes are able to replace N lost due to fire and facilitate community succession (seedling establishment and plant growth) and stability over time. Furthermore, input of organic N from legumes can stimulate recovery of microbial activity and N cycling, this can serve to promote resilience of native ecosystems, but also may create an avenue for invasion (Goergen & Chambers, 2009). In addition, the legumes contribute to the enrichment of soil with organic matter after their senescence because they represent a significant proportion of the biomass. From this perspective, the dominant leguminous species *Hedysarum coronarium* and *Scorpiurus muricatus* may play an effective role in landscape rehabilitation after fire because of their density and spreading vegetative systems for soil protection against erosion and their high biomass and numerous nodules for soil enrichment.

CONCLUSION

The present results constitute the first data on the role of legumes in the regeneration of plant communities after wildfire in the southern part of the Mediterranean basin. No major differences were observed between the two studied communities except that numbers of seedlings and biomass values were slightly higher in the *Q. suber* forest (dominated by *Scorpiurus muricatus*) but richness more important in the *P. halepensis* one (dominated by *Hedysarum coronarium*). In both ecosystems, seedling demography showed two peaks, a major one in December, two months after the fire of low intensity, and a second smaller one in March. This pattern with two peaks rather than only one in seedling demography was likely due to the meteorological conditions during January and February 2005 that were colder than usual whereby germination was slowed down and delayed to March. The legumes richness (19.8 and 24.3 % of species in the *Q. suber* and the *P. halepensis* forests respectively) was far higher than reported for other Mediterranean-type ecosystems (3.8-13.3 %) probably because of the geographic location (lower latitude) of our study area and the overgrazing pressure favouring installation of leguminous species.

ACKNOWLEDGEMENTS

We thank very much Pr. Mokrane Iguerouada (Department of Biology of Organisms and Populations, Abderrahmane Mira University of Bejaïa, Algeria), Mr. Mokhtar Harrat (Cybercafé Harrat, Targa Ouzemmour, Bejaïa, Algeria) for their help with the charts and the three anonymous reviewers upon whose comments the first versions of the manuscript have been much improved.

REFERENCES

ANDREU, V., RUBIO, J.L., FORTEZA, J. & CERNI, R. (1996). — Post-fire effects on soil properties and nutrient losses. *Int. J. Wildland Fire,* 6: 53-58.

ARIANOUTSOU, M. (1998). — Aspects of demography in post-fire Mediterranean plant communities of Greece. Pp 273-295 *in*: P.W. Rundel, G. Montenegro & F. Jaksic (eds). *Landscape degradation in mediterranean-type ecosystems*. Ecological studies 136. Springer-Verlag, Berlin/Heidelberg.

ARIANOUTSOU, M. & MARGARIS, N.S. (1981). — Early stages of regeneration after fire in a phryganic ecosystem (east Mediterranean). I. Regeneration by seed germination. *Biol. Ecol. Méditerr*., 8: 119-128.

ARIANOUTSOU, M. & THANOS, C.A. (1996). — Legumes in the fire-prone Mediterranean regions: an example from Greece. *Int. J. Wildland Fire,* 6: 77-82.

AULD, T.D. & DENHAM, A.J. (2006). — How much seed remains in the soil after a fire? *Plant Ecol*., 187: 15-24.

AULD, T.D. & O'CONNELL, M.A. (1991). — Predicting patterns of post-fire germination in 35 eastern Australian Fabaceae. *Aust. J. Ecol*., 16: 53-70.

BAEZA, M.J. & VALLEJO, V.R. (2006). — Ecological mechanisms involved in dormancy breakage in *Ulex parviflorus* seeds. *Plant Ecol*., 183: 191-205.

BAEZA, M.J., RAVENTOS, J., ESCARRÉ, A. & VALLEJO, V.R. (2006). — Fire risk and vegetation structural dynamics in Mediterranean shrubland. *Plant Ecol*., 187: 189-201.

BAZZAZ, F.A. (1998). — *Plants in changing environments: Linking physiological, population, and community ecology*. Cambridge University Press, Cambridge.

BARCLAY, A.D., BETANCOURT, J.L. & ALLEN, C.D. (2004). — Effects of seeding ryegrass (*Lolium multiflorum*) on vegetation recovery following fire in a ponderosa pine (*Pinus ponderosa*) forest. *Int. J. Wildland Fire,* 13: 183-194.

BEKDOUCHE, F., DERRIDJ, A. & KROUCHI, F. (2008). — Évolution après feu de la composition floristique de la subéraie de Mizrana (Tizi-Ouzou, Algérie). *Sciences & Technologie C*, 28: 19-29.

BEYERS, J.L. (2004). — Post-fire seeding for erosion control: Effectiveness and impacts on native plant communities. *Conserv. Biol.*, 18: 947-956.

BEYERS J.L. (2009). — Non-native and native seeding. Pp 321-336 in: A. Cerda & P.R. Robichaud (eds). *Fire effects on soil and restoration strategies. Land reconstruction and management series, vol.5*, Sciences Publishers, Enfield, Jersey, Plymouth.

BOND, W.J., HONIG, M. & MAZE, K.E. (1999). — Seed size and seedling emergence: an allometric relationship and some ecological implications. *Oecologia*, 120: 132-136.

BORING, L.R., HENDRICKS, J.J., WILSON, C.A. & MITCHELL, R.J. (2004). — Season of burn and nutrient losses in a longleaf pine ecosystem. *Int. J. Wildland Fire,* 13: 443-453.

BUHK, C., MEYN, A. & JENTSCH, A. (2007). — The challenge of plant regeneration after fire in the Mediterranean Basin: scientific gaps in our knowledge on plant strategies and evolution of traits. *Plant Ecol.*, 192: 1-19.

CHAMBERS, J.C., ROUNDY, B.A., BLANK, R.R., MEYER, S.E. & WHITTAKER, A. (2007). — What makes Great Basin sagebrush ecosystems invisible by *Bromus tectorum*? *Ecol. Monogr.*, 77: 117-145.

CROSTI, R., LADD, P.G., DIXON, K.W. & PIOTTO, B. (2006). — Post-fire germination: The effect of smoke on seeds of selected species from the central Mediterranean basin. *For. Ecol. Manage*., 221: 306-312.

DAYAMBA, S.D., TIGABU, M., SAWADOGO, L. & ODEN, P. (2008). — Seed germination of herbaceous and woody species of the Sudanian savanna-woodland in response to heat shock and smoke. *For. Ecol. Manage.*, 256: 462-70.

DE LUIS, M., RAVENTOS, J. & GONZÁLEZ-HIDALGO, J.C. (2005a). — Fire and torrential rainfall: effects on seedling establishment in Mediterranean gorse srublands. *Int. J. Wildland Fire*, 14: 413-422.

DE LUIS, M., RAVENTOS, J. & GONZALES-HIDALGO, J.C. (2005b). — Factors controlling seedling germination after fire in Mediterranean gorse shrublands. Implications for fire prescription. *J. Environ. Manage*., 76: 159-166.

DE LUIS, M., RAVENTOS, J. & GONZALEZ-HIDALGO, J.C. (2006). — Post-fire vegetation succession in Mediterranean gorse shrublands. *Acta Oecol.*, 30: 54-61.

DE LUIS, M., RAVENTOS, J., WIEGAND, T. & GONZÁLEZ-HIDALGO, J.C. (2008a). — Temporal and spatial differentiation in seedling emergence may promote species coexistence in Mediterranean fire-prone ecosystems. *Ecography*, 31: 620-629.

DE LUIS, M., VERDU, M. & RAVENTOS, J. (2008b). — Early to rise makes a plant healthy, wealthy, and wise. *Ecology*, 89: 3061-3071.

DOBIGNARD, A. (2010). — *Base de données de la flore d'Afrique du Nord (BDFAN)*. http://www.tela-botanica.org/page:bdafn

DOUSSI, M.A. & THANOS, C.A. (1994). — Post-fire regeneration of hardseeded plants: ecophysiology of seed germination. Pp 1035-1044 *in*: D.X. Viegas (ed.). *Proceedings of the 2nd International Conference on Forest Fire Research.* Coimbra, Portugal.

DUGUY, B. & VALLEJO, V.R. (2008). — Land-use and fire history effects on post-fire vegetation dynamics in eastern Spain. *J. Veg. Sci.*, 19: 97-108.

EMBERGER, L. (1971). — Considérations complémentaires au sujet des recherches bioclimatologiques et phytogéographiques – écologiques*.* Pp 291-301 *in*: L. Emberger (ed.). *Travaux de Botanique et d'Écologie*. Masson et Cie, Paris.

FERNANDEZ-SANTOS, B., MARTINEZ, C., GARCIA, J.A. & PUERTO, A. (2004). — Postfire regeneration in *Cytisus oromediterraneus*: sources of variation and morphology of the below-ground parts. *Acta Oecol*., 26: 149-156.

FERRANDIS, P., HERRANZ, J.M. & MARTINEZ-SANCHEZ, J.J. (1999). — Effect of fire on hard-coated Cistaceae seed banks and its influence on techniques for quantifying seed banks. *Plant Ecol.,* 144: 103-114.

GARCILLÁN, P.P., EZCURRA, E. & RIEMANN, H. (2003). — Distribution and species richness of woody dryland legumes in Baja California, Mexico. *J. Veg. Sci.*, 14: 475-486.

GOERGEN, E.M. & CHAMBERS, J.C. (2009). — Influence of a native legume on soil N and plant response following prescribed fire in sagebrush steppe. *Int. J. Wildland Fire*, 18: 665-75.

HANLEY, M.E. & FENNER, M. (1998). — Pre-germination temperature and the survivorship and onward growth of Mediterranean fire-following plant species. *Acta Oecol*., 19: 181-187.

HERRANZ, J.M., FERRANDIS, P. & MARTINEZ-SANCHEZ, J.J. (1998). — Influence of heat on seed germination of seven Mediterranean *Leguminosae* species. *Plant Ecol*., 136: 95-103.

HERRANZ, J.M., FERRANDIS, P. & MARTINEZ-SÁNCHEZ, J.J. (1999). — Influence of heat on seed germination of nine woody *Cistaceae* species. *Int. J. Wildland Fire*, 9: 173-182.

JOHNSON, D.W., MURPHY, J.F., SUSFALK, R.B., CALDWELL, T.G., MILLER, W.W., WALKER, R.F. & POWERS, R.F. (2005). — The effects of wildfire, salvage logging, and post-fire N – fixation on the nutrient budgets of a Sierran forest. *For. Ecol. Manage*., 220: 155-165.

JOHNSON, D.W., SUSFALK, R.B., CALDWELL, T.G., MURPHY, J.F., MILLER, W.W. & WALKER, R.F. (2004). — Fire effects on carbon and nitrogen budgets in forests. *Water Air Soil Poll.: Focus,* 4: 263-275.

KAZANIS, D. & ARIANOUTSOU, M. (1996). — Vegetation composition in a post-fire successional gradient of *Pinus halepensis* forests in Attica, Greece. *Int. J. Wildland Fire,* 6: 83-91.

KEELEY, J.E. (2004). — Ecological impacts of wheat seeding after a Sierra Nevada wildfire. *Int. J. Wildland Fire,* 13: 73-78.

KEELEY, J.E. & BOND, W.J. (1997). — Convergent seed germination in South African fynbos and California chaparral. *Plant Ecol*., 133: 153-167.

KEELEY, J.E. & FOTHERINGHAM, C.J. (1998). — Smoke-induced seed germination in California chaparral. *Ecology,* 79: 2320-2336.

KEELEY, J.E., PFAFF, A.H. & SAFFORD, H.D. (2005). — Fire suppression impacts on post-fire recovery of Sierra Nevada chaparral shrublands. *Int. J. Wildland Fire,* 14: 255-265.

KOKKORIS, I. & ARIANOUTSOU, M. (2000). — Flowering and fruiting phenology of four herbaceous species of *Leguminosae* in a burned *Pinus halepensis* forest of Attica, Greece. *J. Mediterr. Ecol.,* 1: 193-200.

LIGHT, M.E., GARDNER, M.J., JAGER, A.K. & VAN STADEN, J. (2002). — Dual regulation of seed germination by smoke solutions. *Plant Growth Regul*., 37: 135-141.

LLORET, F. (1998). — Fire, canopy cover and seedling dynamics in Mediterranean schrubland of northeastern Spain. *J. Veg. Sci*., 9: 417-430.

LUZURIAGA, A.L., ESCUDERO, A., OLANO, J.M. & LOIDI, J. (2005). — Regenerative role of seed banks following an intense soil disturbance. *Acta Oecol*., 27: 57-66.

MADOUI, A., GEHU, J.M. & ALATOU, D. (2006). — L'effet du feu sur la composition des pinèdes de *Pinus halepensis* Mill. dans le nord de la forêt de Boutaleb, Algérie. *Ecol. Mediterr.*, 32: 5-13.

NAVEH, Z. (1975). — The evolutionary significance of fire in the Mediterranean region. *Vegetatio*, 29: 199-208.

OUELMOUHOUB, S. & BENHOUHOU, S. (2007). — Evolution floristique des suberaies incendiées dans la région d'El Kala (nord-est Algérie). *Ecol. Mediterr*., 33: 85-94.

PAPAVASSILIOU, S. & ARIANOUTSOU, M. (1993). — Regeneration of the leguminous herbaceous vegetation following fire in a *Pinus halepensis* forest of Attica, Greece. Pp 19-125 *in*: L. Trabaud & R. Prodon (eds). *Fire in Mediterranean Ecosystems.* Ecosystems Research Report n° 5, Commission of the European Communities.

PARDINI, G., GISPERT, M. & DUNJO, G. (2004). — Relative influence of wildfire on soil properties and erosion processes in different Mediterranean environments in NE Spain. *Sci. Total Environ*., 328: 237-246.

PAUSAS, J.G., LLOVET, J., RODRIGO, A. & VALLEJO, V.R. (2008). — Are wildfires a disaster in the Mediterranean basin? -A review. *Int. J. Wildland Fire*, 17: 713-723.

PÉREZ-FERNANDEZ, M.A. & RODRIGUEZ-ECHEVERRIA, S. (2003). — Effect of smoke, charred wood and nitrogenous compounds on seed germination of 10 species from woodland in central-western Spain. *J. Chem. Ecol*., 29: 237-251.

QUÉZEL, P. & SANTA, S. (1962-1963). — *Nouvelle flore de l'Algérie et des régions désertiques méridionales.* 2 vols, CNRS (eds). Paris.

ROY, J. & SONIÉ, L. (1992). — Germination and population dynamics of *Cistus* species in relation to fire. *J. Appl. Ecol*., 29: 647-655.

RUNDEL, P.W. (1981). — The Matorral Zone of Central Chile. Pp 175-201 *in*: F. Di Castri, D.W. Goodall & R.L. Specht (eds). *Mediterranean-Type Shrublands*. Elsevier, Amsterdam.

SCOTT, K., SETTERFIELD, S., DOUGLAS, M. & ANDERSEN, A. (2010). — Soil seed banks confer resilience to savanna grass-layer plants during seasonal disturbance. *Acta Oecol.*, 36: 202-210.

SPECHT, R.L. (1972). — *The Vegetation of South Australia*. A.B. James Government printer, Adelaide.

TAVŞANOĞLU, Ç. & GURKAN, B. (2005). — Post-fire dynamics of *Cistus* spp. in a *Pinus brutia* forest. *Turk. J. Bot.*, 29: 337-343.

THANOS, C.A., GEORGHIOU, K., KADIS, C. & PANTAZI, C. (1992). — Cistaceae: a plant family with hard seeds. *Israel J. Bot*., 41: 251-263.

THOMAS, P.B., MORRIS, E.C. & AULD, T.D. (2003). — Interactive effects of heat shock and smoke on germination of nine species forming soil seed banks within the Sydney region. *Austral Ecol*., 28: 674-683.

TRABAUD L. (1980). — *Impact biologique et écologique des feux de végétation sur l'organisation, la structure et l'évolution de la végétation des zones de garrigues du Bas-Languedoc*. Thèse de Doctorat d'État en Sciences Naturelles, Université des Sciences et Techniques du Languedoc, Montpellier.

TRABAUD, L. & LEPART, J. (1980). — Diversity and stability in garrigue ecosystems after fire. *Vegetatio*, 43: 49-57.

TRABAUD, L. & OUSTRIC, J. (1989). — Comparaison des stratégies de régénération après incendie chez deux espèces de cistes. *Rev. Ecol. (Terre Vie)*, 44: 207-219.

WILLIAMS, P.R., CONGDON, R.A., GRICE, A.C. & CLARKE, P.J. (2004). — Soil temperature and depth of legume germination during early and late dry season fires in a tropical eucalypt savanna of north-eastern Australia. *Austral Ecol*., 29: 258-263.

Published on: 1st August 2011

MICROPROPAGATION OF ALGERIAN JUVENIL ROOTSTOCKS *CITRUS* SPECIES

FADILA MAIZA BENABDESSELAM[1], BACHRA KHETTAL[1] AND FATIHA BEDJOU[1]

[1] LABORATORY OF PLANT BIOTECHNOLOGY AND ETHNO BOTANY- DEPARTMENT OF PHYSICAL AND CHEMICAL BIOLOGY(BPC)- FSNV- UNIVERSITY OF BÉJIAA- TERGA OUZEMOUR-06000- ALGERIA- DZ.

fadilamaiza@yahoo.fr

ABSTRACT:

Juvenil segments from three Algerian Citrus species, Pineaple orange(C*itrus sinensis*), Eureka lemon (*Citrus limon*) and Carvallah mandarin(*Citrus deliciosa*) , were cultured on eight different media with varying concentrations of phytohormones (auxins and cytokinins) ranged 0 to 2 mg l^{-1}. Best results were obtained with Murashige and Skoog basal medium (MS) supplemented with benzylaminopurine at concentration 1 mg l^{-1} and indole-3-acetic acid, or 2,4-dichlorophenoxyacetic acid at concentrations 0.1 mg l^{-1} in respectively orange and lemon with the first auxin and orange and mandarin with the second one. With mandarin explants a high rate of shoots multiplication was recorded also with Miller medium supplemented with 1-naphthaleneacetic acid at 0.1 mg l^{-1} and good results were observed with limon explants using embryogen medium of Halperin (MSH) without growth factors . Best rooting was obtained, for orange and mandarin, utilizing the basal MS medium MS0/0 (without growth factors) and with MS medium supplemented with 5% of active carbon, BN and BN_{100} media for lemon. After transplanting, the young plantlets were raised in a hotbed frame. Only a limited number of the young plantlets were lost (2%) when we used the above mentioned technique for transferring the plantlets from "*vitro*" to the soil mixture.

[1] Abreviations:

BAP: 6 benzylamino purine ; IAA: indole-3-acetic acid ; IBA: indole-3-butiric acid; NAA: 1-naphthaleneacetic acid ; 2,4-D: 2,4-dichlorophenoxyacetic acid; FYM: decomposed FarmYard Manure

KEY WORDS: Micropropagation, Citrus, Organogenesis, Phytohormones.

INTRODUCTION:

Algeria has a *Citrus* varietal collection of 178 varieties, which represents inestimable genetic resources (Kerboua 2000). Plant tissue culture relies on the fact that many plant cells have the

ability to regenerate a whole plant (Totipotency). Woody species (trees and shrubs) are far more difficult to clone *in vitro* than herbaceous plants (Kalinina and Brown 2007)

Plant regeneration in *citrus* species has been reported by various workers (Singh *et al*., 1994; Parthasarathy *et al*, 2001).In spite of micro-propagation of citrus genotypes reported by several workers, a very few reports of *in vitro* multiplication of citrus rootstocks such as Cleopatra mandarin and Rough lemon are available in the literature (Sharma *et al* 2009).

We report here the *in vitro* conditions required for plant regeneration through direct and indirect organogenesis of citrus plants, with the focus on the effect of various Auxins, IAA(indole-3-acetic acid) , IBA(indole-3-butiric acid), 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA (1-naphthaleneacetic acid) ,in combination with 6 benzylamino purine (BAP) and Kinetin (Kin) at various concentrations.

MATERIALS & METHODES:

Culture of seedlings:

Three Citrus species fruits, Pineaple orange, Eureka lemon and Carvallah mandarin, were provided by the INRA Station of Oued Ghir (Béjaia- Algeria). Fruits were disinfected in ethanol 95° during 15 min. The seeds were taken aseptically, dried 24 H in sterile area and stocked in Petri Disch (20 seeds in a Disch). First and second teguments were removed and seeds were disinfected then by immersion in diluted commercial sodium hypochlorite containing 48% active chlorine (1:10 v:v) for 10 min and rinsed in water. Seeds were sown under germ-free conditions in laminarflow , in Murashige and Skoog basal medium(1962) supplemented with Bacto-agar(7 g l^{-1}) to germinate in light-dark conditions (16 h of light followed by 8 h of darkness). Light (2500 lux) was provided by Sylvania Grolux fluorescent tubes. Temperature was 25 ±1 °C during the day and 19±1 °C at night. Relative humidity was maintained between 50 and 75%.

Obtention and culture of explants:

8 to 10 weeks old seedlings (10 to 12 cm height), were dissected in explants of 1 to 2 cm lengh. Nodal segments, each consisting of a 2 cm-long node with its axillary bud, Young white to yellowish leaves, roots and cotyledons were cut into segments 1 cm in length. Explants were placed in horizontal position (Preliminary tests showed us that horizontal position was the more suitable for proliferation) on the nutritive medium supplemented with Bacto-agar (8 g L^{-1}) and various concentrations of growth factors in the same culture conditions that germination seeds ones.

Medium composition:

Eight media were tested, the basal medium MS of Murashige and Skoog(1962) , P7 and modified P7 (Faure 1990) , MSH medium of Halperin (1964) , MBR medium (Rangan *et al* 1969) , BN_0 and BN_{100} media (Martin *et al* 1979) and Miller medium (Anstis and Northorte 1974). (table1)

The modified P7 media contains the same components but the concentration of 6-BAP was different (0,1mg $l^{-}1$).

In Vitro Propagation:

Instruments such as scalpels, scissors, and forceps used for cutting and transfer of plant pieces are sterilized first by dry heat and by alcohol dip and flaming during manipulations. Explants were aseptically inoculated on various culture media under white light. One explant was inoculated per glass tube and about 200 explants were kept in every set of experiment. All cultures were incubated at 25±20 °C in culture room under 16/8 hrs cycles of light (200 Lux) and darkness. To improve rooting of shoots obtained by micropropagation, proliferated shoots (3-4cm) were inoculated individually on MS media alone or supplemented with active carbon (5%) and or various concentrations of IBA. Regenerated shoots were also subcultured on BN_0 and BN_{100} media which are known to produce the best root development in *Vicia faba* shoots (Martin *et al* 2003).

After 30 days, the rooted plantlets were washed thoroughly and transferred into small pots containing potting media prepared of soil, sand and FYM (decomposed FarmYard Manure) in the ratio of 1:1:1 by volume, and kept at 25±10C. (Sharma *et al* 2009).

RESULTS & DISCUSSION:

Indirect organogenesis :

Effect of the various media and treatments on callus formation from the different explants is reported on Table 2. Maximum callus formation (95.75% and 78.75% respectively) was observed on MS media supplemented with 1mg l^{-1} of BAP and 0.1 mg $l^{-1}$of 2,4-D in orange and mandarin explants.(Fig 1).With the MBR medium callus formation was observed only with mandarin explants (73.33%) and maximum callus formation (100%) was recorded with the stem explants .The interaction between optimum media and explants nature was not significant. The difference in percent callus formation between the three species of *Citrus* might be due to the plant genotype (Sherma *et al* .2009).Ratio of auxin and cytokinin concentrations in culture media is of a great importance in the establishment of *Citrus* callus cultures (Chatuverdi and Mitra 1974).

Indirect caulogenesis (buds formation) (Fig 2) varied with the plant species, the explants nature and the medium composition and treatment (Table 3).Best rates recorded with orange explants were observed with MS medium supplemented with BAP 1mg l^{-1} and Respectively IAA and 2,4D

at 0.1mg l^{-1} and maximum rates (68% and 63% respectively) were observed with stem explants. With orange explants we recorded an average number of buds of 6.13 with stem explants and the number of buds reached ten and more in some explants. Only 12 % of mandarin explants led to the formation of buds when the auxin used was IAA in combination with BAP but when IAA was replaced by 2,4-D , the rate increased to 50% with an average number of buds of 3.16%.

For the explants of Lemon, with MS medium BAP1mg l^{-1}, IAA 0.1mg $l^{-}1$, 92% of stem Explants and 40% of cotyledon ones led to buds formation with respectively average numbers of 2.08 and 2.12.The best rate was recorded with MSH medium (92%) but the number of buds formed was not important (1.11).

Direct organogenesis:

Six various media which didn't led to callus formation gave direct buds formation from roots and stems orange explants (Fig 3). Best results were recorded with roots and stems explants 100% and 80% respectively on MS medium supplemented with NAA and Kin both at 1mg l^{-1},but with a single shoot by explants.

With P7 medium in presence of phytohormones (IBA and BAP at respectively 1mg $l^{-1}$and 0.1mg l^{-1}), the rates recorded with roots and stems explants were relatively highs (70 and 78% respectively) and the number of shoots increase to 3 per explants; and a few number of leaf explants led to direct organogenesis (15%) with appearance of a single shoot on the midribs of the leaf.

Important direct growth of buds from lemon explants was recorded with four media (Fig 4) and the response depended of the explant nature while bests results recorded were respectively with root explants (52%) on MBR medium and with stem explants (50%) with Miller media with or without phytohormones.

Direct organogenesis was observed only on two media with mandarin explants, the best rate recorded was of 68% on Miller medium supplemented with NAA from stem explants with a low number of shoots (2 with stem explants).

Rooting:

Auxins known for their ability to promote adventious root formation, we tested media devoid of cytokinin and with or without IBA wich is by fact the most commonly used auxin to obtain root initiation in conventional cuttings (VanderKricken et al 1992) . Kinetics and intensity (% of rooting shoots) of rooting of the three rootstocks *Citrus* were reported on the Fig 5 to Fig 7 .

The response in MS media without supplementary of growth hormones, proved to be the best medium inducing rooting in both Orange (Fig 5) and Mandarin shoots(fig 6) but the intensities recorded were different with respective percentages of 54% and 93% reached after 8 weeks of

growth. The result with Lemon (Fig 7) is relatively important (67% after 4 weeks of growth) and it was increased to reach 100% with an earlier stage of root formation by supplementation with active carbon.

The addition of auxin (IBA) lowered the percentage of rooted shoots with the three species. The induction of rhizogenesis is usually stimulated by auxin,but sometimes organs or strains of cells are able to grow without any addition of auxin to the medium. They are called "auxin habituated" (Shimizu et al 2006). Maximum rate Lemon rooting (100%) was observed also with BN0 and BN100 media. The ontogeny and physiology of the plant (stock plant or mother plant) has a profound influence on *in vitro* explants response. We observed this influence both in indirect and direct organogenesis than in rhizogenesis responses where significant differences were recorded between the various media reported to the specie and between the responses of the explants of different nature (root, stem, leaf or cotyledonus). Our study revealed that the media which led to callus formations were also the best media for organogenesis and the difference in the number of shoots and in the response of the different organs of the plants among the rootstocks might be due to the difference in their genetic makeup (Chatuverdi and Mitra 1974) .The optimal media for organogenesis was M.S medium BAP 1mg l^{-1}, 2,4-D 0.1mg $l^{-1}$for both orange and mandarin explants and for lemon explants, M.S BAP 1mg l^{-1},IAA 0.1mg l^{-1} and MSH media. Optimal media for rhizogenesis were respectively MS 0/ 0 for orange and mandarin explants and BN100; BN0/0 and MS 0/0 supplemented with active carbon (0.5%) for lemon explants.

The per cent survival of *in vitro* derived plants (Fig 8) in potting media was recorded six weeks after transferring to the pots under greenhouse conditions .we recorded respectively high rates of survival(85; 73 and 77%) in orange, mandarin and lemon with the following potting media soil, sand and FYM (1:1:1). The higher plant survival in this potting mixture can be attributed to the retention of optimum moisture with adequate aeration (Goyal and Arya 1981).

REFERENCE:

Anstis,P.J.P. and Northeote, D.H. 1974. Chlorophyll accumulation by callus tissues of *Glycine max. Planta*.116 (2):105-108.

Chaturvedi, H. C. and Mitra,G.C.1974. Clonal propagation of citrus from somatic callus cultures. *Horticultural Science.* 9:118-120.

Faure,O. 1990. Embryons somatiques de *Vitis rupestris* et embryons zygotiques de *Vitis* sp. : morphologie, histologie, histochimie et développement. *Revue canadienne de botanique*, 68:2305-2315

Goyal, Y. and Arya, H.C.1981. Differentiation in cultures of *Prosopsis cineraria* L. *Current. Science.* 50,(10): 468-469.

Halperin,W.1964. Morphogenetic studies with partially synchronized cultures of carot embryos.*Science*,146:408-410.

Kalinina, A. and Brown, D.C.W. 2007. Micropropagation of ornamental *Prunus* spp. and GF305 peach, a *Prunus* viral indicator. *Plant Cell Rep*. 26:927-935.

Kerboua,M. 2000. L'Agrimuculture en Algérie. In. *Proceedings of the Mediterranean research network on certification of citrus (MNCC): 1998-2001* . D'Onghia A.M. (ed.), Djelouah K. (ed.), Roistacher C.N. (ed.) Bari : CIHEAM-IAMB, 2002. p. 21-26 : 24.

Martin, K.P, Joseph,D., Madassery,J., and Philip, V.J. 2003. Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum* Hort. *In Vitro Cell. Dev. Biol-Plant*, 39: 500-504.

Murashige T, and Skoog F. 1962. A revised medium for rapid growth and bioassays with tob acco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Parthasarathy, V. S., Bose, T.K, Deka, P.C, Das,P., Mitra,S.K and Mohandas, S.2001. Citrus: In Biotechnology of Horticultural Crops Vol. I (Eds.) pp. 119-171.

Rangan,T.S.,Murashige, T.and Bitters,W.P.1968. *In vitro* initiation of nucellar embryos in monoembryonic *Citrus. Horticultural Science*, 3:226-227.

Singh, S., Ray, B.K,. Bhattacharya, S. and Deka, P.C.1994. In vitro propagation of Citrus reticulata Blanco and Citrus limon Burm. *Hort. Science* 29: 214-216.

Sharma,S.,Prakash, A., and Tele,A.2009. In Vitro propagation of Citrus Rootstocks. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*.37(N°1),84-88.

Shimizu, T.,Eguchi, K,Nishida, I, Laukens, K.,Witters, E., Van onchelem, H. and Nagata , T. 2006. A novel cell division factorfrom tobacco2B-13 cells that induced cell division in auxin –starved tobacco BY-2 cells. *Naturwissenschaffen*, 93 (6):278-285.

Van der Krieken, W.M., Breteler, H. & Visser, M.H.M. 1992. Uptake and metabolism of indolebutrinic acid during root formation on *Malus* microcuttings. *Acta Botanica Neerlandica* 41, 435-442.

Table 1 :Composition of the media used for the micro propagation of Citrus plants

Medium Composition (mg l^{-1})	MS	P7	MSH	MBR	Miller	BN0	BN100	
KNO_3	1900	1900	1900	1900	1000	2950	2800	Major salts
NH_4NO_3	1650	1650	1650	1650	1000	-	100	
$Ca(NO_3)_2,4H_2O$	-	-	-	-	500	-	-	
$CaCl_2, 2H_2O$	440	440	440	440	-	440	440	
$MgSO_4, 7H_2O$	370	370	370	370	71,5	370	370	
KH_2PO_4	170	170	170	170	300	170	170	
Na_2Fe EDTA	13.2	-	13.2	13.2	13,2	13.2	13.2	Minor Salts
Fe Cl_3,6 H_2O	-	1	-	-		-	-	
$MnSO_4, 4H_2O$	22,3	0.1	22,3	22,3	14	22,3	22,3	
Zn $SO_4, 4H_2O$	8,6	1	8,6	8,6	3,8	8,6	8,6	
H_3BO_3	6,2	1	6,2	6,2	1,6	6,2	6,2	
Cu $(NO_3)_2,3H_2O$	-	-	-		0,35	-	-	
KI	0,83	0.01	0,83	0,83	-	0,83	0,83	
Na_2 Mo O_4, $2H_2O$	0,35	-	0,35	0,35	-	0,35	0,35	
$(NH_4)_6$ Mo_7O_{24}, H_2O	-	-	-	-	0,10	-	-	
$CuSO_4$, 5 H_2O	0,025	0.03	0,025	0,025	-	0,025	0,025	
Co Cl_2, 6 H_2O	0,025	-	0,025	0,025	-	0,025	0,025	
$NiCl_2,6H_2O$	-	0.03	-	-		-	-	
$AlCl_3$	-	0.03	-	-		-	-	
Inositol	100	2	-	100	100	100	100	Organic coumpounds
Glycine	2	-	-	2	-	2	2	
Thiamin Hcl	1	20	5	0.1	0,1	1	1	
Nicotinic acid	0,5	-	5	0,5	0,5	0,5	0,5	
Pyridoxin Hcl	0,5	-	-	0,5	0,1	0,5	0,5	
Biotin Hcl	-	2	-	-		-	-	
Calcium panthothenate	-	20	-	-		-	-	
adenin	-	-	2	-	-	-	-	
sucrose	35000	35000	35000	50000	35000	35000	35000	
Malt extract	-	-	-	500		-	-	
Difco-agar	9000	9000	9000	9000	9000	9000	9000	

Table 2: Effects of media nature and phytohormones on per cent callus formation in explants of Citrus rootstocks

Media + treatment (mgL^{-1})	Orange					Mandarin					Lemon				
	R	C	S	L	Mean	R	C	S	L	Mean	R	C	S	L	Mean
MS BAP1; AIA 0,1	50	20	80	9	$39.75^{27.66}$ C.V 0.69	1	3	30	2	$9.00^{12.14}$ C.V 1.35	20	1	10	2	$8.25^{7.63}$ C.V 0.92
MS BAP1; 2,4-D 0,1	100	100	93	90	$95.75^{4.38}$ C.V 0.046	100	100	100	15	$78.75^{36.8}$ C.V .0.468	10	2	48	35	$23.75^{18.55}$ C.V 0.78
MS BAP0,1; ANA 1	45	0.0	0.0	13	14.5^{16} C.V 1.103	0.0	0.0	0,0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P_7 BAP0,5; AIB1	3	5	1	1	$2.5^{1.66}$ C.V .0.664	0.0	0,0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P_7 BAP0,1; AIB1	2	1	40	3	$11.5^{16.47}$ C.V 1.43	30	2	2	35	$17.25^{15.3}$ C.V.0.89	0.0	0.0	0.0	0.0	0.0
MSH ad.2; 2,4-D 0,1	100	100	85	90	$93.75^{6.49}$ C.V 0.069	1	1	0	40	$10.5^{18.38}$ C.V 1.75	85	45	93	95	$79.5^{20.27}$ C.V.0.25
MBR	00	00	00	00	00	82	38	100	0	$73.33^{26.04}$ C.V .0.35	0.0	0.0	0.0	00	0.0
Square [(a)]root	50	45.19	34.62	39.55	38.33	41.1	40.2	43.1	15.3	31.42	33.25	20.5	33.92	34.49	30.59
Coef.var (C.V)	0.79	0.99	0.58	1.15	0.89	0.96	1.14	0.74	0.66	0.83	0.87	1.28	0.67	0.89	0.82

Table 3: Effect of media nature and phytohormones on indirect organogenesis (per cent bud formation) and on average number of shoots per explant obtained through the callus in explants of Citrus rootstocks

Plant / Media + Treatment(mgL^{-1})	Oranger			Mandarin			Lemon		
	R	C	S	R	C	S	R	C	S
MS BAP1; AIA 0,1	32	9.5	61	0	0	12	0	40	92
Nb shoots	$5.51^{1.11}$ ±0.54	$4.17^{0.63}$ ±0.31	$6.13^{0.85}$ ±0.42	0	0	$4.11^{2.66}$ ±1.3	0	$2,12^{0.45}$ ±0.22	$2.08^{0.36}$ ±0.17
MS BAP1; 2,4-D0,1	45	25	63	0	50	50	0	0	25
Nb shoots	$5.43^{1.29}$ ±0.63	$6.22^{1.063}$ ±0.52	$4.21^{0.83}$ ±0.41	0	$6.17^{1.16}$ ±0.57	$3.16^{2.86}$ ±1.40	0	0	1^{0}
MS BAP0,1 ANA 1	30	2	35	0	0	0	0	0	0
Nb shoots	$10,12^{1.52}$ ±0.75	0	$5,17^{0.35}$ ±0.17	0	0	0	0	0	0
MS kin1; ANA 0,1	0	0	0	0	0	0	20	0	8
Nb shoots	0	0	0	0	0	0	$2.1^{0.34}$ ±0.17	0	$3.17^{0.63}$ ±0.31
P_7 BAP 0,1; AIB 1	0	0	0	0	0	0	30	5	32
Nb shoots	0	0	0	0	0	0	1^{0}	$0.8^{0.36}$ ±0.18	$2^{0.08}$ ±0.04
Miller 0;0	0	0	0	0	0	0	35	0	53
Nb shoots	0	0	0	0	0	0	$2^{0.29}$ ±0.14	0	1^{0}
Miller ANA 0,1	0	0	0	0	0	0	10	0	5
Nb shoots	0	0	0	0	0	0	$0.9^{0.25}$ ±0.12	0	1^{0}
MSH	2	1	55	0	0	10	0	0	72
Nb shoots	0	0	$1^{0.01}$ ±0.005	0	0	$1.02^{0.003}$ ±0.001	0	0	$1.11^{0.83}$ ±0.41
MBR	0	0	0	14	0	0	55	0	0
Nb shoots	0	0	0	$3^{0.65}$ ±0.32	0	0	$1.23^{0.63}$ ±0.31	0	0

[a]square root C.L :confidence level ±b,*P(0.95)

R : Root explants C : Cotyledon explants S: Stem explant

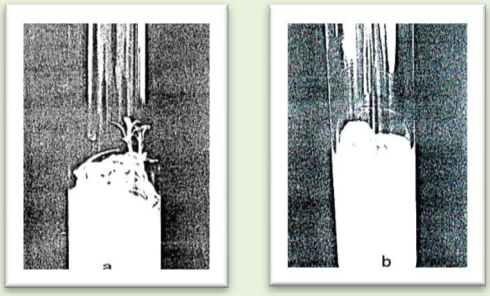
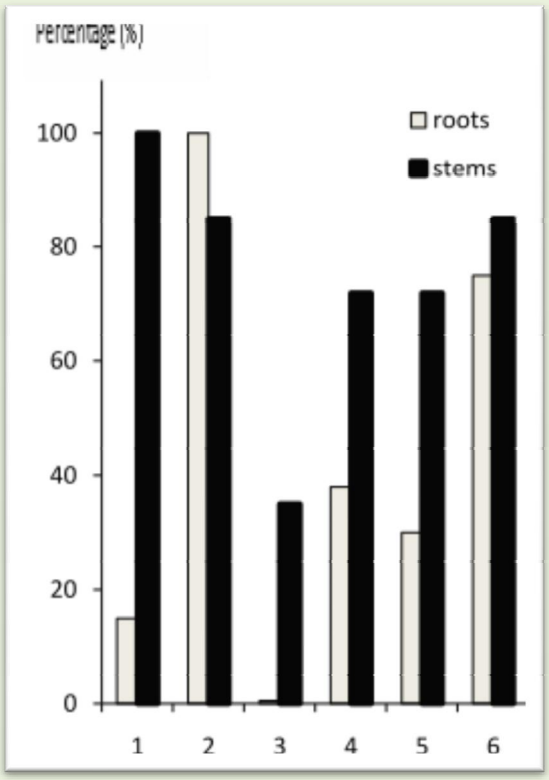


Fig 1 : Callogenesis on stem explants obtained on M.S 2,4-D0,1mg l^{-1} ; BAP 1mg l^{-1}

a- Pineaple orange (*Citrus sinensis*)

Carvallah mandarin (C*itrus deliciosa)*



1: MS ANA 0.01 mg l-1/BAP 1 mg l^{-1} ;
2: MS ANA 1 mg l^{-1}/Kin 1mg ;
3: MS ANA 1mg l^{-1}/kin 10mg l^{-1} ;
4: Miller ;
5:Miller ANA 0.1 mg l^{-1} ;
6: P7 BAP 0.1 mg l-1/AIB 1mg l^{-1}

Fig 3 : Direct organogenesis of orange explants with various media and treatments

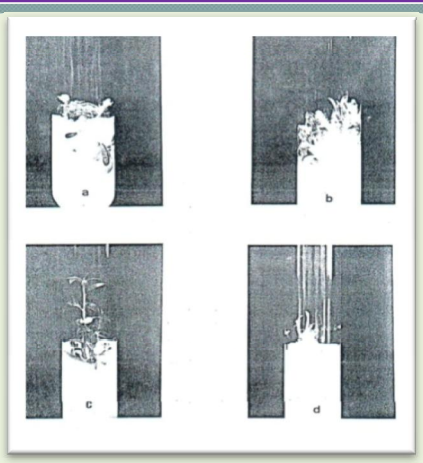
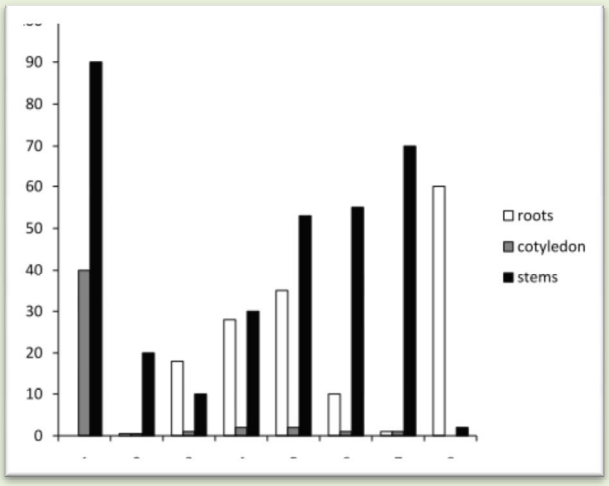


Fig 2 : Indirect organogenesis on stem(a,b,c) and cotyledon (d) explants of Pineaple orange

a- on MS 2,4- D 0.1mg l^{-1}/ BAP 1mg l^{-1}
b- on MS AIA 0.1mg l^{-1}/ BAP 1mg l^{-1}
c- on P_7 AIB 1mgl^{-1}/BAP0.1mg l^{-1}
d- on MS 2,4- D 0.1mg l^{-1}/ BAP1mg l^{-1}



1: MS AIA 0.01mg l^{-1}/BAP 1mg l^{-1};
2: MS 2,4-D 0.1mg l^{-1}/BAP 1mg l^{-1} ;
3: MS ANA 0.1mg l^{-1}/kin 1 mg l^{-1} ;
4: P7 BAP 0. 1mg l^{-1}/AIB 1mg l^{-1} ;
5: Miller 0/0 ;
6: Miller ANA 0.1mg l^{-1};
7: MSH ;
8 : MBR

Fig 4 : Direct organogenesis of Lemon explants with various media and treatments

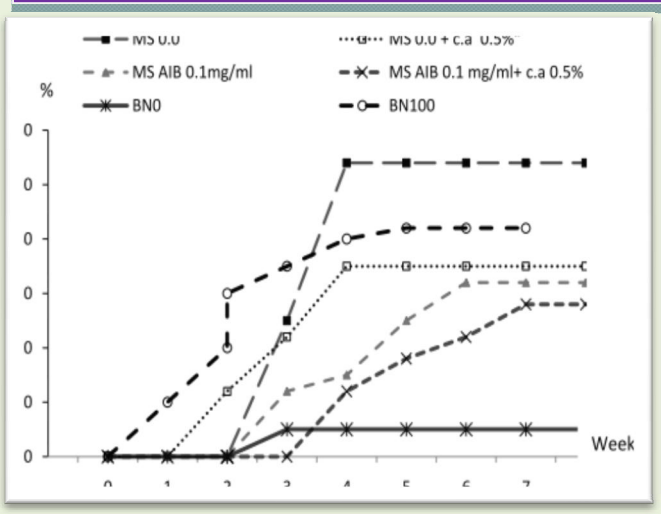


Fig 5: Rooting of Orange shoots on various media culture

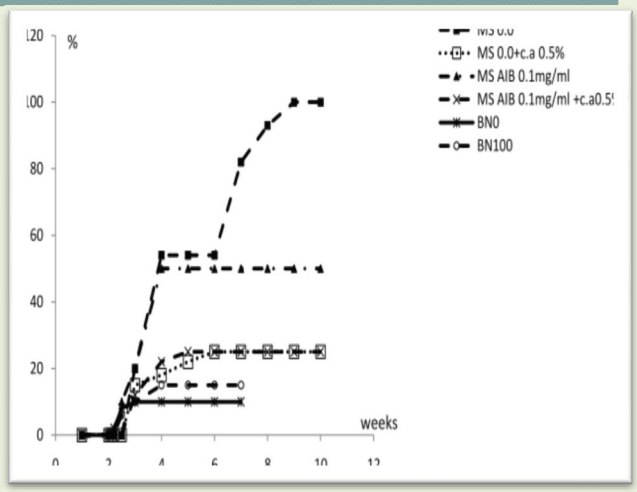


Fig 6 : Rooting of mandarin shoots with various media culture

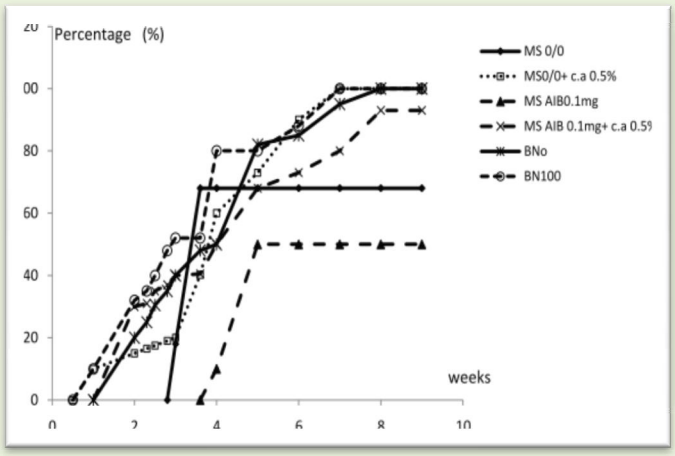
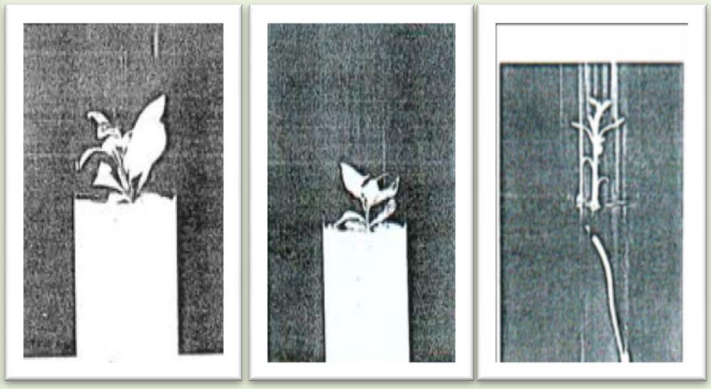


Fig 7 : Rooting of lemon shoots on various media



a **b** **c**

a- Pineaple Orange plantlet Obtained on MS0/0

b- Carvalhal mandarin plant obtained on MS0/0

c- Plantlet of Eureka Limon obtained on MS0/0+ c.a.(0,5%)

Fig 8 : Plantlets obtained by indirect organogenesis(adventitious shoots from callus + Rhizogenesis)

Scientific Research and Essays Vol. 5(17), pp. 2595-2602, 4 September, 2010
Available online at http://www.academicjournals.org/SRE
ISSN 1992-2248 ©2010 Academic Journals

Full Length Research Paper

Malt factor induced flocculation in *Saccharomyces uvarum*

Fadila Maiza Benabdesselam

Department of Physic and chemical Biology, Faculty of Life and Nature Sciences, University of Bejaia – 06000- Bejaia – Algeria. E-mail :fadilamaiza@yahoo.fr. Fax: + 21334215448.

Accepted 8 July, 2010

Tests of fermentability of yeast were carried out with two species of malt; Sympa malt and Nymphea malt, and with their EP (Ethanol Precipitate). Flocculation of *Saccharomyces uvarum* was not observed with any of them. However, Flocculation can be induced by the EP from the bark of another specie of malt Trumpf malt. Analysis of the Trumpf malt EP was carried out using paper chromatography, fractionation on de Cellulose 23, IR (infrared) and UV (ultraviolet) Spectroscopy. Analysis of the effective (F1) fraction revealed the presence of ferrulic acid, arabinose and xylose. Effectiveness of the F1 osidic fraction and the results of IR spectra, which showed the presence of glucosidics bonds, plead for an active role of ferrulic acid and hemi-cellulose ester functions in yeast flocculation process.

Key words: Flocculation, *Saccharomyces uvarum*, malt, EP, osidic fraction, spectrographic analysis.

INTRODUCTION

Flocculation is a well known example of natural, active and reversible aggregation of cells into flocs (Calleja, 1987). The ability of yeast cells to flocculate is very important in industrial processes that are related to fermentation technology like brewing or bioconversion. Yeast flocculation is an important process for the production of beer that causes the yeast to sediment to the bottom of the fermenter at the end of the fermentation. Thus, the yeast can be harvested from the bottom of the fermenter and used for the next fermentation, while the beer may be matured without the need of centrifugation step (Stewart et al., 1975; Stewart and Russel, 1995). The aggregation of cells in flocs generally occurs during the late exponential or early stationary growth phase (Straver et al., 1993). As a result, the majority of cells are separated from the culture medium. However, an interesting yeast strain showed an inverse flocculation pattern (Strauss et al., 2003). The variability in the occurrence of flocculation is a problem (Straver et al., 1993); premature flocculation hampers complete fermentation of the growth medium, whereas, failure of the cells to flocculate at the end of fermentation requires the use of expensive techniques such as centrifugation or filtration to remove cells (Stratford, 1989, 1992; Sadosky and Schawrz, 2002).

Yeast flocculation is a complicated process that is currently only partly understood. It requires the presence of at least two types of molecules on the yeast cell surface. Mannans is one the molecule (carbohydrate chains), which are always present on the cell surface. The other type is flocculins (sugar binding proteins), which are the gene products of the *FLO* genes, that are activated only after depletion of nutrients. (Speers et al., 2006)

Classification of brewer's yeast strains

Brewing strains may be classified in four classes according to their ability to flocculate (Hussain et al., 1986): Non flocculent strains (powdery yeasts, completely dispersed), standard ale strains (Flocculating into small, loose lumps late in fermentation), highly flocculent yeasts (Flocculating into dense masses late in fermentation and producing beer under attenuated and sweet) and very highly flocculent yeasts (Flocculating very early in fermentation owing to non-separation of daughter cells).

Factors affecting flocculation

A number of factors influencing flocculation process have been reported (Jin and Speers, 1998). The genetic aspects

have been recognized since 1950s (Esser et al., 1987) and flocculation is under genetic control (Stratford, 1996; Teunissen and Steensma, 1995; Hidekatsu et al., 2001). The yeast cell wall, the cell age, and other factors of the growth medium can influence the process (Soares et al., 1994).

The flocculation depends on brewery conditions (Johnson et al., 1988) and on the nature of the strain used (Stratford, 1991; Versteepen et al., 2000). It is well established that Wort components influence the ability of the yeast cells to flocculate (Taylor and Orton, 1978). The kinetic approach shows that the calcium content, for example, has an influence on the flocculation process (Stan, 2002), and premature flocculation of yeast is induced by some Wort constituents (Fuji and Horie, 1975; Fujino and Yashida, 1976). The aim of this study was to determine the substance of Wort, which induces flocculation of S*accharomyces uvarum*.

MATERIALS AND METHODS

Organisms and media

The yeast strain used was a « local strain » of *S. uvarum* from the culture collection of « l'IFBM » of Nancy – France. The yeast was cultivated on 1 L of malt wort. 1 ml of the subnatent was added to 50 ml of Wort. The culture, with continuous stirring was incubated at 25°C for 3 days. Appropriate volumes were then transferred to 2 L of malt and the culture was left in the dark at 25°C for 5 - 6 days.

A day before its utilisation, culture was allowed to decant and appropriate volume was then transferred at equivalent volume of new Wort. Before experiments, yeast was dispersed in distilled water and centrifuged twice at 16000 g.

Conservation of yeast strain

Yeast strains were inoculated every 6 months on Y.M agar medium (malt extract 3 g/L, yeast extract 3 g/L, Biotripcase 5 g/L glucose 1 g/L, agar 2 g/L).

Growth experiments

S. uvarum was inoculated in 250 ml Wiame conical flasks using Stewart chemical liquid medium (Stewart et al., 1975) containing amino acids, sugars, vitamins, ions and other growth factors. When the OD (Optic Density) reached 0.85 - 1, appropriate volumes were then transferred to 500 ml conical flasks containing 100 ml of the same medium. The fermentations were carried out in glass vessels, cylinders containing 1000 ml of synthetic medium. Growth was observed at regular intervals by measuring changes in optical density using chemetrix –24- digital colorimeter (red filters) and the degree of flocculation was measured through growth by calculating the decrease in cell turbidity (800 nm) according to the method of Calleja and Johnson (Johnson et al., 1988).

Fermentability tests were performed using synthetic medium in presence of malt extracts of different species (malt Sympa and malt Nymphea), Ethanol Precipitate of each malt (300 ppm of Ethanol Precipitate of Sympa malt, Nymphea malt and Trumpf malt.), and with the different fractions of the Ethanolic precipitate of malt Trumpf.

Preparation of ethanolic precipitate (EP) of malt

The Ethanolic Precipitate was obtained according to Morimoto process (Morimoto et al., 1975). 55 g of malt were ground in MIAG Grinder and supplemented with 250 ml of water at 46°C, while shaking, the mixture was allowed to stand inside a large vat containing water at 45°C for 30 min. Temperature was then increased until it gets to 70°C (1°C by min) and the preparation was boiled during 30 min. After a decline in temperature, the mixture was homogenised and filtered, these were closely followed by the addition of 65 ml of Ethanol to 35 ml of the filtrate. The preparation was left at 0°C overnight. After centrifugation, the precipitate was dissolved in hot water and supplemented with cadmium acetate and mixture was filtered through "hyflo supercel". Ethanol (65%) was added to the filtrate, the precipitate obtained was dissolved in water and dialysed successively against tap water, demineralised water and in fine distilled water and finally lyophilised .

Analysis of malt trumpf barks EP contents

In order to analyse the EP (ethanolic precipitate) malt contents, its chemical degradation was necessary. This was carried out using saponification, acid hydrolysis and enzymatic degradation.

Saponification

Samples of 100 mg of ethanol precipitate were dissolved in 10 ml of NaOH 2 N and then shaken for 2 h. The solution was acidified by HCl 2 N up to pH 2. After extraction with ethylic ether, the organic phase was evaporated and the residue dissolved in 3 - 4 ml of sodium bicarbonate solution (5%) and another extraction was made by 5 - 6 ml of ethylic ether. Organic phase was dried over anhydride sodium sulphate containing phenolic acids salts. The aqueous phase was acidified (HCL 2 N) until pH 2 and added with ethylic ether containing phenolic acids esters. Identification of the acids was achieved by paper and thin layer chromatography.

Acid hydrolysis of the ethanol precipitate

Samples of 100 mg of Ethanol precipitate were hydrolysed by 5 ml of HCL 2 N at 100°C for 1 h. After a decline in temperature, the solution was filtered and extracted with ethylic ether. The acid and aqueous phases contain either sugars or osidic compounds. The acidity was neutralised using anionic resin (DOWEX 2) and the solution was then concentrated by evaporation. Sugars were identified by paper chromatography.

Enzymatic degradation of the ethanol precipitate

Samples of 100 mg of Ethanol precipitate were treated with 10 ml of enzymatic solution at 0.5 mg/L. Enzymes and processes used are shown on Table 1

Paper chromatography and thin layer chromatography

Paper chromatography was used to identify sugars, and phenolic acids were determined by paper and thin layer chromatography. The systems used are shown in Table 2 for sugar identification and Table 3 for phenolic acids identification.

In order to study the sugars, Watmann paper no. 1 was used, and Watmann paper no 4 and plates of cellulose MN 300 (20 × 20 × 0.03 cm) were used to study the phenolic acids.

Table 1. Enzymatic treatments applied to the ethanolic precipitate of trumpf malt.

Enzyme	Characteristic	Treatment
Celulysine TM	Grade B-Calbiochem (amylase+protease Hemicellulase)	At 37°C for 16 h with pH 6.5 and Centrifugation of 300 g for 15 min
Cellulase	Grade B-Calbiochem	At 40°C for 16 h
Hemicellulase	From rhizopus- Sigma chemical company	At 40°C for 16 h
β-Glucanase	Rapidase	At 25°C for 24 h
Esterase	From pig liver – Sigma chemical company	At 25°C for 24 h in phosphate buffer pH 8
α-amylase	Sigma chemical company	At 85°C for 1 h
Pronase	Grade B-Calbiochem	At 25°C for 12 h in water and At 40°C for 12 h in phosphate buffer pH8
Trypsine	Bovin pancreas–Sigma chemical company	At 40°C for 12 h in water and At 40°C for 12 h in phosphate buffer pH8
Papaine	Rapidase	Activation By 5 Mm Of Cystein-Chlorohydrate. At 25°C For 24 h

Table 2. Descendent chromatography of sugars in Trumpf malt EP (wattman paper N° 1). Migration is 24 h in solvent 1; and 24 h in solvent 2. Solvent 1: n-butanol : aceton : water(4:5:1:) ;solvent 2: n-butylacetat : ethanol : pyridine : water (8:2:2:1).

References (standards)	RGL	Revelation anisidine-phtalane
Rhamnose	3.0	Green-yellow
Glucose	1.0	Green- yellow
Arabinose	1.51	Purple
Mannose	1.33	Green-yellow
Xylose	1.82	Prune
Galacturonate	0.066	Orange
Galactose	0.83	Green-yellow

Table 3. Thin layer (cellulose MN300) and paper chromatography (Wathman no 4) of acid phenols of Trumpf malt EP.

Phenolic acids	Solvents: 1: isopropanol :ammoniac water(8:1: 2) 2 : benzene :acetic acid: water(6:7:3)			Fluorescence	Revelation	
	TLC 1	WP 2		UV	*PAN	Na_2CO_3 10%
Ferrulic acid	0.25	0.3	0.87	Blue	Red	Grey-blue
Vanillic acid	0.196	0.23	0.85	-	Yellow	Dark-blue
p-coumaric acid		0.38	0.47	-	Yellow	Black
p-OH benzoic acid	0.30	0.31	0.36	-	Yellow	Red
Salycilic acid	0.79	0.80	-	Violet	-	Yellow
Gentisic acid	0.67	-	0.12	Blue-green	Brown	Yellow
Syringic acid	0.17	0.19	0.93	-	Yellow	Yellow-green
p-OH-p-acetic acid	0.51	0.51	0.316	-	--	Purple
pOH-propionic acid	0.62	0.56	0.517	-	Yellow-blue	Purple
Sinapic acid	0.19	0.188	0.92	Blue	Red	Yellow-green

*PAN: Para Nitro Anilin.

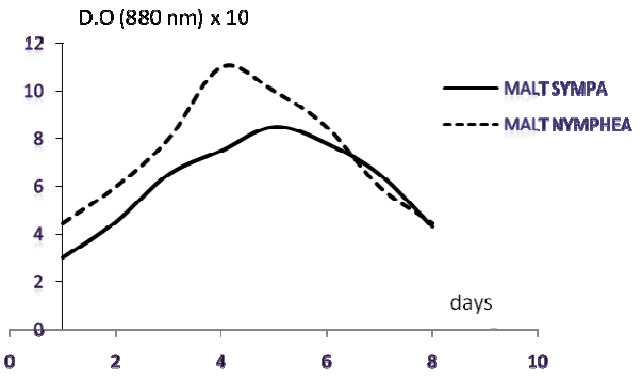


Figure 1. Static fermentation tests of flocculation of *S. uvarum* , comparing fermentation rate with Sympa or Nymphea malt wort, and with incubation time.

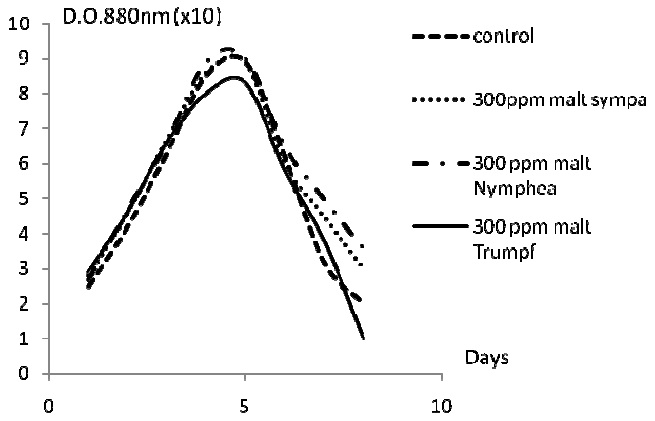


Figure 2. Static fermentation test for flocculation , comparing the effect of 300 ppm of different malt E.P.

The PAN (ParaNitrodiazotAnillin) specific revelatory of phenols and phenolic acids, was obtained by mixing 2 ml of paranitrilaniline (0.5%) with a solution of sodium acetate (20%) and $NaNO_2$(15%).

DE cellulose chromatography

DE –Cellulose-23 used was from W and R. Balston, LTD-n°catal.24231, England. The resin was treated with HCl (0.5 N) for 30 - 120 min, washed twice with NaOH (0.5 N) and H_2O until a pH of 4 - 8 was reached. After sedimentation in the dark, a solution of dissodic boric acid (0.2 M) was added and the mixture was filtered and washed with water. DE-cellulose was then dissolved in water and left for decantation and the columns were then filled.

350 mg of EP of malt Trumpf were fractionated using DE Cellulose 23 chromatography (3.0 × 50 cm). The column was eluted successively by deionised Water, Borax 0.01 M; Borax 0.1 M; NaOH 0.1 N and NaOH 0.3 N. At the same time, sugars were quantified by Dubois method, and proteins by measuring of Optic Density at 280 nm.

IR spectroscopy of bark's trumpf EP and deproteinated EP

200 mg of each sample (EP of malt Trumpf and deproteinated EP by cadmium acetate) were dispersed in 1 mg of K Br. The analysis was carried out following Flemming process (Flemming et al., 1974).

UV spectroscopy of bark's trumpf EP and deproteinated EP

Analysis of phenolic acids was carried out by Ethanol Precipitate, and Ethanol Precipitate deprotinated screening from 200 - 400 nm (spectrophotometer Uvikon 930-Kontron).

RESULTS

Static fermentations experiments

Results of static fermentation experiments, which compare the fermentation rate with yeast in suspension, in presence of wort of two species of malt (Sympa and Nymphea), showed that yeast behavior is the same for the two species (Figure 1). However, the curves obtained by adding Ethanol Precipitate (EP) of Sympha malt, Nymphea malt and Trumpf malt show the presence of flocculation using Trumpf EP (Figure 2).

Ethanol precipitates of trumpf malt contents

Sugars identified by paper chromatography were arabinose, glucose, xylose, galactose and a non identified type of sugar.

Phenolic acids identified by paper and thin layer chromatography are ferulic, isoferulic, paracoumaric, vanilic and syringic acids. The presence of ferulic acid is confirmed by UV (Ultra-Violet) spectrum (Figure 3) where pics at 280 and 300 nm are characteristic of these acids.

IR (Infra-Red) Spectrum (Figure 4) shows the presence of α glucosidics bonds (845 cm^{-1}) and ß glucosidics-bonds (898 cm^{-1}).

Fractionation by DE – cellulose of malt Trumpf EP had led to three fractions (Figure 5). Fraction F1 obtained with water constituted of arabo-xylanes, while arabinose residues are responsible for salvation in water of this fraction. The second fraction F2 (eluted by borax × 0.01 and 0.1 M) contains glycoproteins as a result of the overlapping of sugars and protein's picks. The third fraction F3 (Na OH elute), contains a heterogeneous protein mixture or a mixture of proteins and glycoproteins.

Then the effect of these different fractions of EP on the yeast flocculation was analysed.

Effect of the different fractions obtained by EP bark trumpf malt fractionation

The comparative studies of fermentations of the yeast with 70 ppm of each fraction and 70 ppm of the complete EP shows that the F1 fraction is more efficient than the two other fractions and the Ethanol Precipitate as well

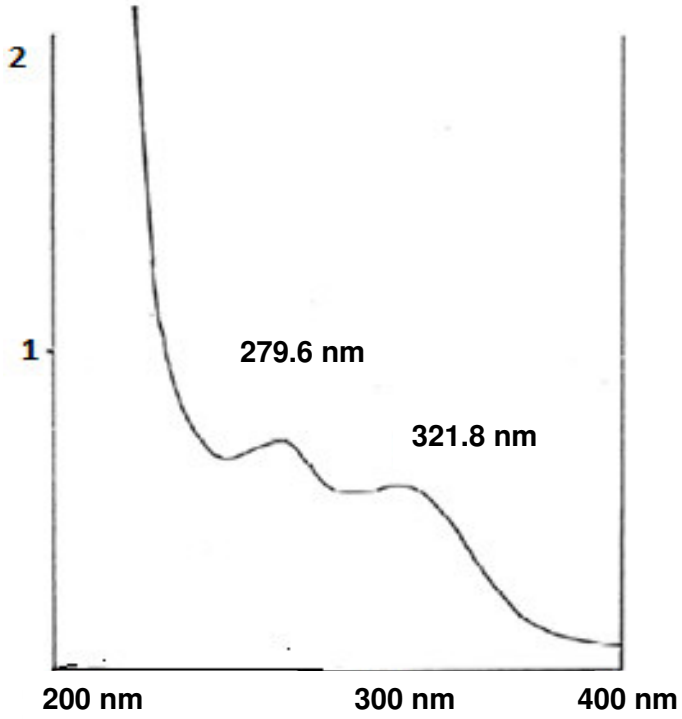


Figure 3. U V Spectrum of deproinated Ethanol Precipitate of Trumpf malt.

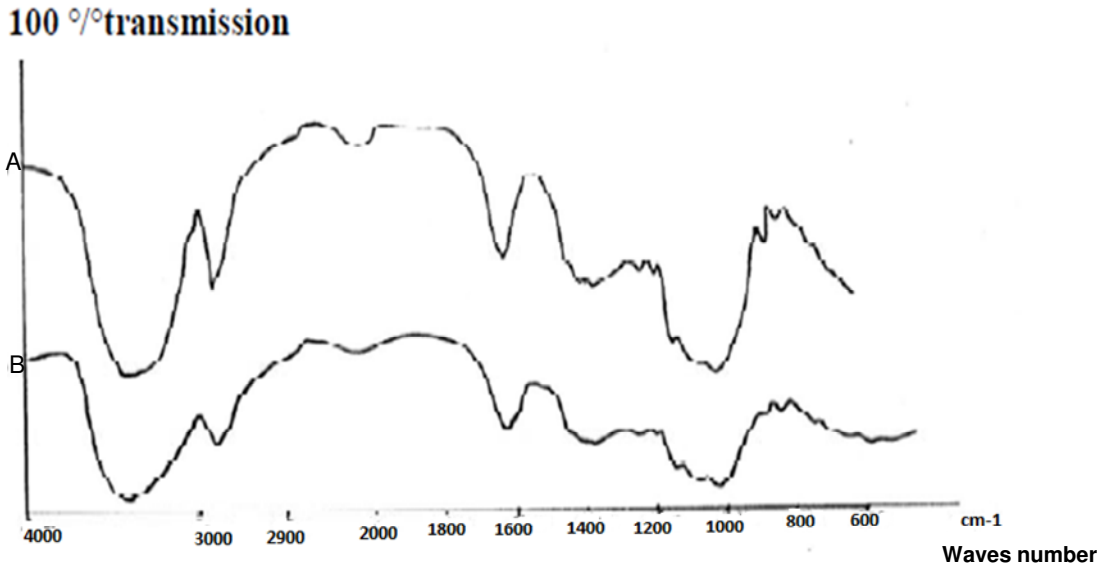


Figure 4. (A) I R spectrum of ethanol precipitate of Trumpf malt. (B) I R spectrum of deproinated Ethanol precipitate of trumpf malt.

(Figure 6). Treatment of the EP by saponification and by α amylase and ß glucanase did not have any effect on flocculation power, but treatment with trypsin or esterase led to the loss of flocculation power of the malt Trumpf. Indeed, saponified Ethanol Precipitate does not induce flocculation (Figure 7) and enzymatic treatments (α amylase, ß glucanase or pronase) did not significantly change the effect of EP.

Maraz and Gelata (2001) suggested that lectine-like cell surface proteins were involved in cell aggregation of

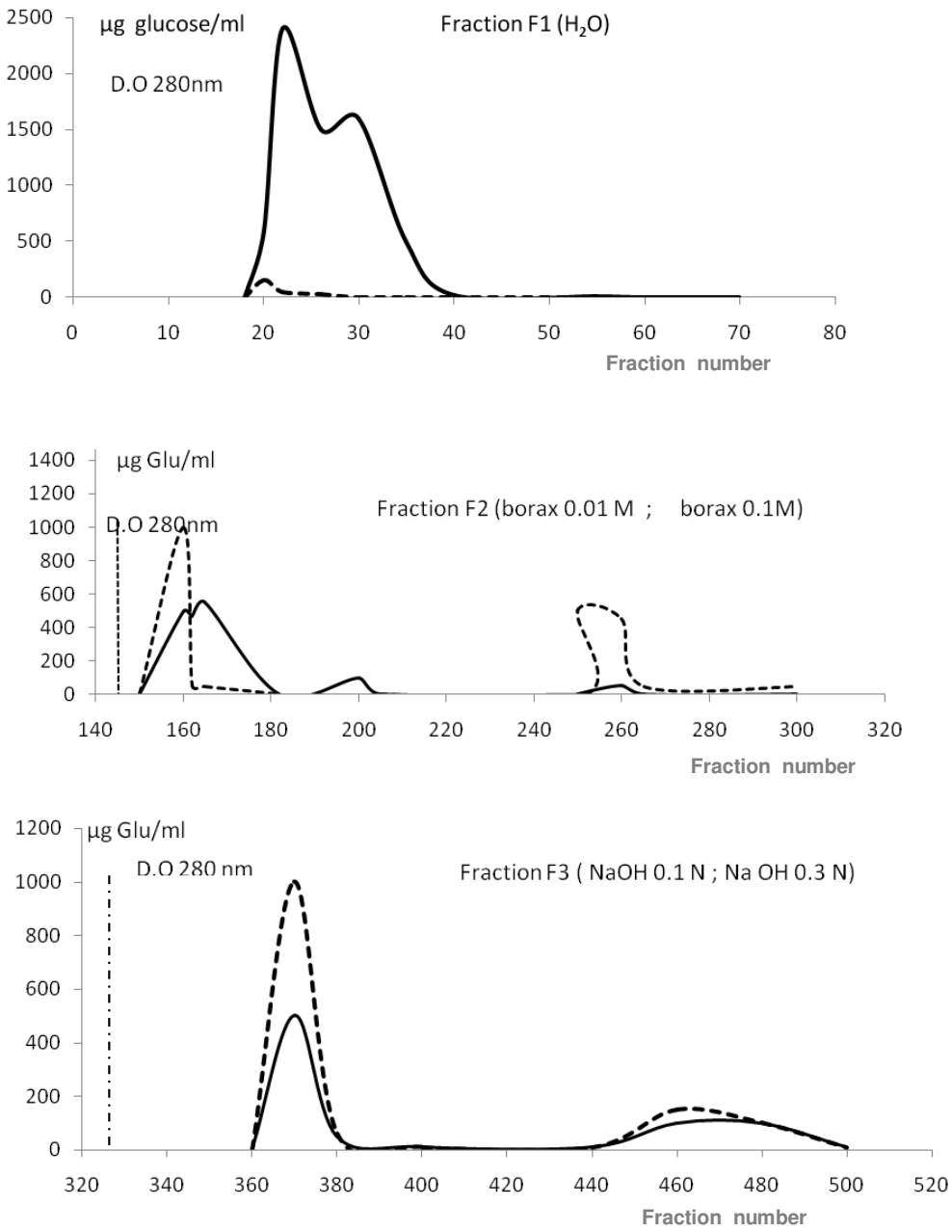


Figure 5. DE cellulose fractionations of the ethanol precipitate from the bark of trumpf malt.

the yeast. One possible role of the compounds of the fraction F1 (sugars, phenolic acids, glucosidic bounds) is that they can provide chemical modification in lectin itself by activating the molecules for binding to the carbohydrate moieties. Further work is needed to see if specific metabolic pathways are induced by those compounds, which directly or indirectly lead to cell aggregation.

The effect of the active compounds could have been caused also by the triggering of a set of events, which finally led to the excretion of bivalent cations (probably ca^{2+}) that are known to have a direct role in flocculation (Startford, 1989).

Conclusion

The efficiency of the osidic fraction F1 of the Ethanol Precipitate of Trumpf malt, which is the malt that appears to be the efficient on flocculation of *S. uvarum* is probably due to the ester-bonds between sugars and phenolic acids. This hypothesis is confirmed by the results obtained with addition of EP after saponification and enzymatic treatment. Indeed, trypsin is an enzyme which contains esterasic activity, and esterase leads to the loss of flocculation power, while the other enzymes do not show any effect on flocculation power.

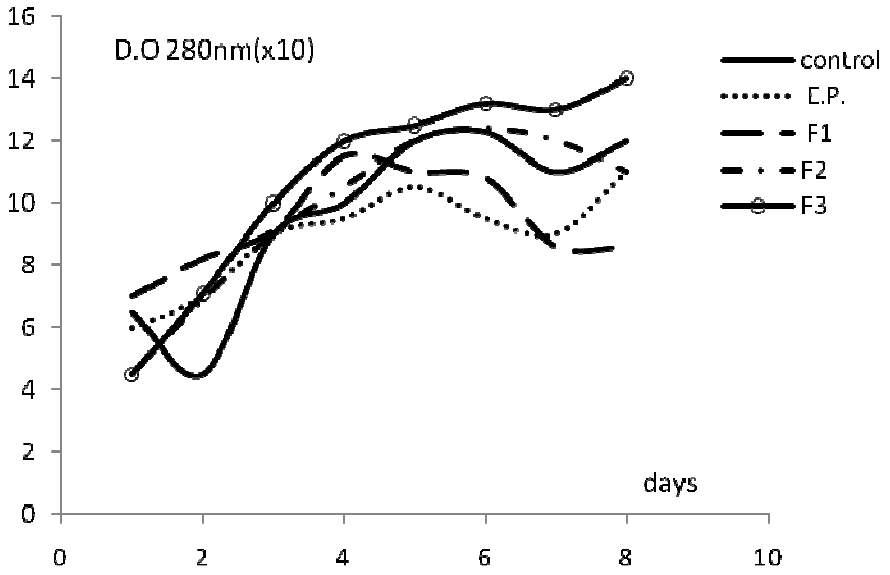


Figure 6. Effect of different fractions of Trumpf malt's EP on flocculation of S. *uvarum*.

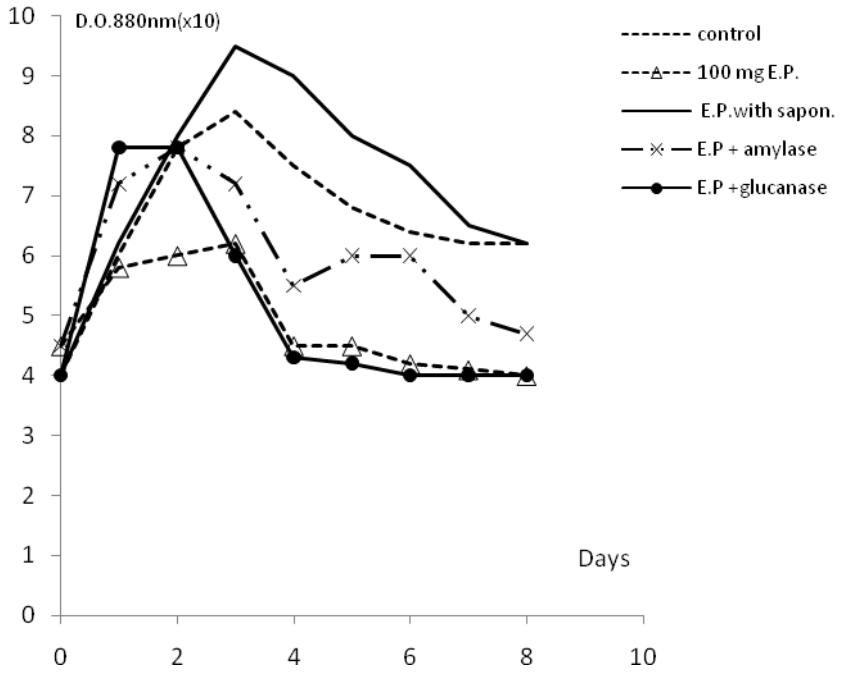


Figure 7. Effect of different treatments of trumpf malt's EP on flocculation induction of *S. uvarum.*

AKNOWLEDGMENTS

The author wish to thank all the staff of the laboratory of biochemistry and microbiology of INPL –ENSAIA- of Nancy – France. Also thanks to Professor Djebbar Atmani who kindly reviewed this manuscript. In memory of my late husband, Dr Abdelhamid Benabdesselam who pioneered this work.

REFERENCES

Calleja GB (1987). Aggregation.The Yeast. In A.H. Rose and JS Harrisson, (ed). Academic Press.London.

Esser K, Hiurich J, Kues U (1987).Genetic control of flocculation of yeast with respect to application in biotechnology. Proc.Technol., 4: 383-398.

Flemming M, Manners DJ, Jackson RM, Cooke S.C (1974).The estimation of ß glucans in barley. J. Inst. Brew., 80: 399-404.

Fuji T, Horie Y (1975). Some substances in malt inducing early

flocculation of yeast . Further investigation on a substance isolated from worth inducing early flocculation. Rept. Res. Lab. Kirin Brew. Co, Ltd., 18: 75-85.

Fujino S, Yoshida T (1976). Premature flocculation of yeast induced by some wort constituents. Rept. Res. Lab. Kirin Brew. Co, Ltd.19: 45-53.

Hidekatsu I, Hideo T, So-ichi Y, Kunio T (2001). Functional co between the nuclear localisation of Fht1p and its flocculation and heat tolerance activities in budding yeast *saccharomyces cereveasiae.* J. Biom. Sci., 8(5): 416-420.

Hussain T, Salhi O, Lematre J, Charpentier C, Bonali R (1986). Comparative studies of flocculation and deflocculating of *Saccharomyces uvarum* and *Kluyveromyces bulgaricus*. Appl. Microbiol. Biotech.*,* 23: 269-273.

Jin YL, Speers RA (1998). Flocculation of *Saccharomyces cerevesiae.* Food Res. Int., 31(67): 421-440.

Johnson BF, Walker T, Calleja JB, Seligy VL (1988). Sexual co-flocculation and asexual self flocculation in budding and fission yeasts : experimental establishment of fundamental difference. Can. J. Microbiol., 34: 1105-1107.

Maraz A, Gelata A (2001). Ethanol induced cell aggregation and its physiological background in *Schizosaccharomyces pombe rive* 4-2-1-Act. Biol. Hung.*,* 52(2-3): 231-239.

Morimoto K, Shimazu T, Fuji T, Horie Y (1975). Some substances in malt inducing early flocculation of yeast .Preliminary investigation of high molecular weight substances in malt and wort. Rept.Res.LabKirin Brew. Co. Ltd., 18: 63-74. .

Sadosky P, Schawrz PB (2002). Effect of araboxylans, beta-glucans and dextrins on the viscosity and membrane filterability of a beer model solution. J. Am. Soc. Brew. Chem., 60(4): 153-162.

Soares EV, Teixeira JA, Mota M (1994). Effects of cultural and nutritional conditions on the control of flocculation expression in *Saccharomyces cerevesia* .Can. J. Microbiol., 40: 851-857.

Speers RA, Wan YQ, Jin YL, Stewart RJ (2006). Effects of fermentation parameters and cell wall properties on yeast flocculation. J. Inst. Brew., 112: 246-254

Stan S (2002). On the doublet formation in the flocculation process of the yeast cells .Htpp://arxiv.org/ps/physics/ 01050602 V 1.

Stewart GG, Russel J, Garrisson IF (1975). Some considerations of the flocculation characteristics of ale and lager yeast strains. J. Inst. Brew., 3(81): 248-257.

Stewart GG, Russel J (1995). Characterization of affinity-purified cell wall binding proteins of *Saccharomyces cerevesiae*: Possible role in flocculation. J. Am. Soc. Brew. Chem., 53: 111-116.

Stratford M (1989). Evidence for two mechanisms of flocculation in *Saccaromyces cerevesiae.* Yeast, 5: 441-445.

Stratford M (1991). Yeast flocculation: reconciliation of physiological and genetic viewpoint. Yeast, 8: 25-38.

Stratford M (1992). Yeast flocculation: a new perspective In : Advances in microbial physiology, A.H., rose, Ed., Academic press: New- York.

Stratford M (1996).Induction of flocculation in brewing yeast by change pH value. FEMS Microb. Lett., 136: 13-18.

Strauss CJ, Kock JLF, Vanwyk PWJ, Viljoen BC, Botes PJ, Hulse G, Nigam S (2003). Inverse flocculation patterns in *Saccharomyces cerevesiae* .UOFS Y-2330. J. Inst. Brew.*,* 109(1): 3-7

Straver MH, Klijne JW, Smith G (1993). Cause and control of flocculation in yeast. J. Biotech., 11: 228- 232.

Taylor NW, Orton WI (1978). Aromatic compounds and sugars in flocculation of *Saccharomyces cerevesia*e. J. Inst. Brew. 84:113-114.

Teunissen A, Steensma HY (1995). The dominant flocculation genes of *Saccharomyces cerevesiae* constitute a new sub-telomeric gene family. Yeast, 11: 1001-113.

Versteepen KJ, Derdelinekx G, Delvaux FR (2000). Late fermentation expression of FLO1 in *Saccharomyces cerevesiae* .J. Am. Soc. Brew. Chem., 59(2): 69-76.

Vet Res Commun
DOI 10.1007/s11259-011-9507-5

ORIGINAL ARTICLE

Modulatory effects of non-steroidal anti-inflammatory drugs on the luminol and lucigenin amplified chemiluminescence of equine neutrophils

H. Benbarek · A. Ayad · G. Deby-Dupont · L. Boukraa · D. Serteyn

Accepted: 4 October 2011

Abstract The purpose of this study was to explore the potential modulation of equine neutrophil oxidative burst by a series of classical NSAIDs which was subsequently monitored by the luminol or lucigenin-enhanced chemiluminescence (CL) technique. A significant dose-dependent inhibition of the luminol CL was observed with the majority of investigated drugs. This inhibition was very significant for phenylbutazone and Indomethacin; while for aspirin, a higher concentration is required. The action of Ketoprofen was significant during the first 5 min and only when the concentration was above 1 mM. Indomethacin and acetylsalicylic acid result in an inhibition dose-dependent of luminol CL. On the other hand, the phenylbutazone showed an inhibiting effect when used either luminol or lucigenin though luminol is slightly better. When the ketoprofen is considered, an inhibiting effect of luminal CL was observed but less significant than the other NSAIDs investigated. The flunixin meglumine enhances strongly the CL.

H. Benbarek (✉)
Faculty of Life and Nature Sciences, University of Mascara,
29000, Mascara, Algeria
e-mail: benbarekh@yahoo.com

A. Ayad
Faculty of Life and Nature Sciences, University A. Mira,
06000, Bejaia, Algeria

G. Deby-Dupont · D. Serteyn
Center of Oxygen Research and Development (CORD),
Chemical Institute, B6a, University of Liège,
Sart Tilman,
4000 Liège, Belgium

L. Boukraa
Veterinary Institute, Ibn Khaldoun University,
14000, Tiaret, Algeria

Keywords Equine neutrophil · Luminol · Lucigenin · Chemiluminescence · NSAID

Introduction

Polymorphonuclear (PMN) leukocytes play an important role in host defense mechanisms (Stossel 1974; Baehner 1975) and are known to cause tissue damage at the inflammatory site (McCord 1974; Salin and McCord 1975). The leucocytes migrate towards tissues following injury or infection (Gallin and Wolff 1975). The adherence and ingestion of cells into phagosomes occur (Stossel 1975), after being destroyed through various pathways, including activation of their oxidative metabolism (Klebanoff 1975). The activation of PMN is characterized by the production of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH) (Carreras et al. 1994).

ROS are known as cytotoxic and implicated in the etiology wide spectrum of human diseases. They can also cause tissue damage by reacting with lipids at the cellular membrane, nucleotides in DNA (Ahsan et al. 2003), sulphydryl groups in proteins (Knight 1995) and cross-linking/fragmentation of ribonucleoproteins (Waris and Alam 1998). Experimental studies have shown the role of ROS in kidneys pathology by exhibiting their mediatory effects (Shah 1989; Greene and Paller 1991). Furthermore, the radical oxygen is thought to be involved in equine post-anesthetic myopathies (Serteyn et al. 1994) in colitis (McConnico et al. 2002) and joint diseases in horses (Dimock et al. 2000). Recently, researchers have suggested the involvement of ROS in lamimitis pathogenesis (Hurley et al. 2006).

Most related studies have used human PMN (Neal et al. 1987; Parij et al. 1998; Mouithys-Mickalad et al. 2000;

Published online: 21 October 2011

Abdullah et al. 2001) in which a number of non-steroidal anti-inflammatory drugs (NSAIDs) were found effective in modulating PMN oxidative activity (Van Dyke et al. 1982; Neumuller and Tohidast-Akrad 1994). The direct effects of NSAIDs on *ex-vivo* isolated equine neutrophils have not been fully investigated, especially in relation to their oxidative activity.

The present investigation aims to explore the potential modulation of equine neutrophil oxidative burst by a series of classical NSAIDs monitored by the luminol or lucigenin-enhanced chemiluminescence (CL) technique.

Material and methods

Isolation of equine neutrophils

Neutrophils were isolated from citrated fresh blood (5.26 g Na citrate/l) drawn from the jugular vein of horses. The donors were healthy horses, fed and bred in identical conditions and under no medical treatment (Faculty of Veterinary Medicine, Liege); aged 2 and 16 years. Each batch of neutrophils was obtained from 400 ml blood of the same horse and used immediately after isolation. A new neutrophil batch was used every day.

The neutrophils were isolated on a discontinuous density gradient formed from two solutions of Percoll (Sigma), a 1.087 kg/1 density solution overlaying a 1.108 kg/1 density solution (Pycock et al. 1987). The anticoagulated blood was layered on the surface of the gradient and centrifuged (400 x *g* for 20 min) at 20°C.

Neutrophils were collected in the interface between two solutions of the discontinuous gradient (>99% neutrophils; >98% viable cells by trypan blue exclusion test). After collection, they were washed and suspended in Hanks' balanced salt solution at pH 7.4 (HBSS: $CaCl_2$. $2H_2O$ 0.185 g/l; KCl 0.4 g/l; Mg $Cl_2.6H_2O$ 0.1 g/l; $MgSO_4.7H_2O$ 0.1 g/l; 14 KH_2PO_4 0.06 g/l; NaCl 8.0 g/l; $NaHCO_3$, 0.35 g/l; Na_2HPO_4 0.45 g/l; analytical grade salts from Merck) and used immediately (1.25 x 10^6 cells per assay). The neutrophils were obtained without plasma which remained on the top of the gradient and was discarded before collecting the cells.

As regards the ethical aspects, the experimental protocol was approved by the Animal Ethics Committee of the University of Liege. The experimental procedure was performed completely in vitro except for blood sampling of the animals, which was performed according to good veterinary practice.

Experimental drugs (NSAID)

The drugs were kindly provided by respective manufacturers: ketoprofen (Rhône-Poulenc Rorer, Paris, France), flunixin meglumine (Schering Corporation, Kenilworth, USA), indomethacin (Pharmacy of the hospital, CHU, Liege, Belgium), acetylsalicylic acid (Merck, VWRI, Belgium) and phenylbutazone (Pharmacy of the faculty, FMV). The poor water soluble drug, as the indomethacin, was dissolved by adding alkaline solution (NaOH). Thereafter, the pH was rapidly adjusted to 7.4. The PMA (phorbol 12-myristate 13-acetate, sigma) was prepared in dimethysulfoxide (1 mg/ml) and then diluted in distilled water.

Chemiluminescence (CL) measurement

The optimal conditions for the measurement of equine neutrophil CL have been previously described (Benbarek et al. 1996). The CL emitted by stimulated neutrophils was measured with a computerized 1251 luminometer (Bio-Orbit Oy-Turku, Finland), in circular polystyrene vials (Bio-Orbit Oy) with the CL amplifier luminol (3-aminophtalic hydrazide, Aldrich) at 10^{-3} M final concentration or lucigenin (bis-N-methyacridinium nitrate, Aldrich) at 0.4 x 10^{-3} M final concentration. The final volume of 600 µl was obtained by adding an adequate volume of HBSS buffer.

The light emission was expressed in mV. Each experimental point was repeated at least five times (*n* value). The neutrophils (1.25 x 10^6 cells per assay) were activated by the PMA. The assays were performed in phosphate buffer (pH 7.4) in presence or absence of drugs. Drug concentrations ranged from 0.1 to 5 mM. Chemiluminescence was monitored for 5 s every 5 min during half-an-hour. Percentage of CL response for each drug concentration was expressed as the percentage of control (PMN+PMA) value and the percentage inhibition was then calculated by subtracting this value from 100.

Statistical analysis

Data (light emission expressed in mV) were given as mean±SD. Statistical analyses were performed with the GraphPad Instat 3.0 software. CL values in presence of NSAIDs were compared to control CL values by using the parametric unpaired *t*-test. Results with $P<0.05$ were considered significant.

Results

A significant dose-dependent inhibition of the luminol CL ($P<0.05$) was observed within the majority of investigated drugs versus control (Fig. 1). This inhibition was very significant above 0.1 mM concentration for phenylbutazone and Indomethacin (Figs. 2 and 3, respectively), whereas

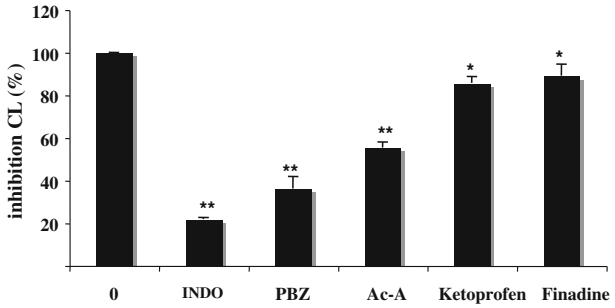


Fig. 1 Luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA. Results are expressed as mean±SD. A significant differences in concentrations NSAIDs vs. control (PMN+PMA+0 mM NSAIDs) are indicated by asterisk (*P<0.05 and **P<0.001)

acetylsalicylic acid requires a concentration of 1 mM (Fig. 4).

The 1 mM concentration was qualified as the strongest inhibitor of luminol CL under similar conditions. However, for lucigenin used as an enhancer, indomethacin showed an activating effect on the PMN ranging from 0.1 mM to 2.5 mM versus control (from 119.9% to 145%, respectively).

On the other hand, the use of acetylsalicylic acid caused an increase of the lucigenin CL (146.6%) (Fig. 4). Whereas, the use of phenylbutazone gave only a moderate activation (0.1 mM) in a concentration dependent inhibition (Fig. 2).

The action of Ketoprofen was significantly (P<0.001) higher than 1 mM during the first 5 min. As shown in Fig. 5, the effect of inhibition becomes visible above 0.1 mM after 10 min of use.

An activating effect of flunixin meglumine was observed during the first 5 min when concentrations from 0.1 to 0.5 were used. In contrast, the inhibiting effect was observed for the concentration of 1 and 5 mM (Fig. 6a). When applying the flunixin meglumine at a concentration of

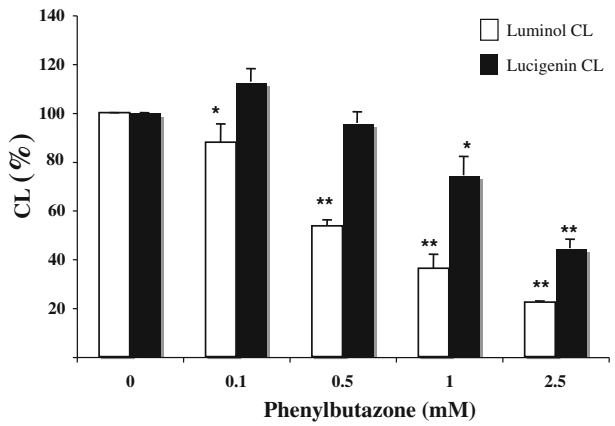


Fig. 2 Lucigenin and luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA. Results are expressed as mean±SD. A significant differences in concentrations phenybutazone vs. control (PMN+PMA+0 mM Phenybutazone) are indicated by asterisk (*P<0.05 and **P<0.001)

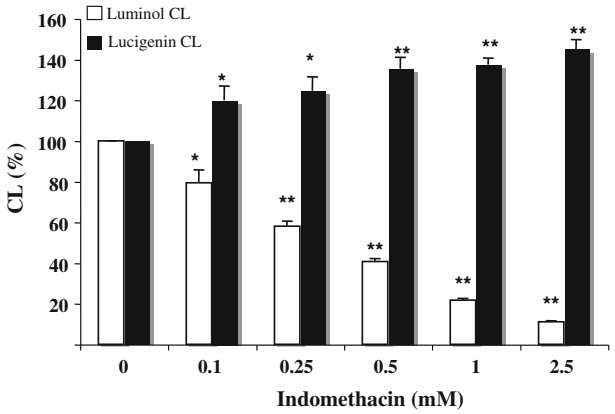


Fig. 3 Lucigenin and luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA. Results are expressed as mean±SD. A significant differences in concentrations indomethacin vs. control (PMN+PMA+0 mM Indomethacin) are indicated by asterisk (*P<0.05 and **P<0.001)

5 mM, about 24.7% of inhibition (versus control) was noticed during the first 5 min. But when the drug was applied for long time, e.g. 25 min, a clear activation effect was found (Fig. 6b).

Discussion

Our results are in accordance with those in literature which show that several NSAIDs were able to modulate the oxidative activity of PMN in a concentration-dependent manner. In fact, this modulation presents an anti or pro-oxidant effect. As a result indomethacin and acetylsalicylic acid generate inhibition of luminal CL which is dose-dependent. In contrast, the use of lucigenin give an activating effect.

On the other hand, the phenylbutazone showed an inhibiting effect when using either luminol or lucigenin.

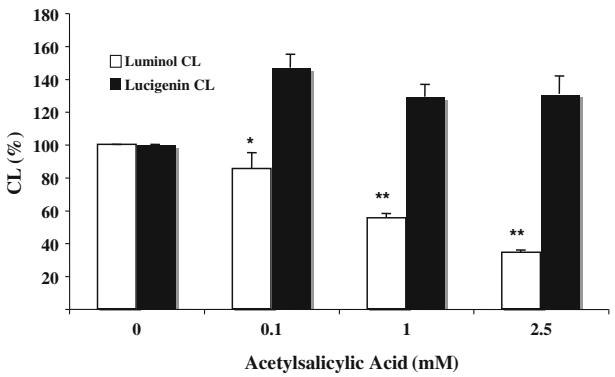


Fig. 4 Lucigenin and luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA. Results are expressed as mean±SD. A significant differences in concentrations acetylsalicylic acid vs. control (PMN+PMA+0 mM Acetylsalicylic Acid) are indicated by asterisk (*P<0.05 and **P<0.001)

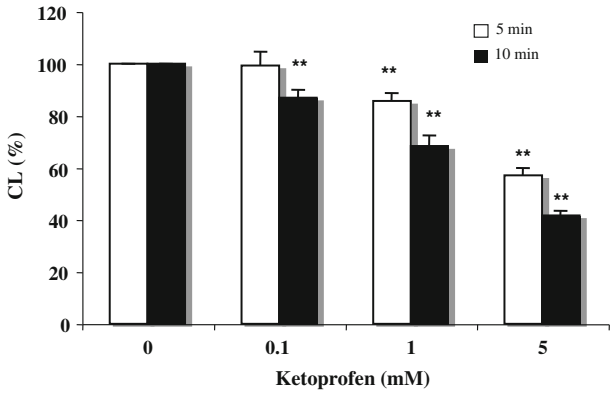


Fig. 5 Luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA: comparison of CL recorded 5 min (*white box*) and 10 min (*black box*) after stimulation. Results are expressed as mean±SD. A significant differences in concentrations Ketoprofen vs. control (PMN+PMA+0 mM Ketoprofen) are indicated by asterisk (**$P<0.001$)

Although, luminol is slightly better. When the ketoprofen is used, an inhibiting effect of luminol CL was observed. Though it was less important than the other NSAIDs investigated (Fig. 1). The flunixin meglumine strongly enhances the CL (Fig. 6b).

Concerning the magnitude and the type of effect (anti or pro-oxidant), luminol and lucigenin CL delivered some discrepancies between the results of both systems. These discrepancies could be explained by the fact that luminol-enhanced CL is the result of the molecular production by the dioxygenation. This happens when an excited amino-phthalate returns to the ground state with the emission of light. Due to its low molecular weight (177 Daltons), luminol enters the cell (Dahlgren and Briheim 1985) and reacts with hydrogen peroxide (H_2O_2) in the presence of myeloperoxidase. Limunol seems to be dependent on the H_2O_2- myeloperoxidase system and would essentially measure the intracellular production of active oxygen species (Allen 1986). Lucigenin-enhanced CL resulted from two-electron reduction linked to a dioxygenation. Because of its molecular weight (510 Daltons), lucigenin cannot enter the cell and reacts preferentially with the active oxygen species produced in the extracellular milieu (Dahlgren and Briheim 1985; Benbarek et al. 1996).

The fact that indomethacin inhibits the luminol CL while increasing the lucigenin has been already reported in human PMN (Minakami et al. 1993; Parij et al. 1998). It was suggested that indomethacin exerts a part of its effect by inhibiting the reaction of the H_2O_2-myeloperoxidase system (Minakami et al. 1993).

Therefore, the increase in the lucigenin CL should be an indirect effect of locking of such system and is thought to reflect an increase in the superoxide anion production (Gay et al. 1984; Minakami et al. 1993). In another study, it was suggested that there is an inhibiting effect of indometacin and acetylsalicylic acid when the PMN are stimulated by the FMLP (receptor mediated). Meanwhile, the PMN have an activating effect when they are stimulated by the PMA (Protein kinase mediated) (Minakami et al. 1993). These authors supported that the inhibiting action is due to the blocking of G protein.

In the present investigation, we attained the same conclusions when PMA has been used with various enhancers. The use of phenylbutazone and ketoprofen has shown the same results reported by several authors (Neal et al. 1987; Breda and Maureen 1992; Angelis-Stoforidis et al. 1998). In another experience, the results showed that ketoprofen has an inhibiting effect on luminal CL which could be related to the difference in concentrations tested (125 μM and 5 mM) (Parij et al. 1998). Unforeseen, the oxidative activity was further increased by the flunixin meglumine, drug widely used in colitis and sepsis treatment, and no inhibitory effect was observed even at high concentrations.

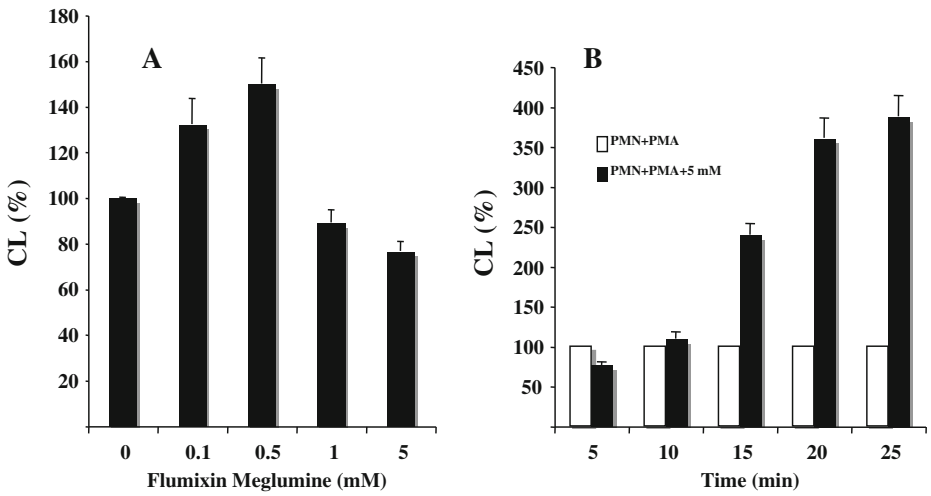


Fig. 6 a Luminol-enhanced chemiluminescence of 1.25 10^6 PMN after stimulation by PMA. **b** Comparison of CL recorded control (*white box*) and treated sample with 5 mM flunixin meglumine (*black box*) after stimulation. Results are expressed as mean±SD

Several authors reported that NSAIDs present a large diversity of effects that are not easy to explain (Neal et al. 1987; Breda and Maureen 1992; Minakami et al. 1993; Parij et al. 1998). It is not possible to give a clear explanation about the results because of the complexity and the non specificity of the measurement systems.

Only global effects of drugs are measured in such systems. They represent the result of complex interactions such as the inhibition of cyclo-oxygenase and prostaglandin synthesis, scavenging of reactive oxygen species produced by PMN, the inhibition of mechanism leading to the activation of the oxidative activity, and the ability of the drug to enter the cell and act on intracellular in addition to the activity on surface receptors.

References

Abdullah AS, Jawad AM, Al-Hashimi AH (2001) In vitro effect of different non20 steroidal anti-inflammatory drugs on human polymorphonuclear leukocyte activity measured by luminol-dependent chemiluminescence of the whole blood. Saudi Med J 22(4):360–365

Ahsan H, Ali A, Ali R (2003) Oxygen free radicals and systemic autoimmunity. Clin Exp Immunol 131(3):398–404

Allen RC (1986) Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis. Methods Enzymol 133:449–493

Angelis-Stoforidis P, Vajda FJ, Christophidis N (1998) Effects of non-steroidal anti4 inflammatory drugs (NSAIDs) on human polymorphonuclear leucocyte function in buffer and plasma. Clin Exp Rheumatol 16(6):703–708

Baehner RL (1975) Microbe ingestion and killing by neutrophils: normal mechanisms and abnormalities. Clinical Haematoogyl 4 (3):609–633

Benbarek H, Deby-Dupont G, Deby C, Caudron I, Mathy-Hartert M, Lamy M, Serteyn D (1996) Experimental model for the study by chemiluminescence of the activation of isolated equine leucocytes. Res Vet Sci 61(1):59–64

Breda MT, Maureen MD (1992) Cyclooxygenase-indepenent effecfs of non-steroidal anti-inflammatory drugs on the neutrophil respiratory burst. Biochem Pharmacol 43(3):413–418

Carreras MC, Pargament GA, Catz SD, Poderoso JJ, Boveris A (1994) Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. FEBS Lett 341(1):65–68

Dahlgren C, Briheim G (1985) Comparison between the luminol-dependent chemiluminescence of polymorphonuclear leukocytes and of the myeloperoxidase-HOOH system: influence of pH, cations and protein. Photochem Photobiol 41(5):605–610

Dimock AN, Siciliano PD, McIlwraith CW (2000) Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. Equine Vet J 32(5):439–22–443

Gallin JI, Wolff SM (1975) Leucocyte chemotaxis: physiological considerations and abnormalities. Clinical Haematology 4 (3):567–607

Gay JC, Lukens JN, English DK (1984) Differential inhibition of neutrophil superoxide generation by nonsteroidal antiinflammatory drugs. Inflammation 8(2):209–222

Greene EL, Paller MS (1991) Oxygen free radicals in acute renal failure. Miner Electrolyte Metab 17(2):124–132

Hurley DJ, Parks RJ, Reber AJ, Donovan DC, Okinaga T, Vandenplas ML, Peroni JF, Moore JN (2006) Dynamic changes in circulating leukocytes during the induction of equine laminitis with black walnut extract. Vet Immunol Immunopathol 110(34):195–206

Klebanoff SJ (1975) Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Seminary Hematology 12:117–142

Knight JA (1995) Diseases related to oxygen-derived free radicals. Ann Clin Lab Sci 25:111–121

McConnico RS, Argenzio RA, Roberts MC (2002) Prostaglandin E2 and reactive oxygen metabolite damage in the cecum in a pony model of acute colitis. Can J Vet Res 66(1):50–54

McCord JM (1974) Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science 185(150):529–531

Minakami K, Watanabe Y, Miyahara M, Kobuchi H, Kurashige T, Utsumi K (1993) Effect of indomethacin and aspirin on the TNF-alpha-induced priming and protein tyrosyl phosphorylation of human neutrophils. Physiology and Chemistry and Physics and Medical NMR 25(1):55–67

Mouithys-Mickalad AM, Zheng SX, Deby-Dupont GP, Deby CM, Lamy MM, Reginster JY, Henrotin YE (2000) In vitro study of the antioxidant properties of non steroidal anti-inflammatory drugs by chemiluminescence and electron spin resonance (ESR). Free Radic Res 33(5):607–621

Neal TM, Vissers MC, Winterbourn CC (1987) Inhibition by nonsteroidal anti2 inflammatory drugs of superoxide production and granule enzyme release by polymorphonuclear leukocytes stimulated with immune complexes or formyl-methionyl4 leucyl-phenylalanine. Biochem Pharmacol 36(15):2511–2517

Neumuller J, Tohidast-Akrad M (1994) Comparative in vitro investigations of the influence of mofebutazone, phenylbutazone and diclofenac on phagocytosis and respiratory burst of human peripheral blood leucocytes. Arzneimittelforschung 44(5):636–641

Parij N, Nagy AM, Fondu P, Neve J (1998) Effects of non-steroidal anti-inflammatory drugs on the luminol and lucigenin amplified chemiluminescence of human neutrophils. Eur J Pharmacol 352 (2–3):299–305

Pycock JF, Allen WE, Morris TH (1987) Rapid, single-step isolation of equine neutrophils on a discontinuous Percoll density gradient. Res Vet Sci 42(3):411–412

Salin ML, McCord JM (1975) Free radicals and inflammation. Protection of phagocytosine leukocytes by superoxide dismutase. J Clin Invest 56(5):1319–1323

Serteyn D, Pincemail J, Mottart E, Caudron I, Deby C, Deby-Dupont G, Philippart C, Lamy M (1994) Direct approach for demonstrating free radical phenomena during 17 equine postanesthetic myopathy: preliminary study. Can J Vet Res 58(4):309–312

Shah SV (1989) Role of reactive oxygen metabolites in experimental glomerular disease. Kidney Int 35(5):1093–1106

Stossel TP (1974) Phagocytosis. N Engl J Med 290(13):717–723

Stossel TP (1975) Phagocytosis: recognition and ingestion. Seminary Hematology 12(1):83–116

Van Dyke K, Peden D, Van Dyke C, Jones G, Castranova V, Ma J (1982) Inhibition by nonsteroidal antiinflammatory drugs of luminol-dependent human-granulocyte chemiluminescence and [3H] FMLP binding. Effect of sulindac sulfide, indomethacin metabolite, and optical enantiomers (+) and (−) MK830. Inflammation 6(1):113–125

Waris G, Alam K (1998) Attenuated antigenicity of ribonucleoproteins modified by reactive oxygen species. Biochem Mol Biol Int 45:33–45

International Journal of Infectious Diseases 15 (2011) e787–e794

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Beneficial effects of a strain of *Lactobacillus paracasei* subsp. *paracasei* in *Staphylococcus aureus*-induced intestinal and colonic injury

Farida Bendali *, Nassim Madi, Djamila Sadoun

Applied Microbiology Laboratory, Nature and Life Sciences Faculty, A. Mira University, Bejaia, Algeria

ARTICLE INFO

Article history:
Received 3 February 2011
Received in revised form 4 July 2011
Accepted 11 July 2011

Corresponding Editor: J. Peter Donnelly, Nijmegen, the Netherlands

Keywords:
Lactobacillus paracasei subsp. *paracasei*
Staphylococcus aureus
Antibiotic-associated diarrhea
Antibiotic resistance
Bacteriocins
In vivo study

SUMMARY

Objectives: The aim of this study was to investigate the in vitro and in vivo anti-staphylococcal activity of a lactic acid bacterial strain and its effect on the intestinal histological damage caused by *Staphylococcus aureus* infection.

Methods: *Lactobacillus paracasei* subsp. *paracasei* was isolated in our laboratory from breastfed newborn feces and identified phenotypically and genotypically. The strain was analyzed by spot-on-lawn and well diffusion assays for the production of bacteriocins against five antibiotic-resistant *S. aureus* strains isolated from the feces of hospitalized patients with antibiotic-associated diarrhea. The anti-staphylococcal activity of this strain was evaluated in fermented milk and in vivo using holoxenic rabbits.

Results: The strain was able to produce a bacteriocin-like substance active against the staphylococcal strains. A reduction of 2 log in *S. aureus* cell numbers was registered in co-culture with *L. paracasei* in fermented milk. Administration of skimmed milk containing *S. aureus* (10^7 cells/ml) to healthy rabbits induced a persistent diarrheal state 5 days after the challenge. Dissection of the rabbits and consequent histological observations showed damage and an atrophy of the intestinal and colonic mucosae of the diarrheal rabbits; in contrast an arrest of the diarrhea concomitant with recovery of the intestinal villi and the colonic crypts was observed in the rabbits treated with *L. paracasei*-fermented milk. Furthermore, the diarrheal state persisted in spite of a decrease in the level of *S. aureus* cells in the feces of the rabbits receiving sterile milk; this was in contrast to the rabbits treated with *L. paracasei*-fermented milk, in which the decrease in the *S. aureus* fecal number was associated with the arrest of the diarrhea.

Conclusions: *L. paracasei* could act as a potential barrier to prevent *S. aureus*- associated injury and might exert its effect on the staphylococcal enterotoxins or their target.

1. Introduction

Acute infectious diarrhea is a major public health concern worldwide, particularly in developing countries.[1] Antibiotic-associated diarrhea (AAD) represents a clinical entity leading to prolonged hospital stays and resulting in additional costs. Most AAD cases result from a transient disturbance of the function of the normal intestinal flora. The incidence of AAD varies between 5% and 25% depending on the antibiotic concerned.[2] *Staphylococcus aureus* can infect a wide range of host systems, and is responsible for about 5–20% of nosocomial infections;[3] it is recognized as one of the most frequent causes of AAD. This species was first suspected to cause AAD in 1954.[4] Since then several studies reporting the occurrence of methicillin-resistant *S. aureus* (MRSA) in the stools of patients with AAD and also data on specific toxin production have been published.[5–7] In two studies, enterotoxin-producing oxacillin-resistant *S. aureus* were found as the cause of nosocomial diarrhea.[8,9] *S. aureus* was first recognized as a cause of enterocolitis in the mid-20th century, and staphylococcal enterocolitis can occur as a complication of antibiotic therapy. It is a severe, acute diarrheal illness characterized by pus, blood, and heavy growth of *S. aureus* in the stools.[10] Early diagnosis and appropriate therapy can reduce the morbidity of such infections.

In recent decades there has been a growing consumer and scientific interest in probiotic bacteria, which are defined as "live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host beyond basic nutrition".[11] Probiotics play an important role in preventing overgrowth of potentially pathogenic bacteria and in maintaining the integrity of the gut mucosal barrier.[12] Lactic acid bacteria (LAB), which are present in large numbers among the normal animal and human gastrointestinal flora, are some of the most widely-used probiotics in the fermented food and beverage industries. *Lactobacillus casei* is already in use commercially to produce a number of fermented

* Corresponding author. Tel.: +213 34 21 43 33/35; fax: +213 34 21 47 62.
E-mail address: kamelea03@hotmail.com (F. Bendali).

1201-9712/$36.00 – see front matter
doi:10.1016/j.ijid.2011.07.003

milk products, and several reports have highlighted its ability to inhibit bacterial infections when orally administered to mice.[13–15] AAD could be prevented through the use of probiotic cultures. In this study, the beneficial effects of oral administration of probiotic *Lactobacillus paracasei* against *S. aureus* infection were investigated using young rabbits as an experimental infection model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. paracasei subsp. *paracasei* was used as the probiotic bacterium. This strain was selected from 38 LAB strains belonging to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Enterococcus* species, on the basis of both its strongest anti-staphylococcal activity and broadest inhibitory spectrum against 123 strains belonging to different Gram-positive and Gram-negative pathogenic bacteria (*Listeria monocytogenes*, *Bacillus cereus*, *S. aureus*, *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*). The strain was classified at the species level according to its carbohydrate fermentation pattern using API 50CH strips (BioMérieux sa, Marcy l'étoile, France), and its identity was further confirmed by sequencing the gene encoding the 16S rRNA. *L. paracasei* subsp. *paracasei* was grown in de Man–Rogosa–Sharpe (MRS) broth and agar (Merck GmbH, Darmstadt, Germany) at 37 °C.

As indicator bacteria for the antibacterial activity assay, antibiotic-resistant *S. aureus* strains screened in our laboratory were used. The strains were grown in brain heart infusion broth (BHIB) and Chapman broth and agar (Difco, Detroit, MI, USA) at 37 °C. For long term storage, the bacteria were kept at −18 °C in 10% glycerol in their culture broths. Before the experiments, each bacterial strain was subcultured at least two times (1%, v/v) at 24-h intervals.

2.2. Isolation of S. aureus strains

S. aureus strains included in this study were obtained from patients at Amizour Hospital, Bejaia, Algeria. Stools from 58 patients with nosocomial AAD (hospitalization for ≥72 h) were collected from December 2008 to March 2009. All samples were investigated for cultured *S. aureus*. All stools were cultured aerobically on Chapman agar (Merck) at 37 °C for 24 h. The bacterial strains were identified by typical phenotypic morphology and with the plasma-coagulase test, and on DNA-agar according to Guiraud and Rosec.[16]

2.3. Screening of antibiotic-resistant strains of S. aureus

Antimicrobial susceptibility was tested by agar diffusion method on Mueller–Hinton agar (Bio-Rad-Diagnostic Pasteur, Marnes la Coquette, France), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[17] Interpretation of antimicrobial susceptibility followed the recommendations of the Antibiogram Committee of the French Microbiology Society (CA-SFM).[18] Nine antibiotics (the most used in the area) were tested: penicillin G, oxacillin, cefotaxime, cefoxitin, gentamicin, erythromycin, pristinamycin, vancomycin, and ciprofloxacin. All antibiotics were from HiMedia Laboratories Pvt. Ltd.

2.4. Detection of anti-staphylococcal activity

L. paracasei subsp. *paracasei* was assessed for its ability to inhibit the antibiotic-resistant *S. aureus* strains using the spot-on-lawn and the well diffusion methods, as described previously.[19]

2.5. Characterization of the antibacterial activity

Filtered supernatant of *L. paracasei* subsp. *paracasei* was divided into three samples. Sample 1 was tested directly; sample 2 was adjusted to pH 6.5 with 1 N NaOH (Merck-Eurolab, Briare Le Canal, France) to rule out acid inhibition. Inhibitory activity from the hydrogen peroxide was ruled out by the addition of catalase (300 U/ml; C-3515, Sigma–Aldrich Chemie, Steinheim, Germany) to sample 3. The antagonistic activity of the three samples was determined in duplicate by the well diffusion assay as described above.

The sensitivity of antibacterial compounds to different proteolytic enzymes was tested. The supernatant of the LAB strain was treated with four proteases (all from Sigma–Aldrich Chemie, Steinheim, Germany): α-chymotrypsin, proteinase K, trypsin, and papain. All samples were adjusted to pH 6.5 with 1.0 mol/l NaOH (Merck), filter sterilized (0.22-μm pore-size Acrodisc syringe filters; Pall Gelman Laboratory, USA), and held for 1 h at 30 °C with the proteolytic enzymes. The treated and control samples (supernatant not treated with the enzymes and enzyme preparations) were heated at 100 °C for 5 min and then immediately cooled at 4 °C in order to inactivate the enzymes. The heat treatment of the control samples was performed in order to test the effect of heat on the bacteriocin-like substance contained in the supernatant and the effective inactivation of the enzymes. The residual activity of treated and control samples was determined by measuring the diameter of the inhibition zones in the well diffusion assay as described above.

2.6. Measurement of the extracellular potassium (K^+) ion concentration

Overnight cultures of *L. paracasei* subsp. *paracasei* strain and one *S. aureus* strain (S_3) in BHIB were diluted into fresh BHIB (10^8 and 10^6 cells/ml, respectively) and incubated at 37 °C, both individually and in co-culture. In the latter case, cultures of *L. paracasei* subsp. *paracasei* (10^8 cells/ml) were incubated in BHIB with *S. aureus* at 10^6 cells/ml. At intervals (1-h), samples were removed, centrifuged (8000 *g*, 20 min, 4 °C), and serially diluted into sterile distilled water. The appropriate dilutions were examined for the external concentrations of K^+ ions using a flame photometer (Jenway, Bibby Scientific France, Nemours, France), and each treatment was performed in triplicate.

2.7. Antagonism in milk

Overnight cultures of the *L. paracasei* subsp. *paracasei* strain and one *S. aureus* strain (S_3) in BHIB were diluted into fresh sterile skimmed-milk (market, Bejaia) and incubated at 37 °C, both individually and in co-culture. In the latter case, cultures of *L. paracasei* subsp. *paracasei* (10^8 cells/ml) were incubated in sterile skimmed-milk with *S. aureus* at 10^6 cells/ml. At intervals (2-h) over 24 h, samples were removed and serially diluted into sterile saline solution. The appropriate dilutions were plated on triplicate Chapman plates, and the average number of colonies obtained after 24–48 h incubation at 37 °C was used to establish the growth and survival curves. pH variation (every 2 h) was also monitored during the incubation period (24 h). The effect of acidified skimmed milk (pH values ranging from 5.5 to 4) on the growth and survival of *S. aureus* S_3 was tested. The pH of milk samples was adjusted stepwise from 5.5 to 4, in steps of 0.5-pH units, by using 1 M lactic acid (Sigma–Aldrich Chemie, Steinheim, Germany). Skimmed milk adjusted to pH 4.8 was also included in the test.

2.8. In vivo study – rabbits

All of the experimental rabbits ($N = 16$) used in this study were 30-day-old rabbits of the same species (*Oryctolagus cuniculus*) purchased from a local farm (Bejaia). The rabbits were brooded under identical conditions (metal cages of 50 cm width, maintained in a well-aired ambiance, at constant temperature

21 ± 1 °C) with a diet consisting of carrots and salads. Water was distributed in adapted feeding-bottles. Cages were cleaned every morning.

2.9. Determination of the staphylococcal intestinal content of rabbits

The 16 rabbits were divided into four groups of four rabbits each. Before proceeding to the study, a numeration of *S. aureus* in the rabbit fecal flora was carried out, and this was undertaken after 3 days of installation in the environment intended for them during the study period.

2.10. Protocol of inoculation of rabbits with the tested bacteria

Four groups of rabbits were subjected to this study. The investigation of each experimental group ($n = 4$) was repeated at least twice. The rabbits in three of the groups received an oral daily dose of 5 ml of skimmed milk containing *S. aureus* S_3 (10^7 colony-forming units (CFU)/ml) for 5 days (until the appearance of the diarrheal state). Each one of these three groups was then subjected to a specific treatment: (1) group 1 (control 1): rabbits did not receive any treatment; (2) group 2 (control 2): rabbits received an oral daily dose of 5 ml of sterile skimmed milk for 7 days; (3) group 3: rabbits received an oral daily dose of 5 ml of fermented milk with *L. paracasei* subsp. *paracasei* (2×10^9 CFU/ml, final pH 5.2) for 7 days. In this case, 24 h after the appearance of the persistent diarrheal state, rabbits were challenged with an *L. paracasei* subsp. *paracasei* strain with a final titer of 2×10^9 CFU/ml. Twenty-four hours after the *L. paracasei* subsp. *paracasei* challenge, the *S. aureus* fecal counts were determined. The fermented milk was prepared as follow: 9 ml of skimmed milk was inoculated with 10^5 CFU/ml of *L. paracasei* subsp. *paracasei* and then incubated for 18 h at 37 °C.

2.11. Analysis of feces

Feces were recovered before proceeding with the study and every day at 4 h post-treatment during the study period (12 days), as follows: during the 5 days of the infection period and the 7 days of treatment. Five fecal samples from a rabbit were collected in bottles and analyzed within 6 h. Approximately 5 g was diluted in 45 ml of peptone water (0.1%) and homogenized for 3 min. Samples were then serially diluted 10-fold using peptone water, and appropriate dilutions were plated on Chapman agar (Merck). Plates were incubated aerobically at 37 °C for 48 h.

2.12. Histological examination

In order to study the impact of the *S. aureus* infection on the intestinal and colonic mucosae, the rabbits in each group were dissected at the end of the study. Samples of the small and large intestine were immediately fixed in 10% neutral-buffered formalin. Preparation of the microscope slides was conducted according to Hould[20] in an anatomy and cytology–pathology laboratory (Bejaia). Tissues were dehydrated through graded ethanol series and acetone, followed by clarification with xylene, before being embedded in paraffin wax. Several 4-μm thick sections were cut from each sample and stained with hematoxylin and eosin. Slides were evaluated using a Zeiss optical microscope.

2.13. Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analysis was performed using the one-way analysis of variance (ANOVA) procedure of Statistica 5.5 (1999 edition) software. Differences among means were detected by paired Student's test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Isolation and screening of antibiotic-resistant S. aureus strains

Five strains of *S. aureus* were isolated and identified from the feces of patients who suffered from AAD. The five isolates were resistant to penicillin G and oxacillin, three were resistant to cefotaxime, and no resistance was detected for gentamicin, erythromycin, pristinamycin, vancomycin, and ciprofloxacin. All the strains were sensitive to cefoxitin except strain S_3, which was resistant and consequently resistant to methicillin and to all the beta-lactams. Strain S_3 was selected for the remainder of the study since it was the only strain possessing resistance to more than three antibiotics.

3.2. Detection of anti-staphylococcal activity

L. paracasei subsp. *paracasei* showed inhibition zones on lawns of *S. aureus* S_3. The inhibition zone diameter was 20 mm (Figure 1). The antagonistic isolate was also active in the well diffusion assay (18 mm).

3.3. Characterization of antibacterial activity

The culture supernatant of *L. paracasei* subsp. *paracasei* was characterized for its antibacterial compound. The supernatant showed an anti-*S. aureus* activity before (pH 4.2) and after neutralization (pH 6.5); nevertheless, the inhibition zone (6 mm) noticed with the neutralized supernatant was smaller than that with the non-neutralized one (18 mm). Inactivation by catalase was not observed and an inhibition zone diameter identical to that of the non-treated supernatant (18 mm) was recorded, which showed that the antibacterial activity did not result from hydrogen peroxide. The antibacterial substance produced by the isolate was completely inactivated by all proteases tested (α-chymotrypsin, trypsin, proteinase K, and papain). Heat treatment of the supernatants at 100 °C for 5 min had no effect on the antibacterial activity.

3.4. K^+ leakage measurement

To determine if the *L. paracasei* subsp. *paracasei* antibacterial activity had an effect on the permeabilization of the plasma membrane of *S. aureus* cells, expressed as external leakage of K^+

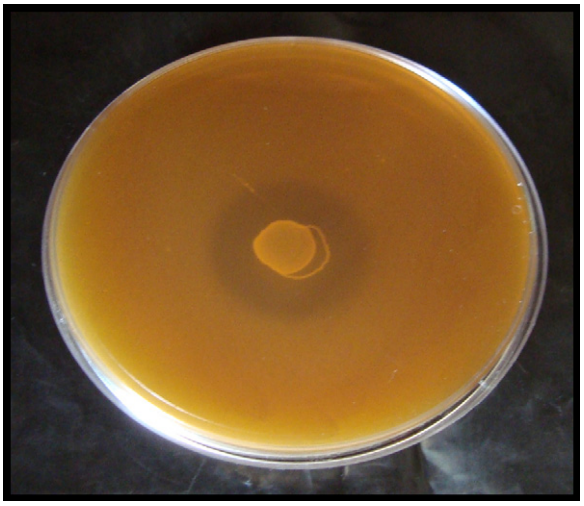


Figure 1. Spot test, *Lactobacillus paracasei* subsp. *paracasei* against *Staphylococcus aureus* S_3 with inhibition zone diameter of 20 mm.

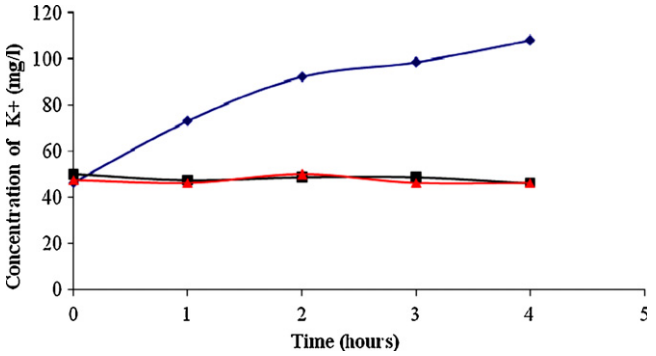


Figure 2. Potassium (K^+) concentration measurement in the culture supernatants (*Staphylococcus aureus* S_3 (▲), *Lactobacillus paracasei* (■), *S. aureus* S_3 + *L. paracasei* (◆).

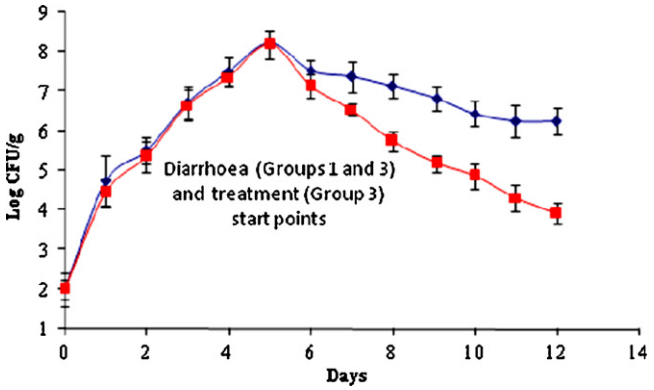


Figure 4. Evolution of *Staphylococcus aureus* numbers in rabbit feces during the in vivo study. ◆: group 1, control (rabbits infected with *S. aureus* but not treated at all during the experiment); ■: group 3, rabbits infected and treated daily with 5 ml of fermented skimmed milk containing *Lactobacillus paracasei* subsp. *paracasei* after the appearance of the diarrhea.

ions, the external concentration of this ion was measured in the culture supernatants of pure and co-cultures conducted in BHIB. During 4 h of incubation (Figure 2), the concentrations of K^+ ions in the pure culture supernatants of *S. aureus* S_3 and *L. paracasei* were unchanged, with values near 40 mg/l. This concentration of K^+ ions measured in pure culture is probably that of the culture medium (BHIB). In contrast, the concentration of K^+ ions in the supernatants of the co-cultures of *L. paracasei* with *S. aureus* S_3 was 61.8 mg/l. Thus, S_3 presents important leakage of the cytoplasmic content of K^+ ions in the presence of *L. paracasei*.

3.5. Co-cultures in skimmed milk

The results reported in Figure 3a show that *S. aureus* S_3 in pure culture presents a normal growth slope, with an exponential growth phase from the beginning of the culture. An increase in the level of cells to 4.5×10^8 CFU/ml with a decrease in the pH value to 6.1 after 24-h incubation were registered in pure cultures, whereas in co-cultures, S_3 showed a different behavior. During the first 4 h of culture, no significant difference was registered between the co-culture and the control ($p > 0.05$). The number of *S. aureus* S_3 cells in co-culture was unchanged between 6 and 20 h (10^7 CFU/ml), however growth was very weak compared to the control ($p < 0.05$), reaching a value of 4×10^6 CFU/ml (2 log lesser) after 24 h of culture. The decrease could be explained by the antagonistic effect of the *L. paracasei* strain towards *S. aureus* S_3; this could be the result of synthesis of inhibitory metabolites as demonstrated in the well diffusion assay. A large decrease in the pH value of the co-culture (to pH 4.8) compared to that of the pure culture (to pH 6.1) was registered after 24 h of incubation (Figure 3b). However this pH value had no inhibitory effect on *S. aureus* S_3, since an acidified skimmed milk adjusted to different pH values (5.5, 5, 4.8, and 4.5) was tested against the strain and no inhibitory effect was observed at pH 4.5 or 4.8.

3.6. In vivo study

The in vivo study was undertaken using holoxenic rabbits, so it was important to estimate the staphylococcal cell number in the rabbit feces before the beginning of the *S. aureus* S_3 infection procedure. After numeration, a mean of 2 log (10^2 cells of staphylococci/g of feces) was registered. The infection of rabbits (three groups) with a daily dose of 5 ml of fermented milk containing 10^7 CFU/ml of *S. aureus* resulted in diarrhea from the 5th day in all of the rabbits. During these 5 days, the *S. aureus* cell number increased from 10^2 to 1.7×10^8 CFU/g of feces (Figure 4), with no significant difference ($p > 0.05$) among the three groups. However, the diarrhea did not appear immediately after the administration of *S. aureus* S_3 (10^7 CFU/ml); this was probably due to the destruction of a large part of the strain cells before reaching the intestinal and colonic mucosae. This decrease in number could have been the result of a gastric acidity effect and the action of bile salts, or a barrier effect exerted by the indigenous intestinal microflora. The number of *S. aureus* reaching the intestine (neutral pH) will increase as a consequence of the adhesion and the multiplication of the strain. Furthermore, the daily administration of the contaminated milk probably allowed the increase in number of enteric *S. aureus* until it reached the infectious dose and consequently the minimal threshold concentration of enterotoxins necessary to induce diarrhea.

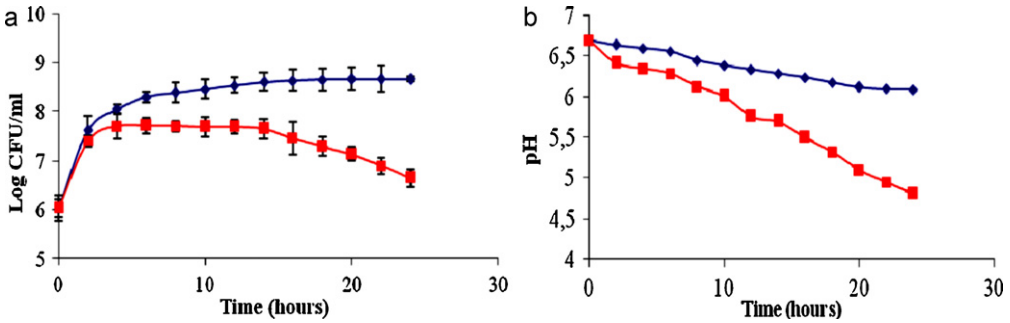


Figure 3. Growth kinetics of *Staphylococcus aureus* S_3 (a) and pH variation (b) in skimmed milk, in pure cultures (*S. aureus* S_3 at 10^6 CFU/ml) (◆) and in co-cultures with *Lactobacillus paracasei* (10^6 CFU/ml of *S. aureus* S_3 + 10^8 CFU/ml of *L. paracasei*) (■).

3.7. Treatment of the rabbits with L. paracasei-fermented milk

From the appearance of persistent diarrhea (day 5), the rabbits in group 2 received 5 ml of sterile skimmed milk daily, and those of group 3 received a daily dose of 5 ml of the same milk fermented by *L. paracasei* (2×10^9 CFU/ml), for 7 days. In the case of the rabbits in group 1 (untreated), the diarrhea persisted for 4 days after the arrest of the infection. During this period, the health state of the rabbits deteriorated (decrease in feeding, behavior changes). At the end of this period, it was noticed that the feces became softer, and the *S. aureus* number in the feces decreased from 1.7×10^8 to 1.7×10^6 CFU/g ($p < 0.05$) by the end of the experiment (day 12) (Figure 4). In the case of the treated rabbits (group 3), the number of *S. aureus* cells decreased at 24-h post-treatment. This decrease became significant (1.7 log; 1.58×10^8 to 3.5×10^6 CFU/g) after 48 h ($p < 0.05$) compared to rabbits in group 1. At the end of this period, the diarrhea disappeared and the sanitary status of the rabbits was improved with a less soft feces. At day 7 of treatment, the feces counts of *S. aureus* of the rabbits in group 3 diminished significantly (2×10^4 CFU/g) and seemed to return to the initial value (10^2 CFU/g) and were significantly ($p < 0.05$) lower than those in group 2 (2×10^6 CFU/g). This difference can be explained by the fact that rabbits in group 3 received milk fermented by *L. paracasei* and those in group 2 were fed only skimmed sterile milk. The results obtained at the end of this study show the beneficial effect of milk fermented by *L. paracasei* towards *S. aureus* diarrhea.

3.8. Histological observations

The lesions caused by *S. aureus* involve both the small intestine and colon. The histopathological evaluation of intestinal and colon tissue sections is shown in Figures 5 and 6. Intestinal and colon sections from rabbits challenged with *S. aureus* (Figures 5b and 6b) showed a high degree of intestinal and colonic injury, with pathological characteristics including severe loss of the mucosa and damage to the intestinal villi and colonic crypts, resulting in abnormal intestinal and colon wall morphology and the loss of intestinal and colonic structural integrity. In contrast, intestinal

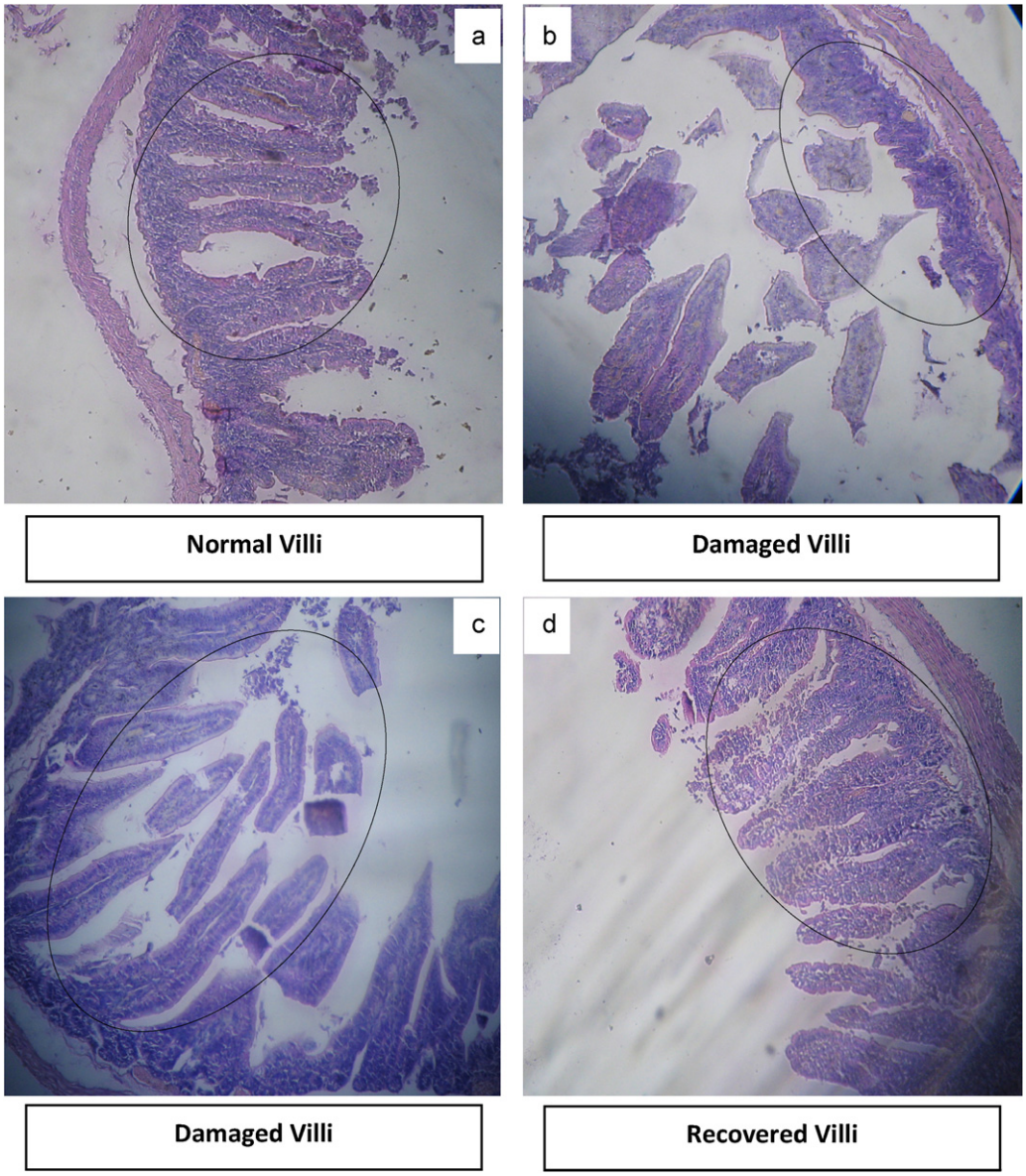


Figure 5. Histological observations of intestinal sections of dissected rabbits (8 × 40 × 5). (a) Healthy rabbit (control). (b) Diarrheal rabbit (group 1, first day of appearance of diarrhea). (c) Untreated diarrheal rabbit receiving sterile skimmed milk (group 2). (d) Treated diarrheal rabbit receiving skimmed milk fermented with *Lactobacillus paracasei* (group 3).

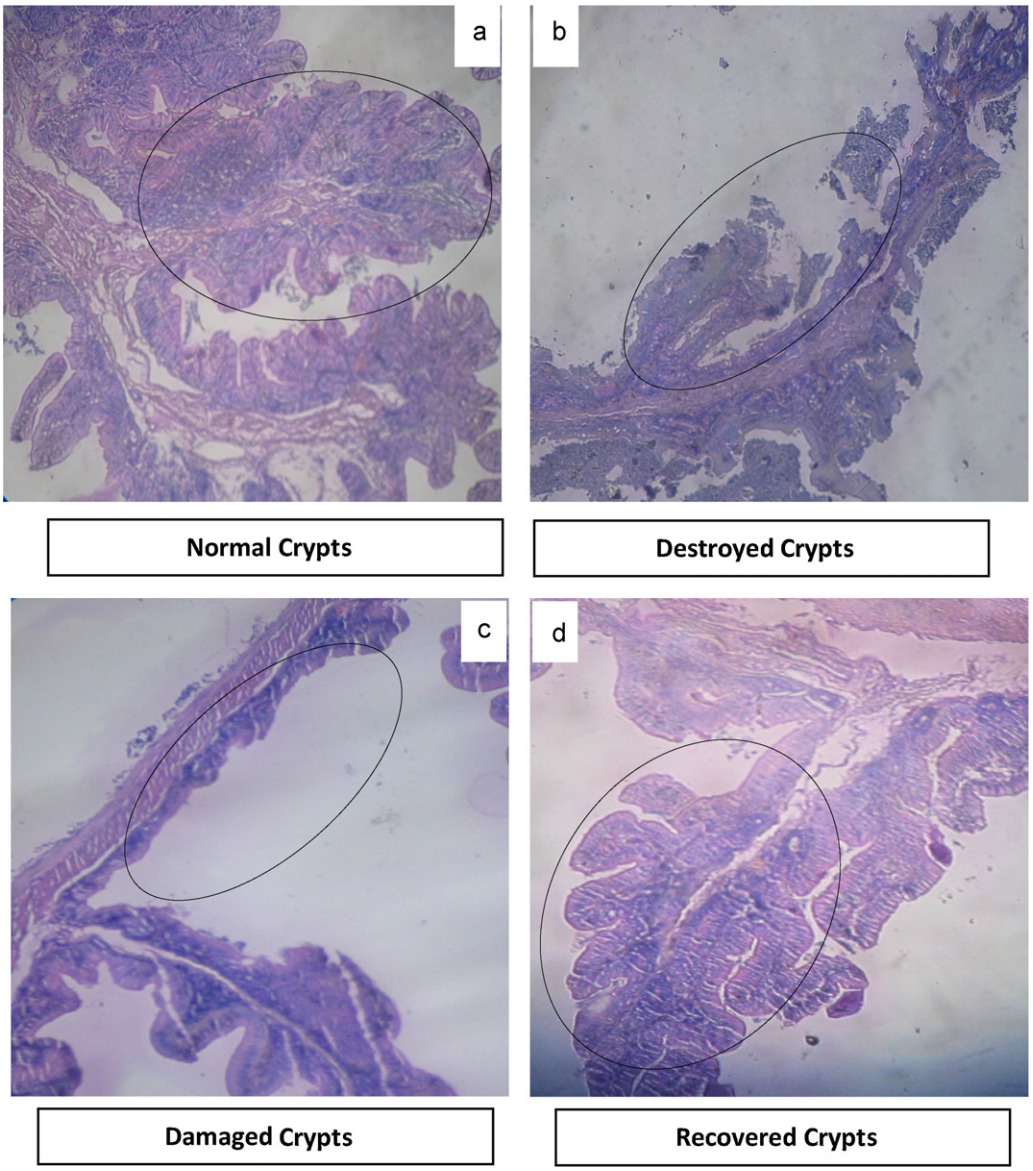


Figure 6. Histological observations of colon sections of dissected rabbits (8 × 40 × 5). (a) Healthy rabbit (control). (b) Diarrheal rabbit (group 1, first day of appearance of diarrhea). (c) Untreated diarrheal rabbit receiving sterile skimmed milk (group 2). (d) Treated diarrheal rabbit receiving skimmed milk fermented with *L. paracasei* (group 3).

and colon sections from unchallenged normal rabbits (Figures 5a and 6a) presented intact structures. As shown in Figures 5d and 6d, rabbits treated with *L. paracasei* after *S. aureus* infection also showed intact intestine and colon, with complete intestinal and colonic mucosae and restructured villi and crypts, which were compact and relatively long when compared with those of skimmed sterile milk-treated rabbits (Figure 5c and 6c). The integrity of the intestinal villi and the colonic crypts is considered an important criterion that reflects the degree of intestinal and colonic injury. As shown in Figures 5b and 6b, the villi and crypts of rabbits of the *S. aureus*-infected control group were significantly different from the normal villi and crypts of the unchallenged rabbits (Figures 5a and 6a). The villi and crypts of rabbits treated with *L. paracasei* were recovered compared to those of the *S. aureus*-infected control group. As reported by Chen et al.[21], intestinal pathological determination depends on the following criteria: intestinal and villous structural integrity, intestinal tissue bleeding, blood vessel dilation, intestinal villous morphology, loss of goblet cells, mucosal damage, intestinal crypt damage, and degree of inflammation. Rabbits treated with *L. paracasei* possessed restructured villi, whereas rabbits from the other two experimental groups – those receiving skimmed sterile milk and those receiving *S. aureus* only – had significantly destroyed villi compared to the unchallenged control group (Figures 5 and 6). Furthermore, histological observations of the intestinal and colon

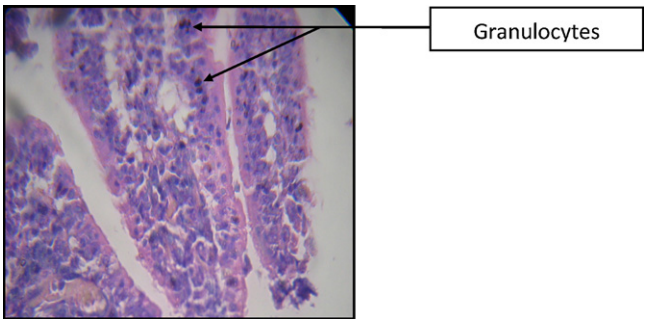


Figure 7. Granulocyte infiltration into the intestinal mucosa of diarrheal rabbits infected with a strain of *Staphylococcus aureus*.

sections of the rabbits in the untreated groups (groups 1 and 2) showed the presence of granulocytes at the site of infection. Figure 7 illustrates an example of an inflammatory reaction at the intestinal mucosa of a diarrheal rabbit.

4. Discussion

Staphylococcus aureus has acquired different types of resistance to anti-staphylococcal agents. Nowadays, 80% to 95% of hospital-associated *S. aureus* strains produce a penicillinase that inhibits the majority of penicillins.[22] In this study *S. aureus* was isolated from stool samples of patients with diarrhea who were hospitalized for ≥72 h at Amizour Hospital in Bejaia, Algeria. *S. aureus* was isolated from five (8.6%) of the fecal samples. All five *S. aureus* strains were identified as penicillin G- and oxacillin-resistant, three were resistant to cefotaxime, and only one strain (S_3) was resistant to cefoxitin and consequently resistant to methicillin and to all the beta-lactams. In the study of Flemming and Ackermann[23] that included 71 patients with *S. aureus*-positive stool samples, data on antimicrobial therapy showed that 18.3% of the patients had been treated with penicillins, and out of 198 *S. aureus*-positive stools, 14.6% of *S. aureus* strains were identified as methicillin-resistant. In another study, Baba-Moussa et al.[24] observed that among 21 *S. aureus* strains from patients with post-AAD, all strains harbored resistance to penicillin G and 95% to oxacillin/methicillin. However, despite the emergence of penicillin resistance, the antibiotic is still being used to treat staphylococcal infections, and despite the recognition of post-antibiotic *S. aureus*-associated diarrhea, treatment of infectious diseases is still based on antibiotic prescription.

Since most AAD cases result from a transient disturbance in the function of the normal intestinal flora, the use of certain probiotics, mainly LAB, could accelerate the reestablishment of the indigenous microflora and reduce adhesion and viability of adherent *S. aureus*. Indeed LAB, one of the groups of probiotics, make up a large group of microorganisms in the gastrointestinal tract of all humans and animals. The basic requirements for a LAB strain which is to be used as a probiotic have been described. It should be tolerant to acid and bile and be able to: adhere to the intestinal epithelium of the host; show an antagonistic activity against pathogenic bacteria; and retain its viability during processing and storage.[25] The strain used in this study (*L. paracasei* subsp. *paracasei*) has already been demonstrated to fulfill these criteria; it was able to adhere to Caco-2 cells and to prevent the adhesion of entero-pathogenic *E. coli* (EPEC) and *Salmonella* Typhimurium, and was also able to overcome simulated gastrointestinal conditions (paper submitted for publication). In this study we demonstrated that this strain is able to produce an antibacterial substance sensitive to proteases; consequently, this strongly suggests that this substance is proteinaceous in nature and can be designated as a bacteriocin-like substance. Furthermore the antibacterial activity was directed towards the plasma membrane, as shown by the leakage of K^+ ions, and this is a characteristic of bacteriocins, which target the plasma membrane of the sensitive strains.[26] Moreover, in another study (in progress), treatment of *L. paracasei* culture supernatant with either proteases or heat (80 °C for 2 h) affects its activities, and purification trials using Sepack cartridges and ion exchange chromatography procedures indicate, respectively, a high hydrophobicity and a cationic nature of the active substance (data not shown here). Nevertheless, further experiments are necessary to identify the chemical nature of the antibacterial compounds.

On the basis of the in vitro results, the *L. paracasei* subsp. *paracasei* strain can be used as a probiotic. In order to evaluate its benefits in the case of an *S. aureus* infection, an in vivo study was carried out. Since staphylococcal enterotoxins primarily affect primates, we used rabbits as the animal model for the study of the *S. aureus* intestinal infection. Due to the use of holoxenic rabbits, an estimation of the normal *S. aureus* intestinal level before contamination was mandatory, and the results indicate a level of 2 log. This level has already been described in healthy subjects as a part of the normal commensal microflora, as stated by Simonova et al.[27] who found that the total counts of staphylococci in the feces of rabbits varied from 1.00 to 1.79 log CFU/g. Administration of skimmed milk containing *S. aureus* (10^7 cells/ml) to healthy rabbits induced a persistent diarrheal state at 5 days after the challenge. Histopathological observations showed destruction of the micro-villi and colonic crypts, with granulocyte infiltration. According to Lin et al.[10] common features of *S. aureus* enterocolitis are acute inflammation and epithelial cell damage with disruption of the usual architecture. Biopsies of the ileum and colon have demonstrated superficial acute inflammation, mild chronic inflammation with increased eosinophils in the lamina propria, and basal plasmacytosis in the colon.[10] These alterations are probably the result of *S. aureus* toxins. Indeed this species can produce a wide variety of toxins, including the toxic shock syndrome toxin (TSST-1), enterotoxins, and the enterotoxin-like proteins that can function as superantigens, with both local and systemic effects.[28] Several enterotoxins have been detected in strains associated with AAD. In the study of Flemming and Ackermann,[23] 114 *S. aureus* strains produced enterotoxins in vitro. Interestingly, an arrest of the diarrhea concomitant with recovery of the intestinal villi and the colonic crypts was observed in the rabbits treated with *L. paracasei*-fermented milk. Probiotics, including lactobacilli, are known to produce organic acids such as lactic acid, which possess potent bactericidal activity.[21] Production of lactic acid, the major metabolite of LAB, is responsible for the associated decrease in pH, which may be sufficient to antagonize many microorganisms. However in this study, the pH value of the fermented milk administered to rabbits was not active against the strain of *S. aureus* tested. Consequently the inhibitory effect observed on *S. aureus* cells could be due to the action of the *L. paracasei* cells or their metabolites.

Our results are in agreement with those of Vesterlund et al.[29] who reported that in displacement in vitro assays, the amount of adherent *S. aureus* in human intestinal mucus was reduced 44% by *Lactobacillus rhamnosus* GG and 41% by *Lactococcus lactis* subsp. *lactis*, and that adherent probiotics reduced the viability of adherent *S. aureus* by 27–36%, depending on the strain, within 2 h. However in our study, the action of the *L. paracasei* subsp. *paracasei* appeared to be directed towards the staphylococcal toxins rather than the cells, since the diarrheal state persisted in spite of a decrease in the *S. aureus* cell level in the feces of the rabbits receiving sterile milk, in contrast to the rabbits treated with *L. paracasei*-fermented milk, in which the decrease in the *S. aureus* fecal number was associated with the arrest of the diarrhea. Indeed, treatment with the sterile milk induced a decrease in the *S. aureus* number in the feces (day 12); this is probably due to the arrest of the infection, but also to the intestinal microflora effect which prevents the adhesion of *S. aureus* ('barrier effect'), gastric pH, and bile salts. However, the persistence of the diarrhea could be explained by the presence of *S. aureus* enterotoxins which continued to act in the gastrointestinal tract of rabbits and induced diarrhea.

Thus, the strain of *L. paracasei* could be used as a potential barrier to prevent infection or as treatment of enteric staphylococcal infection, and might exert its effect on the *S. aureus* toxins or their target, probably by production of bacteriocin-like substances, which might contribute to the antibacterial effect in vivo. In our study we demonstrated that administration of milk containing *L. paracasei* subsp. *paracasei* to rabbits infected by multidrug-resistant *S. aureus* arrests the diarrhea and improves the reestablishment of the intestinal and colonic mucosae.

Acknowledgements

We thank C. Callon, INRA of Aurillac, France, for 16S rRNA sequencing of the *L. paracasei* subsp. *paracasei* strain. We gratefully acknowledge Nadia Khellef (Pasteur Institute of Paris, France) for providing the API 50CH strips. We wish to thank Dr Amrane, anatomo-pathologist, for her help in the preparation, observation, and interpretation of the histological slides. The expert technical assistance of the Amizour Hospital staff is greatly appreciated.

Conflict of interest: No conflict of interest

References

1. Scopetti F, Orefici G, Biondi F, Benini F. *Staphylococcus aureus* resistant to methicillin and gentamicin as a cause of outbreak of epidemic enteritis in a hospital. *Boll Ist Sieroter Milan* 1983;**62**:406–11.
2. Kaltenbach G, Heitz D. Diarrhées associées aux antibiotiques chez le sujet âgé. *Rev Med Interne* 2004;**25**:46–53.
3. Emori TG, Gaynes RP. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev* 1993;**6**:428–42.
4. Oeding P, Austarheim K. The occurrence of staphylococci in the intestinal content after treatment with antibiotics. *Acta Pathol Microbiol Scand* 1954;**35**:473–83.
5. McDonald M, Ward P, Harvey K. Antibiotic-associated diarrhoea and methicillin-resistant *Staphylococcus aureus*. *Med J Aust* 1982;**1**:462–4.
6. Adesiyun AA, Lenz W, Schaal KP. Phage susceptibility, enterotoxigenicity and antibiograms of *Staphylococcus aureus* strains isolated from human wounds and diarrhoea. *Zentralbl Bakteriol* 1992;**277**:250–9.
7. Gravet A, Rondeau M, Harf-Monteil C, Grunenberger F, Monteil H, Scheftel JM, et al. Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhoea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-LukD. *J Clin Microbiol* 1999;**37**:4012–9.
8. Rhee KY, Soave R, Maltz C. Methicillin-resistant *Staphylococcus aureus* as a cause of antibiotic-associated diarrhea. *J Clin Gastroenterol* 2004;**38**:299–300.
9. Boyce JM, Havill NL. Nosocomial antibiotic-associated diarrhea associated with enterotoxin-producing strains of methicillin-resistant *Staphylococcus aureus*. *Am J Gastroenterol* 2005;**100**:1828–34.
10. Holeckova B, Holoda E, Fotta M, Kalinacova V, Gondol J, Grolmus J. Occurrence of enterotoxigenic *Staphylococcus aureus* in food. *Ann Agric Environ Med* 2002;**9**:179–82.
11. Food and Agriculture Organization of the United Nations (FAO); World Health Organization (WHO). Joint FAO/WHO expert consultation on the evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Córdoba, Argentina, 1-4 October 2001. Available at: http://www.fao.org/es/esn/food/foodandfood_probio_en.stm (accessed 27.01.2011).
12. Bengmark S. Ecological control of the gastrointestinal tract. The role of probiotic flora. *Gut* 1998;**42**:2–7.
13. Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, et al. In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. *Appl Environ Microbiol* 2004;**70**:518–26.
14. Yasui H, Kiyoshima J, Hori T. Reduction of influenza virus titer and protection against influenza virus infection in infant mice fed *Lactobacillus casei* Shirota. *Clin Diagn Lab Immunol* 2004;**11**:675–9.
15. Dogi CA, Galdeano CM, Perdigon G. Gut immune stimulation by non pathogenic Gram(+) and Gram(−) bacteria: comparison with a probiotic strain. *Cytokine* 2008;**41**:223–31.
16. Guiraud JP, Rosec JP. Pratique des normes en microbiologie alimentaire. Paris, Association Française de Normalisation (AFNOR); 2004.
17. National Committee for Clinical Laboratory Standards (NCCLS). Performance standards for antimicrobial susceptibility testing. Supplement M100-S12. Wayne, PA: NCCLS; 2002.
18. Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM). Recommandations 2009. Paris, France: Société Française de Microbiologie; 2009.
19. Bendali F, Gaillard-Martinie B, Hebraud M, Sadoun D. Kinetic of production and mode of action of the *Lactobacillus paracasei* subsp. *paracasei* anti-listerial bacteriocin, an Algerian isolate. *LWT Food Sci Technol* 2008;**41**:1784–92.
20. Hould R. Techniques d'histologie et de cytopathologie. Montreal: Décarie; 1984.
21. Chen HL, Lai YW, Chen CS, Chu TW, Lin W, Yen CC, et al. Probiotic *Lactobacillus casei* expressing human lactoferrin elevates antibacterial activity in the gastrointestinal tract. *Biometals* 2010;**23**:543–54.
22. Daurel C, Leclercq R. L'antibiogramme de *Staphylococcus aureus*. *Rev Franc Labo* 2008;**407**:81–90.
23. Flemming K, Ackermann G. Prevalence of enterotoxin producing *Staphylococcus aureus* in stools of patients with nosocomial diarrhea. *Infection* 2007;**35**:356–8.
24. Baba-Moussa L, Ahissou H, Azokpota P, Assogba B, Atindéhou MM, Anagonou S, et al. Toxins and adhesion factors associated with *Staphylococcus aureus* strains isolated from diarrhoeal patients in Benin. *African J Biotech* 2010;**9**:604–11.
25. Fuller R. Probiotics in man and animals. *J Appl Bacteriol* 1989;**66**:365–78.
26. Oscariz JC, Pisabarro AG. Classification and mode of action of membrane active bacteriocins produced by Gram-positive bacteria. *Int Microbiol* 2001;**4**:13–9.
27. Simonová M, Fotta M, Lauková A. Characteristics of *Staphylococcus aureus* isolated from Rabbits. *Folia Microbiol (Praha)* 2007;**52**:291–6.
28. Kotler DP, Sordillo EM. *Staphylococcus aureus* enterocolitis: a review. *Gastroenterol Hepatol* 2010;**6**:115–9.
29. Vesterlund S, Karp M, Salminen S, Ouwehand AC. *Staphylococcus aureus* adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria. *Microbiology* 2006;**152**:1819–26.

Process Biochemistry 46 (2011) 519–525

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Production and partial characterization of xylanase produced by *Jonesia denitrificans* isolated in Algerian soil

Boucherba Nawel[a], Benallaoua Said[a], Copinet Estelle[b], Hebal Hakim[a], Francis Duchiron[b,*]

[a] *Laboratory of Applied Microbiology, Faculty of Nature Science and Life, University of Bejaia, Targa Ouzemmour 06000, Algeria*
[b] *UMR FARE 614 INRA/URCA, Laboratory of Industrial Microbiology, UFR Sciences, University of Reims Champagne-Ardenne, Moulin de la Housse, BP 1039, 51687 Reims, France*

ARTICLE INFO

Article history:
Received 16 June 2010
Received in revised form
20 September 2010
Accepted 3 October 2010

Keywords:
Jonesia denitrificans
Production
Xylanase
Xylan

ABSTRACT

Jonesia denitrificans BN-13 was isolated from a sample collected from Algerian soil. This strain produced extracellular xylanases, the best xylanolytic activity is obtained in a medium made up of xylan (7 mg/ml), extract of yeast (2.5 mg/ml), NaCl (3 mg/ml), NH_4Cl (6 mg/ml), and of $MgSO_4$ (0.3 mg/ml). In fermentor it also proved that the maximum of xylanolytic activity (10.81 U/ml) was observed at the end of the growth phase. Best enzyme activity was observed at the temperature of 50 °C and at pH 7.0. In the presence of metal ions such as Mn^{2+}, the activity of the enzyme increased, the enzyme retained 50% of its activity after 7 h at 50 °C. As such, this enzyme could be considered as a thermotolerant biocatalyst being interesting for biotechnological applications.

At the best of our knowledge, this is the first report on the production of xylanase by this bacterium and especially the genus Jonesia.

1. Introduction

Hemicellulose is the second most abundant renewable biomass and accounts for 25–35% of lignocellulosic biomass which is considered to be a promising biomass feedstock for the production of biofuels, β-1,4-xylan is the major hemicellulose component [22]. Several enzymes are required to complete hydrolysis and assimilation of xylan, including β-1,4-xylanase (1,4-β-D-xylanohydrolase; E.C. 3.2.1.8) that cleaves glycosidic bonds to produce xylooligosaccharides and β-xylosidase responsible for the final breakdown of small xylooligosaccharides into xylose [39].

These xylanolytic enzymes have attracted a great deal of attention because of their biotechnological potential in various industrial processes, such as food industry in order to enhance the digestibility of animal feeding, as well as in the textile industry, pulp and paper industry for bleaching purposes, resulting in a decrease of chlorine utilization and consequently lowering environmental impact [10].

Xylanase in synergism with several enzymes can be used for the generation of biological fuels, such as ethanol and xylitol from lignocellulosic biomass [6,9,32].

Actinomycetes are gram positive filamentous bacteria widely cited in the literature as producers of important industrial enzymes involved in lignocellulose degradation [14,20].

Xylanases observed in mesophilic actinomycetes are mainly of endotype (β-1,4-xylan xylanohydrolase), which have been found, particularly in several species of *Streptomyces* such as *Streptomyces olivaceoviridis* E-86 [12], *Streptomyces viridosporus* T7A [42], *Streptomyces lividans* 66 [21] and *Streptomyces cyaneus* SN32 [40]. In the context of systematic study of biodiversity in Algerian soil we have selected a strain of *Jonesia* sp. Screened from among 20 xylanolytic strains that is able to produce xylanases. This research aimed at optimizing the composition of culture medium (carbon source, nitrogen source and salts), we have also screened the culture condition including incubation times, temperature and initial pH of medium for xylanase production by *Jonesia* sp. Grown on birchwood xylan as a main substrate in liquid-state culture and examine the effects of pH, temperature and metal ions on crude xylanases to just have an idea on the mode of action of xylanases produced by *Jonesia denitrificans* BN13.

Until now, no study on xylanases of any *Jonesia* species has been appeared in scientific literature.

2. Materials and methods

All analytical and media chemicals were purchased from Sigma.

2.1. Isolation and screening of xylanase-producing strains

2.1.1. First screening

Five soil samples were collected from the garden situated in Béjaia in the north of Algeria in September 2006. After removing approx. 3 cm of soil from the surface, samples were taken to a depth of 10 cm. Each soil sample was crushed, mixed thoroughly for isolation of xylanolytic bacteria. Sub samples of 1 g were suspended in 100 ml sterile distilled water.

* Corresponding author. Fax: +33 03 26 91 39 16.
E-mail address: francis.duchiron@univ-reims.fr (F. Duchiron).

1359-5113/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2010.10.003

Mixtures were allowed to settle then serial dilutions were prepared. From each dilution, 0.1 ml was taken and spread on agar plates of medium containing birchwood xylan 7 g/l, yeast extract 2 g/l, NaCl 2.5 g/l, NH_4Cl 5 g/l, KH_2PO_4 15 g/l, Na_2HPO_4 30 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l and agar 15 g/l [43]. The plates were incubated at pH 7.0, 37 °C, for 2 days. Those colonies that grew well under such conditions and showed a clear zone around the colonies were isolated and retained for second screening.

2.1.2. Second screening

Those isolated organisms from the first screening were cultured in liquid media containing the same component but without agar at pH 7.0 in Erlenmeyer flasks. After incubation on a rotary shaker (37 °C, 100 rpm) for 2 days, the culture broth was centrifuged at 10,000 × *g* for 20 min, and the supernatants were collected for enzyme assay. The strain was maintained on nutrient agar and used throughout the study for the production of xylanolytic enzymes.

2.1.3. Physiological and biochemical identification

Physiological and biochemical tests were done using the substrate panel for Jonesia identification, the growth temperature (4, 10, 15, 20, 25, 30, 35, 40, 45, and 50 °C), pH values (4, 5, 6, 7, 8, 9, 10, 11, and 12) and salt tolerance at 2%, 5%, 7.5%, 10%, 12%, 15%, 17.5% (g/l) NaCl were determined [36,37].

2.1.4. 16S rDNA sequencing

Total DNA of the strain BN13 was isolated using Puregene DNA isolation Kit from Gentra. The 16S rRNA region was amplified with Taq DNA Polymerase (Westburg) using the primers PRO-26: AGAGTTTGATCCTGGCTCAG universal forward primer bacterial 16S rRNA = BSF8/20 of UIA database and PRO-27: AAGGAGGTGATCCAGCCGCAG (universal reverse bacterial 16S rRNA = BSR1541/of UIA database). The PCR products were sequenced using the big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.1.5. Analytical methods

Assays for crude xylanase were performed using 0.5% (w/v) birchwood xylan in sodium phosphate buffer (50 mM, pH 7.0). The reaction mixture was composed of 0.9 ml substrate and 0.1 ml crude enzyme. The mixture was incubated in a water bath at 50 °C for 10 min [44]. The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method in which the reaction was stopped by adding 1.5 ml of DNSA acid reagent. A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min [28]. After cooling the reaction tubes to room temperature, absorbance was measured at 540 nm with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 μmol xylose/min/ml under the above mentioned conditions.

The cell growth was estimated by soluble protein estimation following Bradford's method with bovine serum (BSA) as the standard [7] and by measuring the optical density at 600 nm.

To study the properties of the xylanases, the strain was grown in 4-l fermenter at 37 °C on xylan medium, pH 7.0 containing xylan as carbon source. The culture was harvested in the stationary growth phase, after 35 h, and centrifuged (10,000 × *g* for 20 min). The supernatant was ultrafiltered by using a 10,000 MW cut-off ultrafilter (Amicon, USA), the sample was then kept at 4 °C in the refrigerator.

2.1.6. Optimization of culture medium for xylanase production

The concentrations of nutrients such as magnesium (0–0.5 mg/ml), nitrogen (0–10 mg/ml), yeast extract (0–4 mg/ml) and NaCl (0–4 mg/ml) in the culture medium (first screening medium) were varied to optimize xylanase production. Xylanase activity was analyzed after growing the culture for 2 days at 37 °C in the culture medium containing birchwood xylan wherein one of the nutrient concentrations was varied.

2.1.7. Temperature and Initial pH in the culture as a function of growth and xylanase production

The growth and the xylanase activity were assayed at different temperatures ranging from 30 °C to 45 °C.

The effect of varying ranges of initial pH value from 4 to 10 on growth and xylanase production was studied.

The pH in the initial culture was adjusted by using hydrochloric acid (1 M) or sodium hydroxide solution (1 M).

2.1.8. Effect of carbon source on growth and xylanase production

Effect of various carbon sources on the growth and xylanase production was assessed by culturing the isolate in the first screening medium (pH 7.0) at 37 °C. Either of birchwood xylan, oat spelt xylan, wheat bran, delignified and unstarch wheat bran, wheat arabinoxylan, xylose, maltose, arabionose, glucose, lactose, starch, galactose, and carboxymethylcellulose was used as carbon source (0.7%, w/v) individually in liquid medium, After 2 days of the culture growth, xylanase activity was estimated.

2.1.9. Growth and xylanase production profiles

2% pre-culture was used to inoculate 1.5l fermentor containing the xylan defined medium at 37 °C for 2 days. During fermentation, the medium was subjected to 2 vvm of aeration and 250 rpm of agitation, culture samples were collected at 1 h intervals during the cultivation period. Immediately after collection the samples were centrifuged at 4 °C and 10,000 × *g* for 20 min. Supernatants were analyzed for xylanase activity as described.

2.2. Partial characterization of xylanases

2.2.1. Effect of temperature on xylanase activity

Optimal temperature was determined by assaying the enzyme activity between 30 °C and 80 °C, by incubating the enzyme along with the substrate for 10 min at the respective temperature.

2.2.2. Effect of pH on xylanase activity

Relative xylanase activity was determined using 0.5% (w/v) birchwood xylan at various pHs. The pH range used varied from 4 to 10. Three different buffers (0.05 M) were used. Citrate buffer was used for pH 4–6; phosphate buffer was used for pH from 6 to 8 and Tris–HCl buffer for pH 8–10.

2.2.3. Thermostability

The thermal stability was determined at temperatures 30, 40, 50 and 60 °C, after incubation in the absence of substrate for different times (from 0 to 10 h); remaining xylanase activity was measured as described above.

2.2.4. Effect of metal ions and reagents on activity

The effect of various additives on xylanase activity was determined by incubating enzyme at 50 °C for 30 min.

The different monovalent and divalent metals like (1 mM) Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, NH_4^+, Hg^{2+}, K^+, Ni^{2+}, and Cd^{2+}. EDTA, SDS, DTT and PMSF were taken as additives.

Relative activity was expressed as the percentage of the activity observed in the absence of any compound.

3. Results and discussion

3.1. Identification of strain BN 13

Twenty candidates were obtained from the first screening; the strain BN13 showed very high capacity in producing xylanase, this isolate was motile, rod-shaped, catalase-positive and aerobic. Optimal growth temperature was 37 °C; optimal pH was 7; optimal salt concentration was 2–12 g/l NaCl.

From the analysis of the almost-complete 16S rRNA gene sequence, this strain was found to be similar to *J. denitrificans* X83811 (99.6% sequence similarity).

Through the alignment of homologous nucleotide sequence of known bacteria, phylogenetic relationships could be inferred, and the phylogenetic position of the strain and related strains based on the 16S rDNA sequence is shown in Fig. 1.

According to the biochemical criteria and comparison of 16 S rRNA gene sequence, the strain BN 13 was identified as strain of *J. denitrificans* and named *J. denitrificans* BN 13, belonging to actinomycetal order.

3.2. Optimization of culture medium for xylanase production

Nutrient concentrations in the growth medium influenced the production of xylanases by the bacterium. *J. denitrificans* BN-13 showed the highest xylanase production when the growth medium was supplemented with yeast extract (2.5 mg/ml), sodium chloride (3 mg/ml), ammonium chloride (6 mg/ml) and magnesium sulfate (0.3 mg/ml) (Fig. 2a–d).

3.3. Effect of temperature and initial medium pH on growth and xylanase production

The strain showed a greater xylanase production at neutral, slightly acidic pH (6.0 and 6.5) and alkaline pH (8.0–9.5) than at an acidic pH (Fig. 3a). In contrast, the study of the effect of initial pH of growth medium on xylanase of an another actinomycetal *Streptomyces olivaceoviridis* E-86 showed that high xylanase activities were obtained at pH 5.0 and pH 5.5 but very low xylanase activities at pH 6.5 and 7.0 [12].

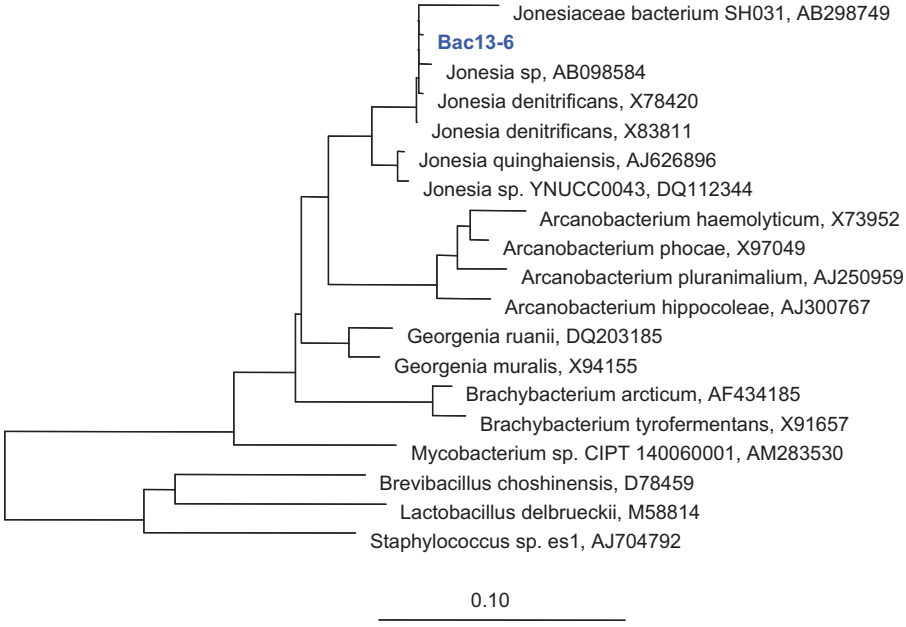


Fig. 1. The phylogenetic dendrogram of *Jonesia denitrificans* BN-13 (Bac-13-6) and related strains based on the 16 S rDNA sequence.

The strain *J. denitrificans* BN-13 grew well at 30 °C, 35 °C and 37 °C (optimum growth), but the growth decrease at 40 °C and 45 °C, The highest xylanase activity was observed when the strain grew at 37 °C (Fig. 3b).

3.4. Effect of carbon source on the growth and production of xylanase

12 carbon sources were evaluated for growth and xylanase production. The results showed that the strain grew well on birchwood xylan, starch, maltose and wheat bran. To optimize the xylanase production by *J. denitrificans* BN13, the same carbon source were used. Among these, birchwood xylan, xylose, lactose and wheat bran were the best carbon sources for enzyme production (2.54, 1.14, 1.78, and 1.51 U/ml respectively) (Fig. 3c). This result is not unusual as commercial xylan was known to induce high levels of xylanase activities in other microorganisms [18,2,23,10,16,26,27,29]. Here in this study wheat bran induced the production of xylanase, there are reports related to the effectiveness of wheat bran as strong inducers of xylanase activity [35], *Streptomyces* sp. CD3 use wheat bran as substrate and produced 2.21 U/ml of xylanase [39].

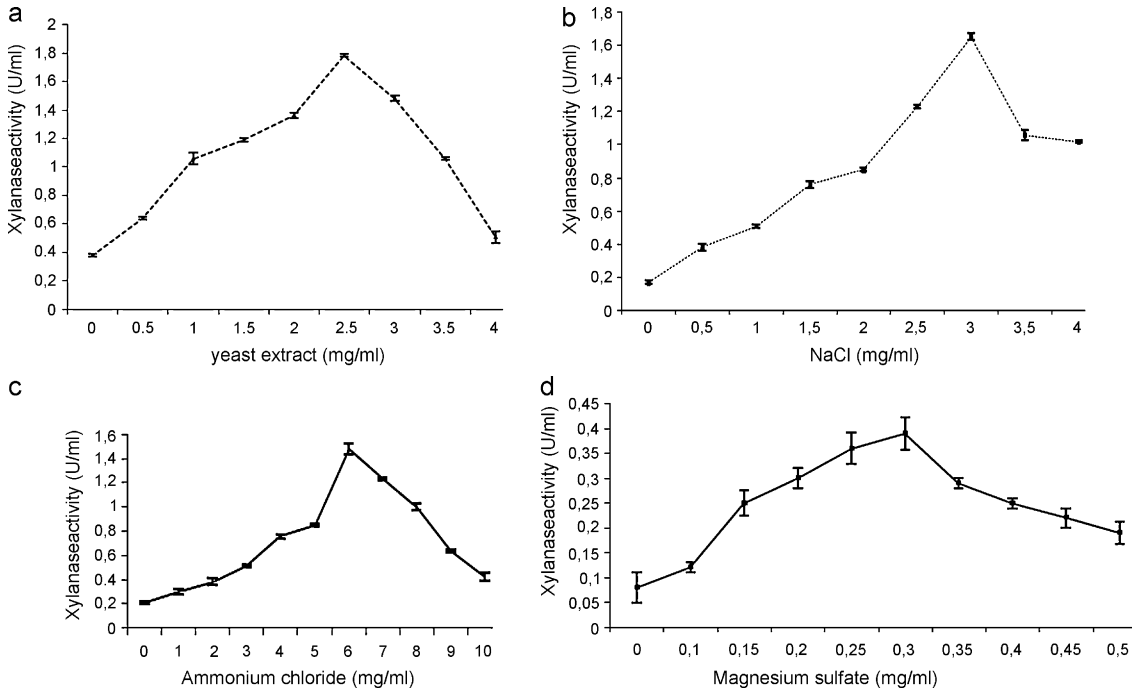


Fig. 2. Effect of yeast extract (a), sodium chloride (b), ammonium chloride (c) and magnesium sulphate (d) on the production of xylanase by *Jonesia denitrificans* BN-13 after 48 h of fermentation in shake flask culture containing xylan medium at 37 °C.

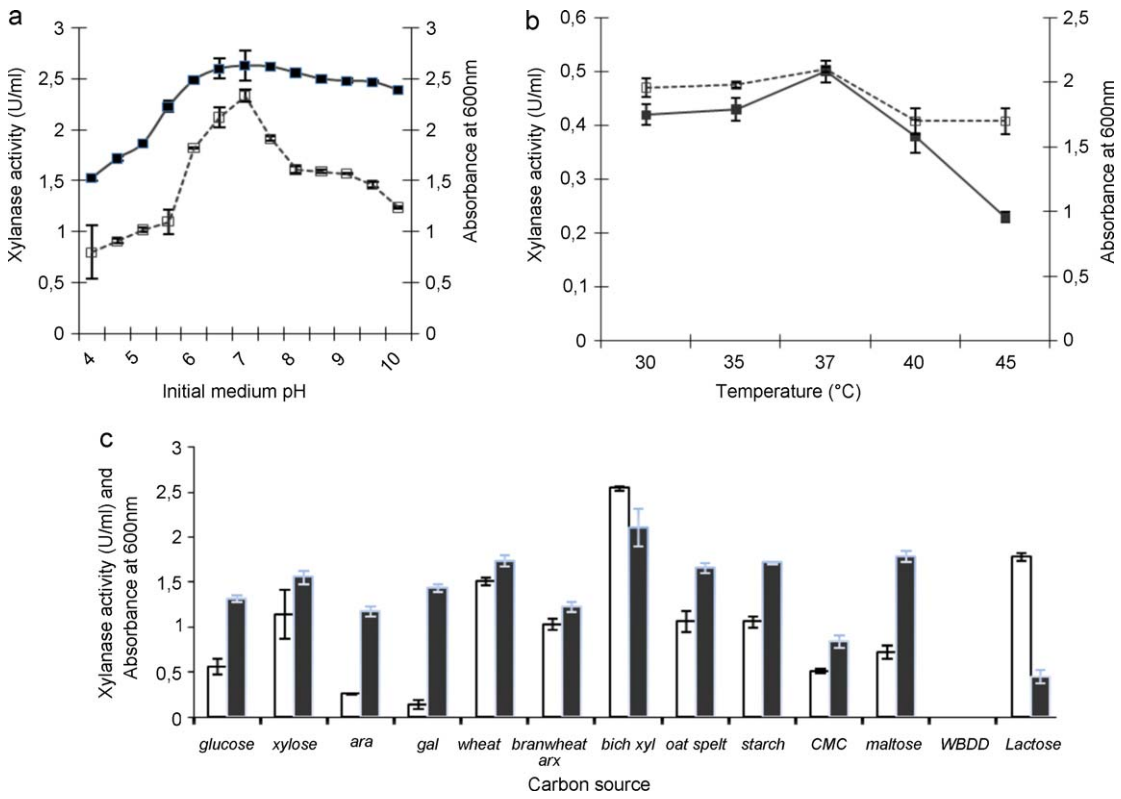


Fig. 3. Effect of initial medium pH (a), temperature (b) and carbon source (c) on growth and xylanase production. (a), (b) and (c) ■: absorbance, □: xylanase activity, (c) ara: arabinose, gal: galactose, Wheat arx: wheat arabinoxylan, Birch xyl: birchwood xylan, CMC: carboxymethylcellulose, WBDD: wheat arabinoxylan delignified destarched. Data are the average of three replicates.

3.5. Growth and xylanase production profiles

The time course of growth and the production of extracellular xylanase were studied using birchwood xylan as a carbon source (Fig. 4).

Soon after inoculation, the culture started growing and reached a plateau at 24 h with a very slow growth up to 33 h where the highest biomass was reached, remaining the same thereafter, as cultivation continued, soluble protein increased and followed a similar trend to that observed for absorbance.

Conversely, xylanase production by the culture increased gradually and reached its highest value at 35 h (10.80 U/ml). The production of extracellular endoxylanases by *J. denitrificans* seems to be partially associated with growth. In most cases, the production of xylanases is growth associated in actinomycetes cultures, including Streptomyces, this was not observed for *Streptomyces* sp. AMT-3 strain, which exhibited a typical non growth associated enzyme production, as observed for other actinomycete ligninolytic systems [26].

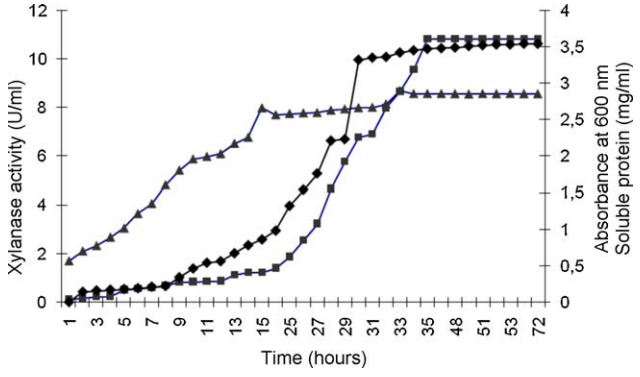


Fig. 4. Time course of growth and enzyme production by *Jonesia denitrificans* BN13 in xylan defined medium (pH 7, 37 °C), ♦: soluble proteins, ■: xylanase activity, ▲: absorbance. Data are the average of three replicates.

Xylanase production using *J. denitrificans BN13* was investigated with birchwood xylan as the substrate, as shown in Table 1, xylanase was produced from birchwood xylan with a maximum activity of 10.8 U/ml in a 4-l fermenter, the activity of 10.8 U/ml was slightly lower than that reported for most other actinomycete [30,44].

However, Nascimento et al. [29] reported 10.3 U/ml of xylanase activity in a 10 days culture of *Streptomyces* sp. strain AMT-3 with birchwood xylan as the substrate. Techapun et al. [41] reported 8 U/ml of xylanase activity in a culture of *Streptomyces* sp. Ab 106 with xylan (1%) as the substrate (55 °C, pH 7.5), xylanase levels from fungal cultures [13,19,38] are generally much higher than *J. denitrificans* BN13.

In general, as exposed in Table 1, the concentration of birchwood xylan used in the growth medium was 10 g/l but the strain *J. denitrificans* BN13 grew in a medium containing only 7 g/l, by reducing the concentration of xylan xylanase production was possibly reduced, the factor which is of great importance for the xylanase assay and comparability of the results is the xylan substrate used, different xylan preparations can differ both in the types and degrees of substitutions and this can result in a significant variation of the activity values measured, also in comparison to the DNS method, the use of the Somogyi–Nelson procedure for the determination of reducing sugars always yields considerably lower apparent activities [33].

Table 1
Production of xylanases in submerged fermentation.

Organism	Substrate	Cultivation conditions	Xylanase activity (U/ml)	References
Jonesia denitrificans BN13	Birchwood xylan, 7 g/l	4 l fermentation, 37 °C, pH 7, 2 days	10.80	Present work
Streptomyces sp. strain AMT-3	Birchwood xylan, 10 g/l	Shake flask, 30 °C, pH 7.0, 10 days	10.30	[29]
Streptomyces sp. QG113	Birchwood xylan, 10 g/l	Shake flask,37 °C, pH 8.0, 5 days	7.50	[5]
Streptomyces sp. Ab106	Xylan, 10 g/l	4 l fermentation, 55 °C, pH 7.5, 5 days	8	[41]
Streptomyces actuosus	Rice bran, 50 g/l	37 °C, pH 5, 4 days	11.60	[44]
Streptomyces malaysiensis	Birchwood xylan, 10 g/l	Shake flask, 30 °C, pH 7, 6 days	11.90	[30]
Bacillus coagulans	Birchwood xylan, 10 g/l	Shake flask, 45 °C, pH 7, 24 h	24.20	[11]
Bacillus subtilis	Birchwood xylan, 10 g/l	3 l laboratory fermentor, 55 °C, pH 9, 2 days	128	[1]
Bacillus pumilus	Wheat bran 10 g/l	Shake flask, 35 °C, pH 9, 3 days	430	[31]
Thermobifida fusca	Wheat bran 20 g/l	Shake flask 50 °C, pH 9.0, 3 days	6.68	[25]
Aspergillus foetidus MTCC 4898	Birchwood xylan, 10 g/l	Shake flask, 30 °C, pH 5, 3 days	210	[38]
Fusarium solani F7	Wheat straw 30 g/l	Shake flask, pH 5.5, 30 °C, 10 days	78.32	[19]
Penicillium kloeckeri NRRL 1017	Birchwood xylan, 10 g/l	Shake flask, 26–28 °C, 5 days	12.20	[13]
Coriolus versicolor	Tomato pomace	Shake flask, 25 °C, 14 days	2.56	[15]

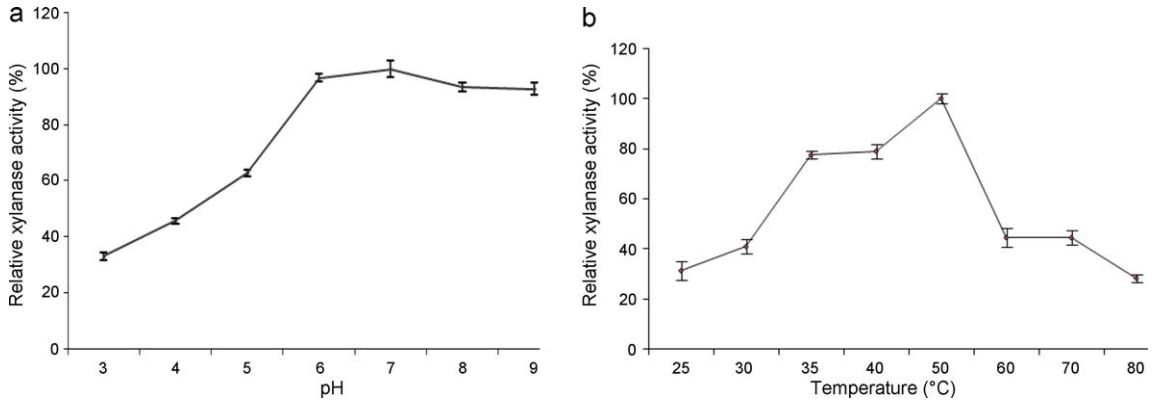


Fig. 5. Influence of pH (a) and temperature (b) on xylanase activity from *Jonesia denitrificans* BN-13. Data are the average of three replicates.

3.6. Properties of the xylanases

3.6.1. Effect of temperature on xylanase activity

The effect of temperature on the xylanase activity from *J. denitrificans* BN-13 is shown in Fig. 5b, for 10 min reaction the optimum temperature was 50 °C, these values are quite close to the results for xylanases from *Streptomyces* sp. *CD3* and *Streptomyces exfoliatus* [45], similar result was obtained for enzyme production with *Bacillus pumilus* [31].

When the temperature reached 80 °C, relative xylanase activity of *J. denitrificans BN13* was only 28.41%.

3.6.2. Effect of pH on xylanase activity

The favourable pH range for xylanase activity of *J. denitrificans BN 13* was 6.0–9.0 (Fig. 5a), these values are in agreement with those values reported for xylanases produced by *Bacillus* sp. strain SPS-0 [3], the enzyme retained considerable activity at higher pH (93.70% and 93.05% at pH 8.0 and 9.0 respectively).

pH for optimum activity was 7.0 and a significant decrease in enzyme activity was observed between pH 3.0 and pH 5.0, the xylanase of *Streptomyces roseisclereoticum* [17], *Streptomyces matensis* [46] and *Streptomyces exfoliates* [45] have the same optimum pH, for *Streptomyces* sp. strain AMT-13, the optimum pH was found to be 6.0 but significant levels of activity were detected between pH 4.5 and 8.0 [29].

3.6.3. Thermostability

Thermal stability was carried out by preincubating xylanase up to 10 h in the range of 30–60 °C (Fig. 6), at 30 °C there was no significant decrease in xylanase activity during 7 h. The enzyme was stable at 50 °C, with a half-life of 7 h, it was sensitive at 60 °C and only an activity of 2.15% was observed after 6 h exposure, the results clearly indicated that the suitable temperature range for industrial application for xylanase from *J. denitrificans* BN13 was 30–50 °C.

This xylanase is more thermostable than the xylanase of *Streptomyces* sp. *QG113* which possess a half-life of 1 h at 50 °C [5] but the *Streptomyces* sp. Ab 106 xylanase exhibited a half-life of 6 h at 60 °C [41], concerning the xylanase produced by *Streptomyces* sp. strain AMT-13, the profile obtained for thermostability showed that 70% of the original activity was retained after 8 h of pre-incubation at 55 °C [29].

3.6.4. Effect of metal ions and reagents on activity

We investigated the effects of metal ions and other reagents on the activities of the enzyme (Table 2).

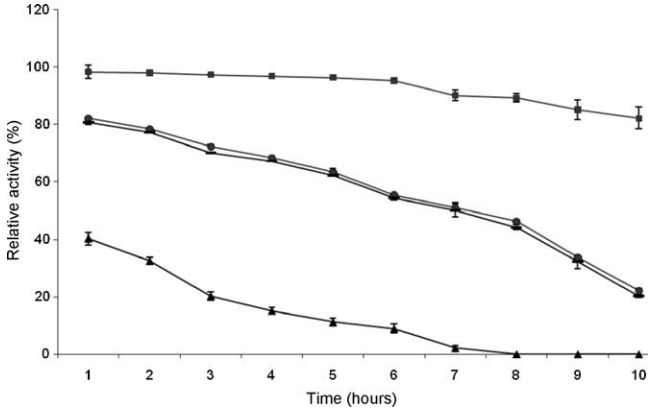


Fig. 6. Thermostability profiles of xylanase (without substrate). ■: 30 °C, ●: 40 °C, –: 50 °C, ▲: 60 °C. Data are the average of three replicates.

Table 2
Effect of metal ions and reagents on the activity of the xylanase produced by *Jonesia denitrificans* BN13.

Metal ions (1 mM)	Relative activity (%)
None	100.00
Ca^{2+}	82.70
Mg^{2+}	89.22
Zn^{2+}	93.13
NH_4	83.50
Hg^{2+}	56.00
Mn^{2+}	110.35
Cu^{2+}	89.90
K^{+}	87.39
Fe^{2+}	91.00
Ni^{2+}	88.00
Cd^{2+}	87.81
SDS	87.57
EDTA	100.00
DTT	89.60
PMSF	89.37

Most of the metal ions (1 mM) tested had little influence on the activity, the same results were obtained with the xylanases produced by Bacillus SPS-0 [4], whereas Mn^{2+} was found to enhance the activity (by 10.35%). Most of the xylanases were found to be inhibited by Mn^{2+} [24,34,10]. However the xylanases of *Bacillus* sp. strain SPS and *Bacillus amyloliquefaciens* [8] are stimulated by Mn^{2+}. It could be supposed that this ion exerts its effect by interacting with some amino acid residues involved in the active site, which causes a change in conformation leading to higher activity, but also higher susceptibility to denaturation at higher temperatures, Hg^{2+} led to the inhibition of the xylanase (by 44%), Arai and co-workers reported that the inhibition of enzymes by Hg^{2+} was attributed to the involvement of tryptophan residues on the active site [44].

Total inactivation due to SDS has already been reported for xylanases of different origins [16], in contrast to the resistance to SDS was found in this study, with 87.57% relative activity after 30 min at 50 °C.

From preliminary study, it can be observed that the strain *J. denitrificans* BN13 was able to grow and produce xylanases using commercial xylan. It could utilise low cost agro industrial residues like wheat bran. The pH and temperature optima of the preparation were 7.0 and 50 °C, respectively, and the enzyme retained 50% of its activity during 7 h at 50 °C. These properties place this enzyme as promising for biotechnological applications especially bioethanol production because the hydrolysis of hemicellulose from lignocellulosic biomass in bioethanol production is important for the recovery of monosaccharides from the residual hemicellulose and for the removal of hemicellulose which otherwise restricts the access of cellulases to cellulose fiber [24].

Acknowledgements

The authors thank K. Bensaid for his help with the figures. Our thanks are also addressed to N. Moussaoui, English teacher at Bejaïa University, for her help in the revision of the language expression. The constructive criticisms from editor and anonymous reviewers are also sincerely appreciated.

References

[1] Annamalai N, Thavasi R, Jayalakshmi S, Balasubramanian T. Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment. Ind J Biotechnol 2009;13:291–7.
[2] Bakir U, Yavascaoglu S, Guvenc F, Ersayin A. An endo-β-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization. Enzyme Microb Technol 2001;29:328–34.
[3] Bataillon M, Nunes Cardinali AP, Duchiron F. Production of xylanases from a newly isolated alkalophilic thermophilic *Bacillus* sp. Biotechnol Lett 1998;20:1065–71.
[4] Bataillon M, Nunes Cardinali AP, Castillon N, Duchiron F. Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. Enzyme Microb Technol 2000;26:187–92.
[5] Beg QK, Bhushan B, Kapoor M, Hoondal GS. Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus Kraft pulp. Enzyme Microb Technol 2000;27:459–66.
[6] Beg QK, Kappor M, Mahajan L, Hondal GS. Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 2001;20:325–33.
[7] Bradford MM. Rapid and sensitive method for the quantitation of microgram quantites of protein utilizing the principale of protein-dye binding. Anal Biochem 1976;72:48–254.
[8] Breccia JD, Sineriz F, Baigori MD, Castro GR, Hatti-Kaul R. Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens*. Enzyme Microb Technol 1998;22:42–9.
[9] Carmona EC, Fialho MB, Buchgnani EB, Coelho GD, Brocheto-Braga MR, Jorge JA. Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor*. Process Biochem 2005:359–64.
[10] Chivero ET, Mutukumira AN, Zvauya R. Partial purification and characterization of a xylanase enzyme produced by a micro-organism isolated from selected indigenous fruits of Zimbabwe. Food Chem 2001;72:179–85.
[11] Choudhury B, Chauhan S, Singh SN, Ghosh P. Production of xylanase of *Bacillus coagulans* and its bleaching potential. World J Microb Biotech 2006;22:283–8.
[12] Ding CH, Jiang ZQ, Li XT, Li LT, Kusakabe I. High activity xylanase production by *Streptomyces olivaceoviridis* E-86. World J Microb Biotech 2004;20:7–10.
[13] Farrel RL, Biely P, McKay DL. Production of hemicellulase by the fungus *Penicillium kloeckeri* in biotechnology in the pulp and paper industry. In: Srebotnik E, Messner, editors. Proceedings of the 6th international conference of biotechnology in the pulp paper industry. Vienna: Facultas-Universitatsverlag; 1996. pp. 485–489.
[14] Flores ME, Pérez R, Huitron C. B-xylosidase and xylanase characterization and production by *Streptomyces* sp. CH-M-1035. Lett Appl Microbiol 1997;24:410–6.
[15] Freixo MDR, Karmali A, Fraza C, Arteiro JM. Production of laccase and xylanase from *Coriolus versicolor* grown on tomato pomace and their chromatographic behaviour on immobilized metal chelates. Process Biochem 2008;43:1265–74.
[16] Fujimoto H, Ooi T, Wang SL, Takizawa T, Hidaka H, Murao S, et al. Purification and properties of three xylanase from *Aspergillus aculeatus*. Biosci Biotech Biochem 1995;59:538–40.
[17] Grabski AC, Jeffries TW. Production, purification and characterization of β(1–4) endoxylanase of *Streptomyces roseiscleroticus*. Appl Environ Microbiol 1991;l57:987–92.
[18] Gessesse A, Gashe BA. Production of alkaline xylanase by an alkaliphilic *Bacillus* sp. isolated from an alkaline soda lake. J Appl Microbiol 1997;83:402–6.
[19] Gupta VK, Gaur R, Gautam N, Kumar P, Yadav IJ, Darmwal NS. Optimization of xylanase production from *Fusarium solani* F7. Am J Food Technol 2009;4:20–9.
[20] Holtz C, Kaspari H, Klemme JH. Production and properties of xylanases from thermophilic actinomycetes. Antonie Van Leeuwenhoek 1991;59:1–7.
[21] Kluepfel D, Vats-Mehta S, Aumont F, Shareck F, Morosoli R. Purification and characterization of a new xylanase (xylanase B) produced by *Streptomyces lividans* 66. Biochem J 1990;267:45–50.
[22] Kumar R, Singh S, Singh OMV. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 2008;35:377–91.
[23] Lee JW, Park JY, Kwon M, Choi IG. Purification and characterization of a thermostable xylanase from the brown-rot fungus *Laetiporus sulphureus*. J Biosci Bioengin 2009;107:33–7.
[24] Liu WH, Zhu W, Lu Y, Kong J, Ma G. Production, partial purification and characterization of xylanase from *Trichosporon cutaneum* SL409. Process Biochem 1998;33:331–6.
[25] Liu WH, Yang SF, Yang CH. Production of xylooligosaccharides from xylans by extracellular xylanases from *Thermobifida fusca*. J Agric Food Chem 2007;55:3955–9.
[26] Lodha SJ, Korus RA, Crawford DL. Production of major extracellular enzymes during lignocelluloses degradation by two streptomycetes in agitated submerged culture. Appl Environ Microbiol 1989;55:1165–8.
[27] Marques S, Alves L, Ribeiro S, Girio FM, Amaral-Collaco MT. Characterization of a thermotolerant and alkalotolerant xylanase from *Bacillus* sp. Appl Biochem Biotechnol 1998;73:159–72.
[28] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:538–42.
[29] Nascimento RP, Coelho RRR, Marques S, Alves L, Girio FM, Bon EPS, et al. Production and partial characterization of xylanase from *Streptomyces* sp. Strain AMT-3 isolated from Brazilian cerrado soil. Enzyme Microbial Technol 2002;31:549–55.
[30] Nascimento RP, Marques S, Alves L, Girio FM, Amaral-Collaco MT, Sacramento DR, et al. A novel strain of *Streptomyces malaysiensis* isolated from brazilian soil produces high endo xylanase titres. World J Microb Biotech 2003;19:879–81.
[31] Prema P, Asha Poorna C. Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. Biochem Engin J 2006;32:106–12.
[32] Rani DS, Nand K. Purification and characterization of xylanolytic enzymes of a cellulase-free thermophilic strain of *Clostridium absonum* CFR-702. Anaerobe 2001;7:45–53.

[33] Royer JC, Nakas JP. Xylanase production by *trichoderma longibrachiatum*. Enzyme Microb 1989;11:405–10.
[34] Saha BC. Production, purification and properties of xylanase from a newly isolated *Fusarium proliferatum*. Process Biochem 2002;37:1279–84.
[35] Sá-Pereira P, Mesquita A, Duarte J, Barros MRA, Costa-Ferreira M. Rapid production of thermostable cellulase free xylanase by a strain of *Bacillus subtilis* and its properties. Enzyme Microb Technol 2002;30:924–33.
[36] Seeliger HPR, Jones D. Genus listeria pirie. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology, vol. 2. Baltimore: Wiliams and Wilkins; 1986. pp. 1235–1245.
[37] Schumann P, Cui X, Stackebrandt E, Kroppenstedt Reiner M, Xu L, Jiang C. *Jonesia quinghaiensis* sp. nov., a new member of the suborder Micrococcineae. Inter J Syst Evol Microbiol 2004;54:2181–4.
[38] Shah AR, Madamwar D. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem 2005;40:1763–71.
[39] Sharma P, Bajai BK. Production and partial characterization of alkalitolerant xylanase from an alkali-tolerant xylanase from an alkalophilic *Streptomyces* sp. CD3. J Scient Indust Resear 2005;64:688–97.
[40] Suchita N, Kapoor M, Kubad RC. Purification and characterization of extracellular xylanase from *Streptomyces cyaneus SN3*. Bioresour Technol 2008;99:1–5.
[41] Techapun C, Charoenrat T, Poosaran N, Watanabe M, Sasaki K. Thermostable and alkaline-tolerant cellulase-free xylanase produced by thermotolerant *Streptomyces sp.* Ab106. J Biosci Bioeng 2002;93:431–3.
[42] Timothy S, Magnuson D, Crawford L. Purification and characterization of alkaline xylanase from *Streptomyces viridosporus* T7A. Enzyme Microb Technol 1997;21:160–4.
[43] Viet DN, Kamio Y, Abe N, kaneko J, Izaki K. Purification and Properties of β-1,4-Xylanase from *Aeromonas caviae* W-61. Appl Environ Microbiol 1991;57:445–9.
[44] Wang SL, Yen YH, Shin IL, Chang AC, Chang WT, Wu WC, et al. Production of xylanases from rice bran by *Streptomyces actuosus* A-151. Enzyme Microb Technol 2003;33:917–25.
[45] Wong KKY, Tan LU, Saddler JN. Multiplicity of β-1,4-xylanase in microrganisms: function and applications. Microbiol Rev 1988;52:305–17.
[46] Yan Q, Hao S, Jiang Z, Zhai Q, Chen W. Properties of a xylanase from *Streptomyces matensis* being suitable for xylooligosaccharides production. J Mol Catal B: Enzymatic 2009;58:72–7.

Bird Study
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbis20

Breeding biology of Grey Wagtails *Motacilla cinerea* at the southern edge of their breeding range (region of Béjaia, Algeria)

Abdelazize Franck Bougaham [a], Riadh Moulaï [a] & John O'Halloran [b]
[a] Faculté des sciences de la nature et de la vie, Université de Béjaia 06000 Béjaia Algérie
[b] School of Biological, Earth and Environmental Sciences, University College Cork, Ireland

Available online: 21 Apr 2011

To cite this article: Abdelazize Franck Bougaham, Riadh Moulaï & John O'Halloran (2011): Breeding biology of Grey Wagtails *Motacilla cinerea* at the southern edge of their breeding range (region of Béjaia, Algeria), Bird Study, 58:3, 357-360

To link to this article: http://dx.doi.org/10.1080/00063657.2011.575928

Bird Study (2011) **58**, 357–360

SHORT REPORT

Breeding biology of Grey Wagtails *Motacilla cinerea* at the southern edge of their breeding range (region of Béjaia, Algeria)

ABDELAZIZE FRANCK BOUGAHAM[1], RIADH MOULAÏ[1] and JOHN O'HALLORAN[2]*
[1]*Faculté des sciences de la nature et de la vie, Université de Béjaia 06000 Béjaia Algérie;* [2]*School of Biological, Earth and Environmental Sciences, University College Cork, Ireland*

Capsule The first breeding data for this population of Grey Wagtails *Motacilla cinerea* at the southern edge of their breeding range in North Africa show smaller clutches and the production of fewer young compared with populations in the core of the breeding range in Europe. Overall, the breeding success at hatching was 4.33 chicks per nest. Over 62% of broods were successful and predation accounted for most of the losses. Grey Wagtails used up to 17 different plant species for nest construction, although the number of different plant species varied from 10 to 14.

The breeding biology of Grey Wagtails *Motacilla cinerea* is well described in Europe (Tyler 1972, Flousek 1987, Ormerod & Tyler 1991, Smiddy & O'Halloran 1998). Breeding territories of Grey Wagtails are restricted to running water. Although up to three clutches may be laid between the end of March and the end of July, typically there are one or two breeding attempts per pair per year (Klemp 2000). Complete clutch size varies from three to seven eggs, with clutches of five the most common. This is the first study on the breeding biology of Grey Wagtails on the African continent at the southern edge of their breeding range. Here we describe the habitat, nesting sites, nest materials and key breeding biology parameters of Grey Wagtails in North Africa and compare them with studies of European populations.

STUDY AREA

The study area was located in the southeast of the city of Béjaia near Souk-El-Tenine, in the foothills of the north slope of Djebel Tababort. The study site is 4 km from Tameridjet (36°34′ N, 5°22′ E), and lies between 210 and 600 m a.s.l.; a region that is humid to per-humid (Gharzouli & Djellouli 2005) and characterized by a network of streams that join the main river, Ighezer n'reha. These streams flow over a substrate formed by Liassic limestone (Duplan 1955) and are usually permanent, rising in the north slope of Tababort mountain (1969 m) with riparian forest (maximum height = 8 m), composed mostly of *Alnus glutinosa*, and some *Populus alba* and *Nerium oleander*.

METHODOLOGY

A systematic search for nests was conducted mainly along the rivers during the 2009 breeding season. We recorded the height of the nest above water, first egg date (day 1 = 1 January), clutch size, the breeding success and characteristics of nests (e.g. location, substrate and nest composition). The date of first egg was back-calculated from the estimated average duration of incubation of eggs (13 days; Flousek 1987, Ormerod & Tyler 1987). Clutch size was recorded when the number of eggs in the nest did not differ between two successive visits (1–5 days). Broods damaged (especially by children) or discovered late were not included in calculating the mean clutch size. The breeding success at hatching was estimated, based on the number of chicks per nest (1–3 days post-hatching). The breeding success at fledging was determined by the ratio of fledged young to the total number of successful broods (at least one hatched chick). Although birds were not ringed

*Correspondence author. Email: j.ohalloran@ucc.ie

Table 1. Mean weight of plant material used in nest-building by Grey Wagtails in Béjaia.

Nests	Average mass of nests (g)	Average mass of plant material (g)	Herbaceous (in % of plant material)	Woody (in % of plant material)	Various materials (in % of total)
Nest 1	75.90	61.50	55.93	39.67	6.91
Nest 2	52.30	49.00	85.71	11.22	5.16
Nest 3	53.30	49.50	80.80	16.16	7.87
Means (± sd)	**60.5 ± 13.34**	**53.33 ± 7.07**	**74.14 ± 15.96**	**22.35 ± 15.20**	**6.64 ± 1.37**

individually, each breeding attempt was classified as first, second or replacement clutch.

At the end of the breeding season, we collected three nests and each was weighed separately. The materials used for nest-building were separated into plant material and other materials (feathers, hair and wool). The plant material was then sub-divided into herbaceous plant material origin and woody plant material origin, and each fraction weighed separately. The plants used for nest-building were identified where possible. Spearman's rank correlation was used to explore any associations between breeding parameters and height of the nest location above water.

RESULTS AND DISCUSSIONS

We recorded 16 nesting attempts of 13 pairs of which 10 nests (62.5%) were found along the main stream. Most nests (87%) were located very close to running water and situated in the exposed parts (without vegetation) of streams. Two nests were located under cover of the riparian forest and a further two on man-made structures. Most nests were located in cracks or holes in blocks of rocks overhanging the water (75%). The height of the nest location ranged between 0.55 and 5.50 m, with an average of 2.07 m.

We recorded 13 clutches as first breeding attempt and the first egg date was 23 March (day 82). Two second clutches were found on 10 June (day 161) and a single replacement clutch was located on 7 July (day 188). The mean first-egg date was 17 May (day 137 ± 30). The clutch size varied between 3 and 6 eggs, with a mean of 4.64 (± 0.84, $n = 14$). Clutches of 5 and 4 eggs are the most common ($n = 6$ and 5, respectively), with the remainder clutches of 3 ($n = 1$) and 6 ($n = 2$), respectively. Height of the nest location showed significant correlation with clutch size ($r_S = 0.57$, $P < 0.05$, $n = 12$).

Overall, the breeding success at hatching was 4.33 chicks per nest. Over 62% of broods were successful (a total of 10 chicks reached fledging). Predation explained most losses (about 19%) compared with damaged or abandoned nests (respectively, about 12% and 6%). Breeding success at fledging was 2.5 chicks fledged per pair ($n = 12$). A significant correlation was found between height of above-water nest location and breeding success at fledging ($r_S = 0.60$, $P < 0.05$, $n = 10$).

The mass of Grey Wagtail nests ranged from 52.3 to 75.9 g, with an average of 60.5 g (± 13.3) (Table 1). Analysis of the three nests ($n = 3$) recovered shows a dominance of plant material (Table 1). Grasses were used more than woody plants (Table 1). The plant materials used for nest-building were abundant in the immediate area surrounding the nest. Grey Wagtails used up to 17 different plant species for nest construction, although the number of different plant species varied from 10 to 14 (Table 2).

Like its congeners in Europe, Grey Wagtails in the region of Béjaia show a marked (87% of nests) preference for nesting near rivers. Most nests are built in the crevices of rock faces (75%). This contrasts with most

Table 2. Plant species used for nest-building by Grey Wagtails in Béjaia.

Nest 1	Nest 2	Nest 3
Quercus suber	*Quercus suber*	*Quercus suber*
Salix sp.	*Salix* sp.	*Salix* sp.
Rubus ulmifolius	*Rubus ulmifolius*	*Rubus ulmifolius*
Adiantum capillus-veneris	*Adiantum capillus-veneris*	*Adiantum capillus-veneris*
Ampelodesma mauritanica	*Ampelodesma mauritanica*	*Ampelodesma mauritanica*
Bryum sp.	*Bryum* sp.	*Bryum* sp.
Pteris aquilina	*Pteris aquilina*	*Pteris aquilina*
Populus nigra	*Populus nigra*	*Dactylis glomerata*
Phillyrea media	*Phillyrea media*	*Nerium oleander*
Trachelium coeruleum	*Dactylis glomerata*	*Pistacia lentiscus*
–	*Nerium oleander*	*Calycotome spinosa*
–	–	*Alnus glutinosa*
–	–	*Erica arborea*
–	–	*Origanum glandulous*
10 species total	11 species total	14 species total

European studies where the nests are mostly located in or below bridges (72% in Great Britain, Tyler 1972; 97% in Ireland, Smiddy & O'Halloran 1998). We also recorded nesting on human habitations. A similar pattern has been observed elsewhere in its distribution (Tyler 1972, Rodríguez & Rodríguez 2007), or in Algeria, where Bellatréche (1994) found a nest with two chicks inside the ruins of a house in the woods of Aïn Setta in Taza (Jijel). The average density (2 pairs/1 km) of Grey Wagtails in Béjaia is greater than that found by Tyler (1972) (less than 1 pair/1 km) in Great Britain, or Ormerod & Tyler (1987) (1–15 pairs/10 km) in Wales. The average height of the nest location is close to those recorded in Ireland (2 m, Smiddy & O'Halloran 1998) and Canary Islands (about 1.6 m, Rodríguez & Rodríguez 2007). This contrast with nests observed in Great Britain (Tyler 1972) or Morocco (Thévenot *et al.* 2003), where nests are generally over 3 m above river level.

As reported elsewhere in Europe and North Africa (Tyler 1972, Ormerod & Tyler 1987, Smiddy & O'Halloran 1998, Thévenot *et al.* 2003), the first-egg date occurs towards the end of March and the season extends to the end of July. This contrasts with timing of breeding on the Canary Islands, where the breeding season extends from late February to early June (Rodríguez & Rodríguez 2007). Most clutches in Béjaia are laid in mid-May. The European populations of Grey Wagtails have a peak in egg-laying in mid-April (Tyler 1972, Ormerod & Tyler 1987, 1991, Smiddy & O'Halloran 1998). The difference of nearly a month between the first-egg date in Europe and that of Béjaia, and in the peak of egg-laying, is most likely explained by the fact that typically there is heavy rainfall in April in Béjaia. The spring floods change the structure of micro-habitats and reduce the abundance of food, because the insect larvae are displaced by high water flow (Riederer 1981, Imhof 1994). This may alter the phenology of egg-laying, inducing a delay in egg-laying (Klemp 2003). However, the Grey Wagtails in Béjaia do have second clutches following a previous successful nesting attempt. It seems that the quantity and quality of food available for Grey Wagtail in early spring to late summer allows a second and a third brood in the one season (Tyler 1972).

The mean clutch size (4.64 ± 0.84) is similar to that obtained in Ireland, but lower than other European populations (4.79, Smiddy & O'Halloran 1998). Indeed, the average clutches size of 4.93 was greater for Great Britain (Tyler 1972), and 5.27 eggs for Czechoslovakia (Flousek 1987), or for Wales, where clutch size varies between 5 and 5.07 eggs (Ormerod & Tyler 1987, 1991). However, in Béjaia, Grey Wagtails lay on average more eggs than the island population of the Canary Islands (3.96, Rodríguez & Rodírguez 2007). This relatively small clutch size could be interpreted as an adaptation to environmental circumstances there. In mountain habitats, as in our study area, the conditions are generally more variable than those of lower altitudes (Klemp 2003). It is commonly accepted that in a stable climate the variation in food availability is low (Smiddy & O'Halloran 1998, Rodríguez & Rodríguez 2007), resulting in an increase in local population density, reflecting a low mortality of adults in winter. This leads in part to intraspecific competition during the breeding season (Rodríguez & Rodríguez 2007).

It seems that the breeding success at hatching calculated in Béjaia (4.33) is relatively close to that of other populations being 4.40 for Wales (Ormerod & Tyler 1987) and 4.59 in Ireland (Smiddy & O'Halloran 1998). In contrast, in the Canary Islands, the hatching success of Grey Wagtail is lower compared to this study (3.86, Rodríguez & Rodríguez 2007). These authors suggest that the survival of broods is closely associated with the presence of potential predators and human disturbance. The fate of broods seems to depend on the nature of the immediate environment of the nest. The absence of shelters from tufts or tussocks of grass (obstacles plants) near the nest, resulted in chicks being exposed to climatic factors (weather, sun). These features may have a pronounced effect on survival of broods when the eggs and chicks aged less than 3 days stay too long after exposure to human disturbance.

The number of young fledging (2.5, $n = 12$) for each nest is within the range of known values for the Canary Islands (2.85, Rodríguez & Rodríguez 2007) and for Morocco (2–5, Thévenot *et al.* 2003). The breeding success at fledging of population in Algeria, Morocco and Canary Islands seems lower than populations for northwest Europe (Smiddy & O'Halloran 1998). This difference reflects subtle changes of local eco-climatic conditions (altitude and hydrologic regime) and/or the availability of food resources. In addition, human disturbance could have a significant effect on the survival of young.

It is likely that the nature of the nesting materials used plays an important role for nest building. Nests sampled were generally homogeneous with Grey Wagtails generally selecting the same plant species to consolidate the essential architecture of nests. Among the plant fragments, there was a clear preference for

the use of herbs (74.1% $\pm$ 15.9, $n = 3$), while the woody plants represented only 22.3% ($\pm$ 15.2). It appears that the fragments from herbs are more conducive to design the shape of the nest. Plant species found in the nests were generally collected in the vicinity.

Finally, the Grey Wagtails of Béjaia lay smaller clutches and produce fewer young than other populations. A similar pattern has also been noted in the Canary Islands. This difference could be interpreted as an adaptation to environmental circumstances (latitudinal and altitudinal gradients, climate).

ACKNOWLEDGEMENTS

We are grateful to Michel Thévenot, Sverre Klemp and Hichem Azafzaf for assistance with references and Airam Rodríguez for comment on an earlier draft.

REFERENCES

Bellatrèche, M. 1994. Ecologie et biogéographie de l'avifaune forestière de la Kabylie des Babors (Algérie). Thèse doctorat, Université de Bourgogne (Dijon).

Duplan, L. 1955. *La région de bougie. Le XIX^e Congrès international de géologie, monographie régionale.* 1^e série, 17, Alger, p. 45.

Flousek, J. 1987. Breeding biology of the Grey Wagtail, *Motacilla cinerea* (Passeriformes: Aves). *Acta Univ. Carolinea-Biologica* **1985:** 253–300.

Gharzouli, R. & Djellouli, Y. 2005. Diversité floristique de la Kabylie des Babors (Algérie). *Sécheresse* **16:** 217–223.

Imhof, A. 1994. Habitatansprüche und Verhalten von *Perla grandis* Rambur (Plecoptera: Perlidae) und anderen räuberischen Steinfliegenlarven. PhD Thesis, ETH Zürich.

Klemp, S. 2000. Effects of parental effort on second brood, moult and survival in the Grey Wagtail *Motacilla cinerea*. *Ardea* **88:** 91–98.

Klemp, S. 2003. Altitudinal dispersal within the breeding season in the Grey Wagtail *Motacilla cinerea*. *Ibis* **145:** 509–511.

Ormerod, S.J. & Tyler, S.J. 1987. Aspects of the breeding ecology of Welsh Grey Wagtail *Motacilla cinerea*. *Bird Study* **34:** 43–51.

Ormerod, S.J. & Tyler, S.J. 1991. The influence of stream acidification and riparian land use on the feeding ecology of Grey Wagtails *Motacilla cinerea* in Wales. *Ibis* **133:** 53–61.

Riederer, R.A.A. 1981. Die Eintags- und Steinfliegenfauna (Ephemeroptera und Plecoptera) im Mittellauf der Töss: Eine demökologische Untersuchung. PhD Thesis, ETH Zürich.

Rodríguez, B. & Rodríguez, A. 2007. Breeding biology of Grey Wagtail *Motacilla cinerea canariensis* on Tenerife, Canary Islands. *Acta Ornithol.* **42:** 195–199.

Smiddy, P. & O'Halloran, J. 1998. Breeding biology of the Grey Wagtail *Motacilla cinerea* in southwest Ireland. *Bird Study* **45:** 331–336.

Thévenot, M., Vernon, J.D.R. & Bergier, P. 2003. *The Birds of Morocco*. British Ornithologist Union Checklist Series **20,** p. 594.

Tyler, S.J. 1972. Breeding biology of Grey Wagtail *Motacilla cinerea*. *Bird Study* **19:** 69–80.

(MS received 21 February 2011; revised MS accepted 23 March 2011)

QUALITATIVE STUDY OF THE VASCULAR FLORA OF THREE INSULAR ENVIRONMENTS CONCERNING THE WEST COAST OF JIJEL

(GRAND CAVALLO ISLAND, PETIT CAVALLO ISLAND AND GRAND CAVALLO ISLET) (ALGERIA)

Samira HANIFI-BENHAMICHE[1] (*), Riadh MOULAÏ[2], A-F.BOUGAHEM[3] and H.BOUYAHMED[4]

[1,2,3,4] Laboratoire d'écologie et environnement,
Faculté des sciences de la nature et de la vie,
Université de Bejaia, 06000 Bejaia, Algérie.
E-mail : [1] benhamichesam@yahoo.fr [2] moulai741@hotmail.com

Abstract

The particular microclimate covering the islands allows the development of an adapted flora to the extreme conditions. This study aims to characterize qualitatively the vascular vegetation of three insular environments of the West coast of Jijel (Grand Cavallo, Petit Cavallo, Grand Cavallo islet).The results show that the island Petit Cavallo is the richest environment in vegetable species (77 species). Its vegetable cover is very similar to that of the island Grand Cavallo (60.13%). The therophytic taxa characterize the three islands with respective rates 54.4%, 38.96 % and 35.29% for the island's Grand Cavallo, Petit Cavallo and the Grand Cavallo islet. The whole of the islands and islets is covered with low vegetation, herbaceous, ruderal, with predominance of therophyts because of the multiple disturbances in particular by the invasive presence of breeding seabirds including the Yellow-legged-gull (*Larus michahellis*) (the number of registered couples varies from 53 to 610 depending on the study site). These affect the dissemination of diaspores, where a vegetation rather zoochory (41.55% to 52.94%). This avian population contributes by their several displacements to change the vegetation's quality also the quantity.

Keywords: Island Flora, diversity, Jijel, Yellow-legged-gull.

I. INTRODUCTION

Islands are considered as "natural laboratories", which are undeniable to study the ecological processes, and more especially those relied to termination or implantation of species. The small island ecosystems are very fragile, because of their reduced specific diversity and the presence of rare species. They usually suffer from disturbances that affect them. The latter are responsible for profound changes in stands structures (new, invasive or endangered species) [Whiteheard and Jones (1969)]. The threats to biodiversity in the world and specifically in insular areas are indisputable. Islands of Algeria are also concerned, despite their weak areas. In this context, any attempt to protect these systems, requires a prior knowledge of their

biodiversity, and specifically the phytodiversity [Patrick (2002)]. This work aims to characterize the flora of the islands, in order to_evaluate of the site, and to study its evolution compared to the different disturbances, including those caused by colonies of nesting seabirds, like Yellow-legged-gull *(Larus michahellis*), whose number is becoming increasingly important.

II. PRESENTATION OF THE STUDIED AREA

The Algerian coast stretches over 1200km of varied enough coastline: rocky coasts, sandy coasts and some lagoons; however it's characterized by scarcity of islands [Moulai (2005)]. There are 38 islands and islets most of which is not sufficiently studied (physiographic parameters, fauna and flora interests, threats, status, history and resources) [Jacob and Courbet (1980)] and [Boukhalfa (1990)]. The investigated area covers 60 km of linear coastal; it contains three insulars stations that are Grand Cavallo island, Grand Cavallo islet and Petit Cavallo island (Fig.1).

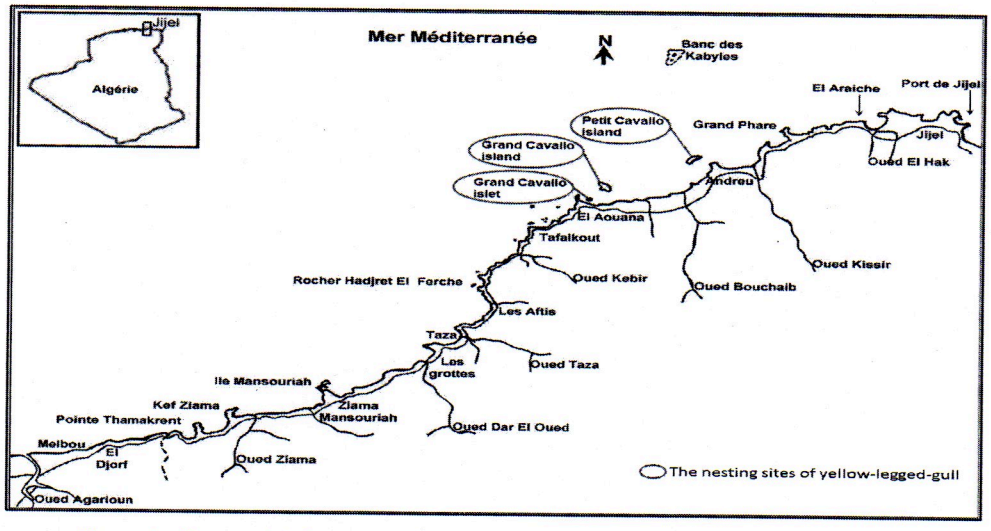


Figure1: Geographic location of the investigated area with the three insular sites

The three insular sites are refuge and nesting sites for the Yellow-legged-gull (*Larus michahellis*) [Bougaham (2008)].

-Grand Cavallo island: located at 950m of the shore and covering 6ha of area. It's maximum altitude does not exceed 50m. It's vegetation is "matorral type" mainly composed of *Pistacia lentiscus*, Phillyrea *angustifolius* and a nitrophile flora as *Lavatera cretica* and *Urtica membranea.*

- Petit Cavallo Island: located at 750m of the shore and covering 4ha of area, with a maximum altitude of 10m (fairly flat terrain). It's vegetation is of high matorral type, composed of *Pistacia lentiscus, Phillyrea angustifolius, Olea europea, Ficus carica, Opuntia ficus- indica* and a nitrophile flora like *Crithmum maritimum, Myrtus communis* and *Daucus carota.*

- Grand Cavallo Islet: at 50m of the shore, it has an area of 0.15ha and a maximum altitude of 30m. Vegetation: Clumps on the tops, composed of *Pistatia lentiscus, Phyllirea angustifolius* and *Chamaerops humilis*. Herbaceous Vegetation, located lower: *Inula viscosa, Bellis annua, Ariplex prostata, Halimione portulacoïdes*

1-Climatological aspects

As the islands climatic data do not exist, we use those of the nearest continental stations, considering the weak distance of our little islets from the shore.

- Pluviometry: 185mm in annual average with a minimum of 19mm in summer.

- Wetness (Humidity): quite stable, its values fluctuate around 75 percent (in marine environment).
- Temperatures: fluctuate between 26° for the warmer summer months and 10° for the coldest winter months.

1-1-Sampling

The earliest inventories of vascular plants have been performed in 2006 then completed in 2008 by Bougaham [Bougaham (2008)]. The sampling was systematic for the whole island and prospecting are distributed between March and June. The taxa identification is based on the New flora of Algeria [Quezel and Santa (1963)]. The reported taxa are then characterized with vital attributes, according to the approach of Medail and Vidal (1998). For each kind of taxa, we tried to give the following information: biogeographically type [Gamisans and Jeanmonod (1963)], biological type [Raunkiaer (1934)], spread mode [Vander Pigl (1982)] and demographic strategy C S R of Grime [Grime (1974)].

III. RESULTS

1-Specific richness

Table1: Specific richness of the studied sites

Sites / Parameters	Petit Cavallo Island (site1)	Grand Cavallo Island (site2)	Grand Cavallo Islet (site3)
Number of taxa	96	79	21
Number of families	40	31	12
Class	76 Dicots 20 Monocots	61 Dicots 18 Monocots	17 Dicots 4 Monocots

The whole composition of vascular flora in those sites currently contains 96, 79 and 21 species, for Petit Cavallo Island, Grand Cavallo Island, and Grand Cavallo Islet, respectively (table1). All the listed species are angiosperms, related to 40, 31 and 12 families, according to sites 1, 2 and 3, respectively. Thus, the richest families are the Asteraceaes, the chenopodiaceaes, the Fabaceaes, Poaceaes, Liliaceaes and Oleraceaes. Note that Gymnosperms are totally absents.
Despite their small size, our studied areas show an interesting specific richness. It reflects the habitat diversity, particularly in relation to disturbances generated by seabirds (especially by Yellow-legged-gull).
These results are similar to those obtained by Paradis and Piazza for the Corsica islands [Paradis and Piazza (2002)] and by Medail and Vidal for the islands of Marseille (France) [Medail and Vidal (1998)].

2- Functional analysis

2-1-Biological type

The flora structure of a station can be characterized by its biological spectrum that indicates the rate of each biological type. Their main interest is that they reflect the environmental conditions by the structure of vegetation.

The general appearance of our sites is characterized by rather low vegetation.

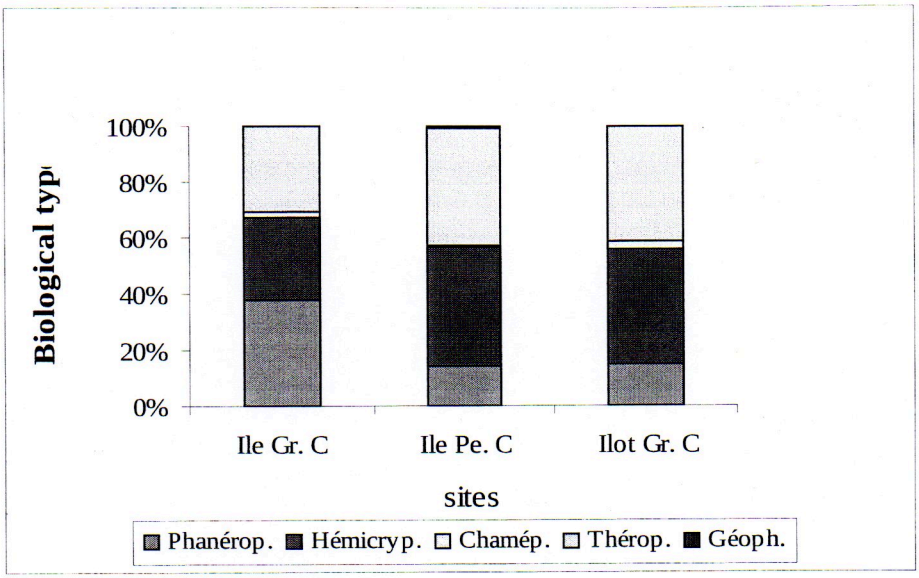


Figure 2: Biological type frequency for the three studied sites

Therophyts *(Fumaria capreolata, Stachys ocymastrum, Hordeum murinum, Coronopus didymus, Lotus ornithopoides, Hyoseris radiata, Hyoscyamus albus...)* and the hemicryptophyts(*Urginea maritima, Arundo donax, Lavatera cretica, , Lobularia maritima, Crithmum maritimum, Daucus carota* …) show a co-dominance, with the rates of 42.1 % and 35.33% respectively, followed by phanerophyts represented by *Smilax aspira, Pistacia lentiscus, Ficus carica et Phyllirea angustifolia* (fig.2). Therophyts and hemycryptophyts taxa are conventionally considered as privileged by disturbances caused by zoopopulations, particularly because of the openness of the environment [Noy-Meir and Al (1989) in Vidal (1998)]. According to [Emberget (1966)], hemicryptophyts dominate among the most exposed environments to disturbances. The degradation of these environments would be accompanied by an enrichment of the vegetation coverage in therophyts (annuals species), that resist to the hard conditions of the environment. They have a limited ecological interest, because of their short life cycle, which take only a few weeks or days [Chaeb (2003)]. [Bonnet et Al (1999)] found similar results in the archipelago of Frioul, with a dominance of therophyts.

2-2-Biogeographically type

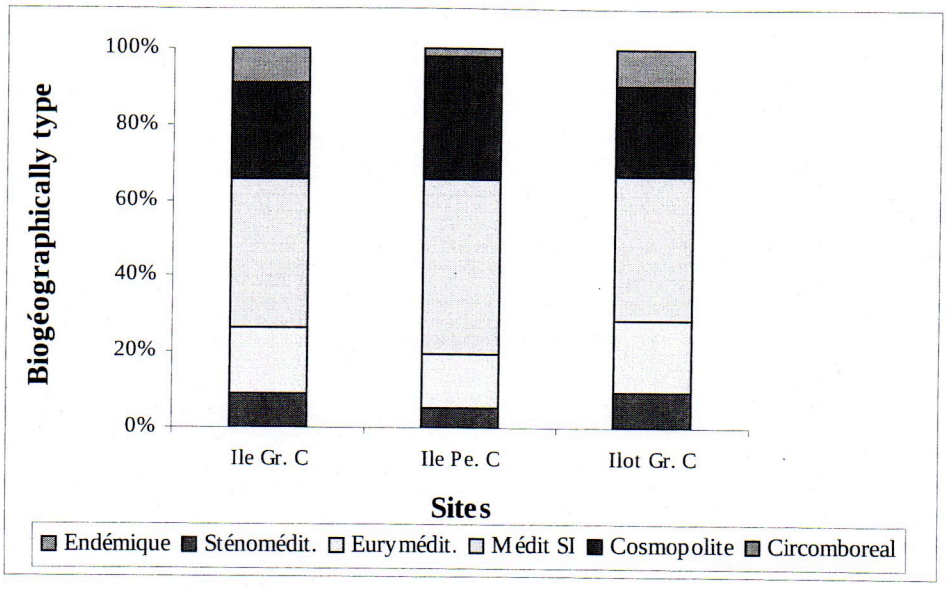


Figure 3: Biogeographically type frequency for the three studied site

From a phytogeographical point of viw taxa with Mediterranean affinity reign throughout our sites (45.83%, 39.24% and 38.09%) (Fig.3). This particularity impact on the type of vegetation that settle on these sites. Indeed, Mediterranean climate accentuates the drastic nature of the disturbed areas by birds, drought and evaporation. Cosmopolitans are also quite important, they are very good colonizers of any type of environment.

2-3-Spreading mode

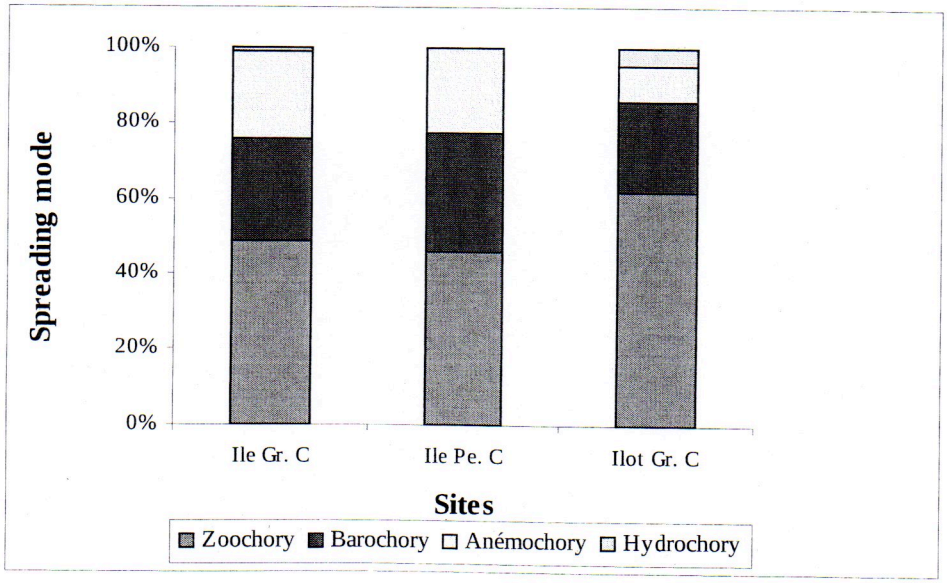


Figure 4: Spreading type frequency for the three studied sites

We note a striking dominance of the zoochory (dispersed by animals) spreading mode (51.94 %) compared to barochory (dispersed by gravity) ones which are positioned after with 27.22%, followed by the anemochory (dispersed by wind) (18.41%); hydrochory (water-

dispersed plants) (2.01%) are insubstantial (Fig.4). Zoochory dominance can be explained by the large number of Yellow-legged-gull (*Larus michahellis*) that attends to these island environments to get shelter and to reproduce safely. These vertebrates carry and disperse diaspores by their countless trips between the island sites and the continental environments. Transportation may be external (external zoochory or epizoochory), when diaspores are attached to plumage, legs or to surface of birds. That transportation type represents the highest rate [Duhautois and Hoff (2000)].

Transportation is internal (endozoochory or active zoochory), when diaspores are ingested by birds. Yellow-legged-gull constitutes a very important active agent. Indeed, by [Bougaham (2008)], wastes of plants with many types of diaspores (related to the reported flora on the studied sites), are found in the Yellow-legged-gull regurgitation balls. According to the same author, the composition of these birds' regurgitation balls is dominated by inorganic and vegetal wastes, with frequencies ranging from 68.4% to 100%. The wind vector (anemochory) found in some taxa can be explained by the ease of movement of diaspores between the islands and the continent, knowing the weak distance that separates them. No taxa with anthropochory spreading have been recorded, this may be due to the fact that these environments are not manned and therefore poorly attended.

2-4- Adaptative strategy of Grime

For all our small islands, ruderal vegetation is dominant, and it represents 45.57%, 42.71% and 23.81% for the Grand Cavallo Island, Petit Cavallo Island, and Grand Cavallo Islet, respectively (Fig5).

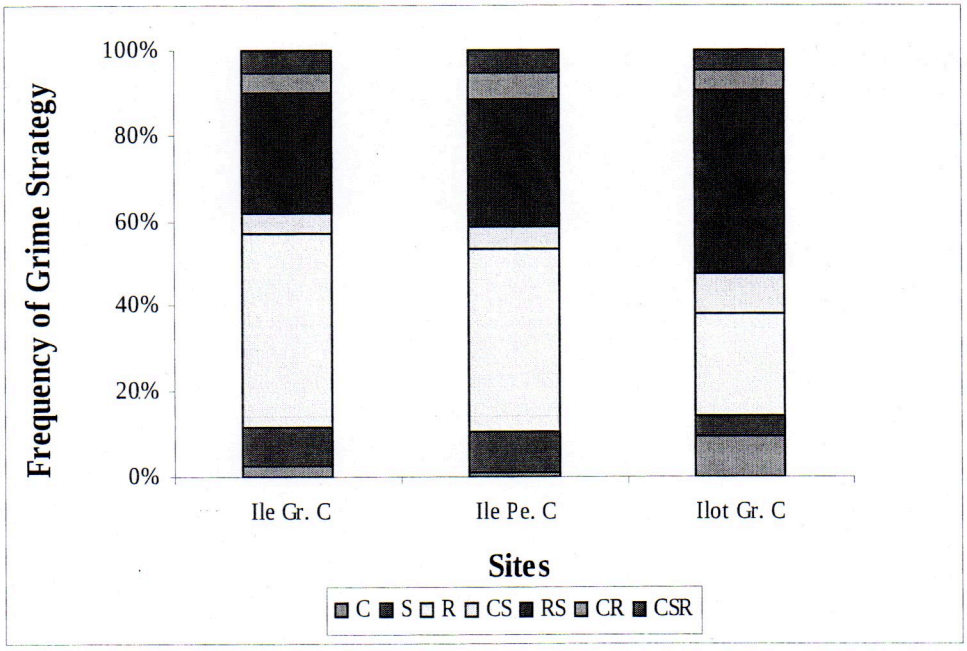


Figure5: Frequency of Grime Strategy type for the three studied sites

According to [Grime (1974)], the ruderals species grow in habitats subjected to frequent and intense disturbances. They present a growth rate and a rapid life cycle associated with a significant production of diaspores. Moreover, due to the disturbances related to climate, the action of nesting seabirds, in particular yellow-legged-gull (*Larus michahellis*) is undeniable. Thus, the coastal areas where the yellow-legged-gull are stationed know the extension of

ruderal species, halo-resistant like *Lavatera arborea*, *Lobularia maritima*, etc. The proliferation of some vegetal species like *Sonchus oleraceus* and *Urtica urens* is characteristic of degraded environments. Furthermore, the maritime influence (salinity gradient), characterizing the island environments, encourages the development of typical salt-tolerant species like *Crithmum maritimum*, and halo-resistant species like *Asteriscus maritimus*. This fact is justified by the large zoopopulation, including the avian population, by repeated trampling and soil enrichment with nitrogen and phosphorus substances, and finally under the effects of excreta and Guano's rejects.

IV. CONCLUSION

The biology of conserving some sites, especially insular areas, is a global concern. In this context, the development of conservation strategies necessarily involves information on the status of biodiversity. Plants are very good indicators of environmental quality, their trend of evolution and their specific ecological situations [Duautois and Hoff (2000)].

The plant communities identified in our sites are fairly common to other small Mediterranean islands. We most often find low halophytic vegetation that grows at the expense of high vegetation*: Crithmum maritimum, Atriplex prostrata, Chenopodium album, Chenopodium chenopoïdes, Halimoine portulacoide, Lotus angustissimus, Lotus cytisoide, Daucus carota, Dactylis glomerata*, etc.

In this paper, we used some recent botanical inventories, and this could not lead to clearly define the dynamics of vegetation over time. However, our floristic data are used in order to analyse, through several parameters, the relationship between the vegetation type and the fauna that lives there. Our data also allowed studying the nesting seabirds like Yellow-legged-gull that knows a rapid and constant growth. Yellow-legged-gull is the most abundant seabird in the Mediterranean [Thibault and Al (1996)]. [Moulai (2006)] shows in his work, that Yellow-legged-gull is the most frequent and common bird on the coast of Jijel. It is the only kind of seabird that nests on the coast of Jijel (Algeria). Furthermore, the important growth of the Yellow-legged-gull number is clear and surprising. Thus, the number of couples increased from 32, 22 and 0 in 1978, to 610,395 and 53 in 2007, for Grand Cavallo Island, Petit Cavallo Island and Grand Cavallo Islet respectively [Bougaham (2008)]. The Yellow-legged-gull proliferation generates a set of ecological disturbances that deconstructs the original vegetation [Vidal and Marthe (2003)].

As we could analyze the organic substances contained in their waste, we show that Yellow-legged-gull cause the nitrification of the areas that they attend. They are also responsible for a significant trampling and uprooting of plants for making their nests. Furthermore, some authors like [Bioret and Al (1991)], underline the effect of droplets of salt water made by the bird feathers. The resulting vegetation is then less specific, as this is the case for our sites. Understanding the organization of plant communities in our sites is far from guaranteed. However, the obtained results show a trend to the therophytisation and ruderalisation of vegetation.

V. REFERENCES

Bioret, F.; Cuillandre, J.P. and Fichaut B. (1991): *Degeneration processes of a micro insular ecosystem put through gulls influence: The isle of Banneg (Finistère, France).Essay of ecological integrated cartography*. In RAVERA O. and HORWOODE. éd. Terrestrial and aquatic ecosystems: perturbation and recovery. New-York, pp.276-283.

Bonnet, V. ; Vidal, E. ; Medail, F. et Tatoni, T. (1999) *: Analyse diachronique des changements floristiques sur un archipel Méditerranéen périurbain (îles du Frioul, Marseille)*. Rev. Ecol. (Terre et vie), Vol.54 (1). pp.3-18.

Bougaham, A.F. (2008): *Contribution à l'étude de la biologie et de l'écologie des oiseaux de la côte à l'ouest de Jijel*. Mémoire de Magister. Université A.MIRA, Bejaia (ALGERIE).103p

Boukhalfa, D. (1990*):Observations de quelques espèces d'oiseaux de mer nicheurs sur la côte d'Oran (Algérie).* Rev. L'oiseau et R.F.O., Vol.60 (3). pp.248-251.

Chaeb, M. (2003) : *Caractéristiques floristiques des îles KNEISS*. Projet de préservation de la biodiversité dans la réserve naturelle des îles KNEISS.

Duhautois, L. et Hoff, M. (2000) : *La flore de France, enjeu majeur de la politique de conservation de la nature*. Les données de l'environnement N° 54.pp.1-4.

Emberget, L. (1966) : *Réflexion sur le spectre biologique de RAUNKIAER*. Mém .Soc. Bot. Fr., 1966.pp.147-156.

Gamisans, G. and Jeanmonod, D. (1934): *The life-forms of plants and statistical plant geography*. Clarenlon Press. Oxford. 632p.

Gillham, M.E. (1961): *Alteration of the breeding habitat by sea-birds and seals in Western Australia*. J. Ecol., 49.pp.289-300.

Grime, J.P (1974): *Vegetation classification by reference to strategies*. Nature.pp. 26-31.

Jacob, J.P. et Courbet, B. (1980) : *Oiseaux de mer nicheurs sur la côte en Algérie*. Le Gerfaut 70.pp.385-401.

Moulai, R. (2005): *Contribution à l'évolution de la biodiversité biologique des îlots de la côte occidentale de Bejaia (Algérie)*.1er séminaire International sur l'environnement et ses problèmes.4p.

Moulai, R. (2006) : *Bioécologie de l'avifaune terrestre et marine du parc national de Gouraya (Bejaia), cas particulier du Goéland leucophée, Larus michahellis Naumann,* ***1840.*** Thèse de Doctorat d'état, Sci. Agro. Inst.Nat.Agro.,EL HARRACH.185p.

Medail, F. et VidaL, E. (1998): *Rôle des Goélands leucophées dans l'implantation et l'expansion d'espèces végétales allochtones sur l'archipel de Riou (Marseille, France)*.Biocome, Mesgeen, Nice, 15(1).pp.123-140.

Paradis, G. et Piazza, C. (2002): *Contribution à l'étude de la flore et de la végétation des îlots satellites de la Corse*. Le monde des plantes N°477.

Patrick, B. (2002) : *Etre plantes à l'ombre des forêts tropicales*. Nathan/VUEF, Paris, France, 432 p.

Quezel, P. et Santa, S. (1963*): La nouvelle flore de l'Algérie et des régions désertiques méridionales*. Ed. du centre national de la recherche scientifique.

Raunkiaer, C. (1934): *The life forms of plants and statistical plant and geography*. Clarenlon Press. Oxford, 632p.

Thibault, J-C.; Zotier, R.; Guyot, I. and Bretagnolle, V. (1996): *Recent trends in breeding marine birds of the Mediterranean region with special reference to Corsica*. Colonial Water birds, 19.pp.31-40.

-Vander Pigl, L. (1982): *Principles of dispersal in higher plants*. Springer Verlag. Berlin, Heidelberg, New York.

Vidal, P. et Marthe, A. (2003) : *Les îles de Marseille : patrimoine naturel et problématique de conservation. Extrait de Méditerranée autour de ses îles*.128ème Congrès National des sociétés historiques et scientifiques. Bastia.

Vidal, E. (1998) : *Organisation des phytocénoses en milieux insulaires méditerranéen perturbé : Analyse des inter -relations entre les colonies de Goélands leucophées et la végétation des îles de Marseille*. These doctorat. 155p.

Whiteheard, D.R. and Jones, C.E. (1969): *Small islands and equilibrium theory of insular biogeography*.Evolution, 23:pp. 171-179.

This book collects edited and revised versions of papers and abstracts that have been scheduled to be presented at the first International Conference on the Conservation and Sustainable Use of Wild Plant Diversity (CSUWPD), held in May 4-8, 2010, at the Orthodox Academy of Crete, Kolympari, Chania.

The high number of proposals confirms the growing interest for the issues of biodiversity conservation. About forty four (44) abstracts proposal were submitted from twenty nine (29) countries, including *Algeria, Austria, Australia, Bangladesh, Bulgaria, Ethiopia, Faulkland, France, Hungary, Germany, Greece, India, Indonesia, Iraq, Iran, Ireland, Italy, New Zealand, Poland, Romania, Serbia, Sierra Leone, South Africa, Soudan, Sweden, Tanzania,* Turkey, United Kingdom and the United States of America. From these submissions, thirty nine (39) were selected for presentation and publication in this book. More than hundred participants were expected but due to the volcano ashes of Ireland and the general strike in Greece mainland, only one third of the registered participants managed to come to Crete. Therefore, this book stands as a relief for those who could not join the conference.

The CSUWPD event brings together multidisciplinary and multicultural approaches of biodiversity conservation. The themes were classified into six categories namely:

- Botanic Gardens, Reserves and Case Studies for wild plants,
- Eco-Theology and Plant Ethics,
- Wild plant in Scientific Research and Development,
- Taxonomic Investigations and Conservation Methods for Wild plants,
- Models and Issues of Sustainable Use of Wild plants,
- Wild plant in health science, cosmetics and pharmacology, and
- Wild plant in natural ecosystem and climate change.

The selected papers and abstracts present a useful perspective to evaluate the core of biodiversity management and the conference itself revealed the diversity of approaches to the issues of biodiversity conservation. However, the goal was not to collect academic papers but to raise awareness for the protection of life and to call the community to celebrate together the year of biodiversity. The outcome of the conference is summarized in the "***2010 Declaration from Crete on Plant Biodiversity***" which is stated at the end of this book.

I wish the most constructive inspirations for all readers of this book and I look forward to organizing the second CSUWPD conference in 2013 at the same place (OAC) – God Willing.

Dr. Lucas Andrianos
CSUWPD organizer
Institute of Theology and Ecology
Orthodox Academy of Crete (OAC)
www.oac.gr

ISBN 978-960-86383-6-5

Caractérisation de dix variétés d'huile d'olive algérienne: étude du profil en composés phénoliques par HPLC

R. Laribi[1]*
F. Laincer[1]
A. Tamendjari[1]
S. Keciri[3]
L. Arrar[4]
S. Venturini[2]
P. Rovellini[2]

[1]Faculté des Sciences de la Nature et de la Vie, Laboratoire de Biochimie Appliquée, Université A/Mira, Bejaia, Algérie.
[2]Stazione Sperimentale per le Industrie degli Oli e dei Grassi Milano Italy
[3] Institut Technique de L'arboriculture Fruitière et de la Vigne, Takarietz, Bejaia, Algérie
[4]Laboratoire de Biochimie Appliquée, Département de Biologie, Université Ferhat Abbas, Sétif, Algérie

**AUTEUR CORRESPONDANT*
Lehouche Rahima née Laribi
Faculté des Sciences de la Nature et de la Vie
Département des Sciences Alimentaires
Université A/ Mira,
Bejaia 06000, Algérie.
tel. +213 34 21 43 33 à 35
fax +213 34 21 60 98
e-mail: laribi.rahima@yahoo.fr

La présente étude est effectuée pour une caractérisation physico-chimique de dix variétés d'huile d'olive algérienne**.** Les paramètres de qualité (acidité, indice de peroxyde, K_{232} et K_{270}), la composition chimique des huiles en acides gras, le dosage des composés phénoliques totaux et *ortho*-diphénols ainsi que le profil en composés phénoliques par HPLC sont déterminés. Les résultats obtenus montrent que les dix variétés d'huile appartiennent à la catégorie d'huile d'olive vierge extra. Des variations cultivar-dépendantes ont été relevées concernant la composition en acides gras et en composés phénoliques.
Mots clés: Huile d'olive, caractérisation, variétés algériennes, qualité, composés phénoliques, HPLC

Studio del profilo dei composti fenolici mediante HPLC: caratterizzazione di dieci varietà di oli di oliva algerini

Con il presente studio è stata effettuata una caratterizzazione fisico-chimica di dieci varietà di olio di oliva algerino.
In particolare sono stati determinati i parametri di qualità (acidità, numero di perossidi, K_{232}, K_{270}), la composizione chimica degli acidi grassi, il dosaggio dei composti fenolici totali e degli orto-difenoli, oltre al profilo di tutti i composti fenolici ottenuto per HPLC.
I risultati ottenuti mostrano che le dieci varietà di olio appartengono alla categoria degli oli extra vergini di oliva.
Alcune differenze cultivar-dipendenti sono state individuate per quanto riguarda la composizione in acidi grassi e in composti fenolici.
Parole chiave: Olio di oliva, caratterizzazione, varietà algerine, qualità, composti fenolici, HPLC.

1. INTRODUCTION

La culture de l'olivier revêt une importance non négligeable pour l'Algérie. Elle couvre plus de 200.000 ha et représente 49% du verger arboricole. Cette oléiculture est localisée principalement en Kabylie et dans la région oranaise. Le nombre d'arbres plantés est estimé à 32 millions, avec une production moyenne, sur les cinq dernières campagnes (2003/2004 à 2008/2009) de 39800 tonnes (soit 1,4% de la production mondiale) [1]. L'oléiculture algérienne est caractérisée par une large gamme de variétés dont une collection (36 variétés) existe au niveau de l'institut technique d'arboriculture fruitière et de la vigne de la wilaya de Bejaia. Cette wilaya est considérée comme le premier bassin oléicole national, avec plus de 50000 hectares de vergers et 4 millions d'arbres. Les variétés les plus répandues dans cette willaya sont *Chemlal*, *Limli*, *Azeradj* et *Takesrit* [2].
La caractérisation des variétés de l'huile d'olive est une préoccupation primordiale pour les pays producteurs notamment ceux du Bassin Méditerranéen. D'ailleurs, plusieurs travaux de recherches sont publiés dans ce domaine [3-11]. En Algérie, il existe plusieurs travaux de recherches traitant différents aspects sur l'huile d'olive [12-17]. Parmi ces travaux, peu d'entre eux ont étudié la fraction phénolique des huiles. Cette fraction est importante en raison de sa contribution aux propriétés nutritionnelles, sensorielles et à la stabilité de l'huile d'olive [18]. L'objectif de ce présent travail consiste en la caractérisation d'une dizaine de variétés algériennes à travers l'étude des paramètres de qualité d'une part et d'autre part la composition chimique des huiles en acides gras et en biophénols.

2. MATERIEL ET METHODES

2.1 MATÉRIEL VÉGÉTAL

Les échantillons d'olive de dix variétés ont été récoltés à la main durant la saison de récolte 2008/2009. Neuf variétés (*Aghenfas*, *Akerma*, *Blanquette de Guelma*, *Bouricha*, *Chemlal*, *Ferkani*, *Limli*, *Neb Djmel* et *Tabelout*) ont été récoltées à partir d'une ferme pilote de l'institut technique de l'arboriculture fruitière et de la vigne (ITAFV), situé à Takarietz, daïra de Sidi Aich, wilaya de Bejaia, Algérie. La dixième variété a été récoltée à partir d'une ferme pilote située à Tazmalt (Bejaia). Les quantités d'olives récoltées sont d'environ 7 Kg pour chaque variété. Après la cueillette, les olives ont été nettoyées de toutes impuretés et ensuite transportées au laboratoire.
Après avoir déterminé l'indice de maturation des olives selon la méthode d'Uceda et Frias [19], les olives nettoyées ont subi l'extraction afin de récupérer l'huile. Cette étape a été réalisée à l'aide d'un oléodoseur de laboratoire (Levi-Deleo-Lerogsame). L'extraction consiste en un broyage des olives, un malaxage de la pâte et une séparation de l'huile par centrifugation. Une quantité d'eau tiède (30°C) à raison de 50 ml pour 920 g de pâte d'olive est ajoutée pour faciliter l'extraction. Les huiles sont recueillies dans des flacons en verre fumé, remplis, étiquetés et conservés à une température de 4°C en attendant d'être analysées.

2.2 LES INDICES DE QUALITÉ

L'acidité et l'indice de peroxyde sont déterminés suivant les protocoles de l'U.I.C.P.A [20]. Les absorbances dans UV à 232 nm et 270 nm sont déterminées suivant la méthode du Conseil Oléicole International [21]. Trois essais ont été effectués pour chaque paramètre.

2.3 COMPOSITION EN ACIDES GRAS

Les esters méthyliques sont préparés suivant la méthode de la Commission Européenne [22]. Les esters méthyliques sont injectés dans un chromatographe en phase gazeuse de type Chrompack C 9002 équipé d'un détecteur FID. Une colonne capillaire DB 23 (60 m, 0,25 mm, 0,25 µm) a été utilisée. Le gradient de température suivant a été utilisé: de 130°C à 170°C (maintenu pendant 1 mn) à 6,5°C/mn, de 170°C à 215°C (maintenu pendant 12 mn) à 2,75°C/mn et de 215°C à 230°C (maintenu pendant 3 mn) à 40°C/mn. L'injecteur est du type split, avec un débit de 49,9 ml/mn et il est porté à une température de 270°C. Le gaz vecteur utilisé est l'azote à un débit de 45 ml/mn. La température du FID est de 250°C et le volume injecté est de 1 µl.

2.4 DOSAGE SPÉCTROPHOTOMÉTRIQUE DES COMPOSÉS PHÉNOLIQUES TOTAUX

L'extraction des composés phénoliques est réalisée suivant le protocole de Tsimidou *et al*. [23] modifié. Cinquante grammes d'huile sont dissous dans 50 ml d'hexane, cette solution est introduite dans une ampoule à décanter et 30 ml du mélange méthanol/eau (80/20) sont ajoutés, le mélange est agité vigoureusement durant 5 min puis laissé décanter, la phase polaire (phase méthanolique) contenant les composés phénoliques est récupérée, tandis que la phase apolaire subit une 2ème et une 3ème extraction pour récupérer la fraction phénolique restante. Chaque fraction polaire récupérée subit un lavage avec 50 ml d'hexane. Les solutions sont concentrées sous vide à l'aide d'un rotavapor (K-IKA Labortechnik), à une température de 40°C, jusqu'à avoir des volumes d'extraits d'environ 2 ml. Le séchage des extraits est complété dans l'étuve à 40°C jusqu'à l'obtention d'un poids constant.
L'estimation de la teneur en composés phénoli-

ques a été réalisée selon la méthode au Folin-Ciocalteu. L'absorbance est mesurée au spectrophotomètre à 765 nm. Trois essais ont été effectués.
La concentration en composés phénoliques des extraits de l'huile est déterminée en se référant à la courbe d'étalonnage obtenue en utilisant l'acide gallique comme standard.

2.5 DOSAGE SPÉCTROPHOTOMÉTRIQUE DES *ORTHO*-DIPHÉNOLS

La concentration en *ortho*-diphénols des extraits méthanoliques des échantillons d'huile est déterminée suivant la méthode de Bendini *et al*. [24]. L'absorbance est mesurée à 370 nm. Les teneurs en *ortho*-diphénols des échantillons sont déterminées à partir d'une courbe d'étalonnage réalisée avec de l'acide caféique comme standard. Trois essais ont été effectués.

2.6 DÉTERMINATION DES COMPOSÉS PHÉNOLIQUES DES HUILES D'OLIVE PAR CHROMATOGRAPHIE EN PHASE LIQUIDE À HAUTE PERFORMANCE (HPLC)

La méthode se base sur une extraction des composés mineurs polaires de nature phénolique directement à partir de l'huile d'olive au moyen d'une solution méthanolique «méthanol/eau (80/20)», suivie de leur dosage par HPLC au moyen d'un révélateur UV à 280 nm. L'étalon interne est l'acide syringique [25].
Un volume de 20 µl d'échantillon est injecté dans une colonne C18 de type Spherisorb ODS-2 (4,6 mm x 250 mm, taille des particules : 5 µm). La phase mobile est composée de: eau/acide orthophosphorique (99,8 : 0,2 V/V), méthanol et acétonitrile.
Les teneurs correspondantes aux polyphénols totaux, individuels et oxydés sont exprimées en mg/kg.

2.7 ANALYSE STATISTIQUE

Une étude statistique a été réalisée concernant l'indice de maturité, les paramètres de qualité et les dosages colorimétriques des composés phénoliques totaux et *ortho*-diphenols en appliquant une analyse de variance (ANOVA). Le test de Newman-Keuls est utilisé pour la comparaison intergroupe. Le logiciel utilisé est STATISTICA 5.5.

3. RESULTATS ET DISCUSSION

3.1 INDICE DE MATURITÉ

L'indice de maturité est un paramètre qui pourrait nous renseigner d'une façon globale sur la maturité des fruits. Les résultats obtenus (Tableau I) montrent des valeurs de l'indice de maturité qui varient entre 3,38 pour la variété *Aghenfas* et 6,79 pour la variété *Tabelout*. Cette variation peut être liée à la variation de la période de récolte étant donné que la variété *Tabelout* est récoltée à une époque tardive par rapport aux variétés *Limli*, *Bouricha*, *Aghenfas* et *Neb Djemel*. L'analyse statistique permet de répartir nos variétés en groupes homogènes (*Aghenfas*, *Bouricha* et *Limli*), (*Akerma* et *Neb Djemel*) et (*Akerma*, *Blanquette de Guelma* et *Ferkani*). Bien que les variétés *Chemlal Tazmalt*, *Ferkani* et *Tabelout* soient récoltées à la même période, elles enregistrent un écart significatif (p<0,05) de l'indice de maturité, ceci est probablement lié à l'effet variétal suite à des facteurs génétiques, certaines variétés entrent en maturation plus vite que d'autres. El Antari *et al*. [7] ont noté que la variété *Manzanilla* se distingue par sa vitesse élevée d'entrée en maturation.

Tableau I - Valeurs de l'indice de maturité des olives des différentes variétés

Variété	Indice de maturité
Aghenfas	3,38±0,17[a]
Akerma	4,73±0,08[d]
Blanquette de Guelma	4,85±0,01[d]
Bouricha	3,88±0,17[ab]
Chemlal	5,55±0,44[e]
Chemlal Tazmalt	4,01±0,01[b]
Ferkani	5,02±0,09[d]
Limli	3,72±0,07[ab]
Neb Djemel	4,40±0,06[c]
Tabelout	6,79±0,12[f]

***** les valeurs portant les mêmes lettres ne présentent aucune différence significative (p<0,05)

3.2 LES INDICES DE QUALITÉ

Les valeurs d'acidité (Tableau II) sont très faibles et inférieures à la limite établie par le COI [26] qui est de 0,8% pour l'huile d'olive extra vierge. Les différences significatives relevées entre les variétés peuvent être liées à l'indice de maturité des olives. En effet, l'acidité augmente avec la maturité du fruit d'olive [27], suite à une augmentation de l'activité enzymatique spécialement l'enzyme lipolytique [28, 29].
Les huiles des variétés étudiées sont moins acides que les huiles des variétés tunisiennes analysées par Zarrouk *et al*. [30], pour lesquelles l'acidité libre est comprise entre 0,38 et 0,41% d'acide oléique, elles sont proches des huiles des variétés espagnols *Picual*, *Cornicabra*, *Manzanilla*, *Arbequina* et *Local* dont les valeurs sont comprises entre 0,10 et 0,25% [31] et des variétés d'huiles européennes introduites en Tunisie ainsi que la variété tunisienne *Chemlali* dont les teneurs varient entre 0,11 et 0,28% [32].

Tableau II - Les indices de qualité des différentes variétés d'huile d'olive

Indice / Variété	Acidité (% d'acide oléique)	Indice de peroxyde (méq O_2/Kg)	K_{232}	K_{270}
Aghenfas	0,16 ± 0,00^{d}	4,75 ± 0,25^{c}	1,968 ± 0,008^{d}	0,155 ± 0,002^{c}
Akerma	0,08 ± 0,00^{b}	5,75 ± 0,25^{d}	2,461 ± 0,017^{i}	0,146 ± 0,006^{c}
Blanquette de Guelma	0,18 ± 0,008^{e}	8,25 ± 0,25^{g}	1,484 ± 0,000^{b}	0,206 ± 0,006^{d}
Bouricha	0,15 ± 0,00^{d}	4,25 ± 0,25^{bc}	2,205 ± 0,043^{f}	0,191 ± 0,003^{c}
Chemlal	0,11 ± 0,00^{c}	8,25 ± 0,25^{fg}	2,443 ± 0,009^{j}	0,150 ± 0,005^{c}
Chemlal Tazmalt	0,05 ± 0,008^{a}	7,25 ± 0,25^{e}	1,882 ± 0,000^{c}	0,151 ± 0,002^{c}
Ferkani	0,11 ± 0,00^{c}	4,25 ± 0,25^{b}	2,342 ± 0,011^{g}	0,125 ± 0,002^{b}
Limli	0,15 ± 0,00^{d}	8,75 ± 0,25^{g}	2,428 ± 0,000^{h}	0,143 ± 0,007^{c}
Neb Djemel	0,18 ± 0,008^{e}	7,75 ± 0,25^{f}	2,113 ± 0,000^{e}	0,165 ± 0,002^{c}
Tabelout	0,23 ± 0,00^{f}	3,25 ± 0,25^{a}	1,155 ± 0,005^{a}	0,107 ± 0,005^{a}

* les valeurs portant les mêmes lettres ne présentent aucune différence significative (p<0,05)

Concernant l'indice de peroxyde, les huiles analysées montrent des valeurs qui varient entre 3,25 meq O_2/Kg pour la variété *Tabelout* et 8,75 meq O_2/Kg pour la variété *Limli.* Les valeurs atteintes sont inferieures à la norme du COI [26] pour les huiles d'olives de catégorie extra-vierge (20 meq O_2/Kg). Les huiles de nos variétés présentent des indices de peroxyde proches de ceux enregistrés pour les huiles des variétés tunisiennes *Chétoui*, *Jarboui*, *Ain Jarboua*, *Neb Jmel*, *Rekhami*, *Regregui* qui varient entre 2,63 et 7,90 meq d'O_2/Kg [33], mais inferieures à certaines variétés turques (entre 7,37 et 16,08 meq d'O_2/Kg) [34].

Concernant l'extinction spécifique dans l'UV, des différences significatives (p<0,05) sont enregistrées entre les variétés (Tableau II) sauf entre les variétés *Akerma* et *Chemlal* pour le coefficient K_{232} et entre les huiles *Aghenfas*, *Akerma*, *Bouricha, Chemlal, Chemlal Tazmalt, Limli* et *Neb Djemel* pour le coefficient K_{270}.

Les valeurs les plus élevées du coefficient K_{232} sont relevées pour la variété *Akerma* (2,46) et *Chemlal* (2,44) et la valeur la plus basse pour la variété *Tabelout* (1,15). Quant au coefficient K_{270}, les valeurs se situent entre 0,107 et 0,206. Les échantillons d'huile présentent tous des coefficients d'extinction spécifique dans l'UV (K_{232}, K_{270}) inférieurs aux limites établies par le COI [26] pour une huile d'olive extra-vierge ($K_{232} \leq 2,5$ et $K_{270} \leq 0,22$).

Nos résultats des coefficients d'extinction spécifique dans l'UV (K_{232}, K_{270}) sont proches de ceux enregistrés pour les variétés européennes introduites en Tunisie *Ascolana Tenera, Koroneiki et Picholine* qui varient entre 1,6 et 2,8 (pour le coefficient K_{232}) et entre 0,1 et 0,2 (pour le coefficient K_{270}) [35].

Les résultats d'analyses (acidité, indice de peroxyde et des coefficients d'extinction spécifique dans

Tableau III - Valeurs moyennes des acides gras totaux des différentes variétés d'huile d'olive (en % des acides gras totaux)

	Aghenfas	*Akerma*	*Blanquette de Guelma*	*Bouricha*	*Chemlal*	*Chemlal Tazmalt*	*Ferkani*	*Limli*	*Neb Djemel*	*Tabelout*
C16 :0	13,59	14,89	12,51	18,45	17,02	19,15	14,21	18,97	19,55	16,27
C18:0	2,85	2,35	2,65	2,70	1,75	1,82	2,52	2,57	1,88	2,47
C18:1	72,13	67,38	61,00	66,26	65,51	65,71	67,88	65,11	64,06	70,97
C18:2	9,31	13,88	21,67	10,77	14,39	11,48	13,25	11,5	12,75	8,93
C18:3	0,94	1,00	0,86	0,81	0,65	0,71	0,52	0,81	0,79	0,75
C20:0	0,43	0,40	0,49	0,45	0,38	0,40	0,46	0,46	0,37	0,38
C20:1	0,30	0,10	0,43	0,26	0,29	0,35	0,30	0,27	0,23	0,24
AGI	82,68	82,36	83,96	78,1	80,84	78,25	81,95	77,69	77,83	80,89
AGS	16,87	17,64	15,65	21,6	19,15	21,37	17,19	22	21,8	19,12
AGI/AGS	4,90	4,67	5,36	3,61	4,22	3,66	4,76	3,53	3,57	4,23
C18:1/C18:2	7,75	4,85	2,81	6,15	4,55	5,72	5,12	5,66	5,02	7,95

AGI : acides gras insaturés, **AGS** : acides gras saturés.

l'UV (K_{232}, K_{270}) effectuées sur les huiles produites à partir des dix variétés étudiées s'inscrivent tous parfaitement dans les limites définies par le COI [26] pour une huile d'olive extra vierge, ce qui nous permet de classer les huiles issues de ces variétés dans cette catégorie. Ces résultats peuvent être expliqués en partie par les conditions appropriées de récolte et d'extraction des huiles.

3.3 COMPOSITION EN ACIDES GRAS

L'analyse de la composition des acides gras totaux (Tableau III) est qualitativement similaire entre les échantillons. Quantitativement, toutes les huiles des variétés étudiées présentent des teneurs en différents acides gras répondant aux normes établies par le COI [26] pour les huiles d'olives extra-vierges.

L'acide oléique est l'acide gras dominant de la composition des huiles d'olive. Toutes les variétés étudiées ont des proportions supérieures à 60%. Ces proportions peuvent distinguer entres les variétés, les valeurs les plus élevées sont enregistrées respectivement pour les variétés *Aghenfas* et *Tabelout* (72,13% et 70,97%) suivies des variétés *Akerma, Bouricha, Chemlal, Chemlal Tazmalt, Ferkani, Limli* et *Neb Djemel,* la plus faible valeur étant notée pour la variété *Blanquette de Guelma* (61%). Ces taux sont très proches de ceux obtenus pour des variétés marocaines [7], des variétés tunisiennes [36] et des variétés turques [37]; supérieurs aux teneurs des variétés *Chemlali* et *Chétoui* de Sousse et Sfax [38] et de la variété espagnol *Arbequina* [39] et inferieurs aux taux enregistrés par Pardo *et al*. [31] pour des variétés espagnoles. Les variétés *Aghenfas* et *Tabelout* se distinguent des autres variétés par les taux les plus faibles en acide linoléique soit 9,31 et 8,93% respectivement. Ces variétés se caractérisent ainsi par les rapports acide oléique/acide linoléique les plus élevés (7,75 et 7,95).

Des résultats similaires sont rapportés par Oueslati *et al*. [40] pour la variété tunisienne *Fakhari Douirat.* La variété *Blanquette de Guelma* présente le taux le plus élevé en acide linoléique (21,67%), elle se caractérise ainsi par le rapport acide oléique/acide linoléique le plus faible. Des résultats proches sont relevés pour la variété *Chétoui* cultivée à Sousse ville de la Tunisie [38]. Le rapport acide oléique/acide linoléique est utilisé comme paramètre de stabilité et plusieurs études ont montré qu'un rapport élevé engendre une stabilité oxydative importante [36].

Certains acides gras minoritaires peuvent également être utilisés comme indicateur variétal. La variété *Akerma* se distingue du reste des variétés par les plus faibles proportions en acide gadoléique (0,10%), par rapport aux autres variétés dont les taux oscillent entre 0,23 et 0,43%.

Les taux d'acides gras saturés et insaturés nous permettent de distinguer entre les variétés. La variété *Blanquette de Guelma* présente un pourcentage en acides gras saturés de 15,65% et un total en acides gras insaturés de 86,18%. Tandis que les variétés *Limli, Neb Djemel, Bouricha* et *Chemlal Tazmalt,* enregistrent respectivement, un total en acides gras saturés de 22%, 21,8%, 21,6% et 21,37% et un total en acides gras insaturés de 77,69%, 77,83%, 78,1% et 78,25%.

Des variations cultivar-dépendantes en profils d'acide gras des variétés d'huile d'olive sont relevées par plusieurs auteurs [3, 39, 41]. Bien que les variétés soient cultivées dans la même parcelle expérimentale mais elles conservent leur propre rythme de biosynthèse des lipides, la composition d'acide gras varie sensiblement d'un cultivar à un autre, selon leur patrimoine génétique.

3.4 LES POLYPHÉNOLS TOTAUX

Les teneurs en polyphénols totaux diffèrent significativement (p<0,05) d'une variété à une autre (Tableau IV). Ces résultats sont en accord avec les résultats de Haddada *et al*. [36] et Zarrouk *et al*. [30] quant à l'influence de la variété sur la composition quantitative en composés phénoliques dans l'huile d'olive.

Tableau IV - Teneurs en composés phénoliques totaux et en *ortho*-diphénols des différents extraits méthanoliques des huiles d'olives par méthode colorimétrique

Variété	Teneur en composés phénoliques totaux (mg/Kg)	Teneur en *ortho*-diphénols (mg/Kg)
Aghenfas	161,78 ± 6,24^{c}	13,15 ± 0,00^{d}
Akerma	130,92 ± 7,68^{b}	10,07 ± 0,00^{c}
Blanquette de Guelma	365,24 ± 8,40^{f}	23,37 ± 0,59^{g}
Bouricha	369,79 ± 6,88^{f}	15,65 ± 0,86^{e}
Chemlal	234,38 ± 6,42^{d}	15,49 ± 0,18^{e}
Chemlal Tazmalt	115,73 ± 4,35^{a}	7,54 ± 0,00^{b}
Ferkani	243,23 ± 2,87^{e}	19,30 ± 0,00^{f}
Limli	123,43 ± 0,79^{ab}	5,68 ± 0,00^{a}
Neb Djemel	420,95 ± 2,02^{g}	29,67 ± 0,73^{h}
Tabelout	239,79 ± 1,69^{e}	19,36 ± 0,97^{f}

***** les valeurs portant les mêmes lettres ne présentent aucune différence significative (p<0,05)

Les teneurs les plus élevées sont enregistrées pour la variété *Neb Djemel* (420,95 mg/kg) suivie des variétés *Bouricha* (369,79 mg/kg) et *Blanquette de Guelma* (365,24 mg/kg), tandis que les teneurs les plus faibles sont obtenues pour les variétés *Akerma* (130,92 mg/kg), *Limli* (123,43 mg/kg) et *Chemlal Tazmalt* (115,73 mg/kg). Le reste des variétés présentent des teneurs comprises dans l'intervalle de 161,78 à 243,23 mg/kg.

D'après Tsimidou [42], on peut classer les variétés étudiées par rapport à leurs teneurs en polyphénols comme suit: les variétés *Aghenfas, Akerma*, *Chemlal Tazmalt, Limli* sont classées dans la catégorie à teneur faible en polyphenols totaux, tandis que les variétés *Blanquette de Guelma, Bouricha, Chemlal, Ferkani, Neb Djemel* et *Tabelout* sont à teneur moyenne en polyphénols totaux.

Nos variétés présentent des teneurs en polyphénols relativement proches de celles des variétés italiennes étudiées par Baiano *et al*. [43], pour lesquelles les teneurs en polyphenols sont comprises entre 133,68 et 322,18 mg/kg, et des variétés turques étudiées par Ocakoglu *et al*. [34] qui varient entre 75,46 et 333,37 mg/kg; mais supérieures aux teneurs des variétés espagnoles analysées par Cerretani *et al*. [44], qui présentent des teneurs en polyphenols totaux comprises entre 37,2 et 93,2 mg/kg, et aux variétés *Picual, Barnea, Empeltre, Manzanilla Californiana et Manzanilla Criolla* cultivées en Argentine, dont les teneurs varient de 25,3 à 92,7 mg/kg [45].

D'après Tovar *et al*. [46], au cours de la maturation, l'activité de l'enzyme PAL diminue et l'activité des estérases et des glucosidases augmente entrainant ainsi une réduction des taux des polyphénols totaux, ce qui peut expliquer les faibles concentrations obtenues pour les huiles des variétés *Tabelout* et *Chemlal* pour lesquelles nous avons noté des indices de maturités de 6,79 et 5,55 respectivement, par rapport aux variétés *Neb Djemel*, *Bouricha* et *Blanquette de Guelma* dont les teneurs en polyphenols sont élevées et un indice de maturité qui varie entre 3,38 et 4,85.

3.5 LES *ORTHO*-DIPHÉNOLS

Les teneurs en *ortho*-diphénols sont représentées dans le Tableau IV. Des différences significatives (p<0,05) sont relevées entre les variétés d'huiles étudiées sauf entre les huiles *de Chemlal* et *Bouricha* et entre *Ferkani* et *Tabelout*.

Les huiles des variétés de *Neb Djemel* (29,67 mg/kg) et *Blanquette de Guelma* (23,37 mg/kg) sont les plus riches en *ortho*-diphénols. En revanche, les variétés *Limli* et *Chemlal Tazmalt* donnent des huiles à teneurs inférieures (5,68 mg/kg, 7,54 mg/kg respectivement). Le reste des variétés présentent des teneurs comprises dans l'intervalle de (10,07 à 19,36 mg/kg). Comparé aux teneurs en polyphénols totaux enregistrées pour les différentes huiles analysées, on remarque que c'est l'huile la plus riche en polyphénols totaux (*Neb Djemel*) qui enregistre le taux le plus élevé en *ortho*-diphénols, mais ce n'est pas le cas pour les variétés *Ferkani* et *Tabelout* qui enregistrent des teneurs plus importantes en *ortho*-diphénols par rapport à *Chemlal* et *Bouricha* malgré que ces dernières enregistrent des teneurs plus élevées en polyphenols totaux. Un coefficient de corrélation significatif (p<0,05) est relevé entre le taux de polyphenols totaux et *ortho*-diphénols (r=0,88). Nos résultats sont en accord avec ceux de Di Giovacchino *et al*. [47] et ceux de Cerretani *et al*. [10] qui ont enregistré un coefficient de corrélation de 0,88 et 0,82 respectivement.

Les teneurs enregistrées en *ortho*-diphénols pour nos variétés sont relativement proches des variétés espagnoles *Picual, Hojiblanca* et *Arbequina* dont les teneurs varient entre 3,99 et 18,92 mg/kg [48], mais largement inférieures aux variétés tunisiennes telles rapportées par Zarrouk *et al*. [30] dont les teneurs varient entre 188,12 et 213,24 mg/kg.

3.6 DÉTERMINATION DES COMPOSÉS PHÉNOLIQUES DES HUILES D'OLIVES PAR CHROMATOGRAPHIE EN PHASE LIQUIDE À HAUTE PERFORMANCE (HPLC)

L'analyse des chromatogrammes des extraits phénoliques des huiles étudiées montre une composition qualitative en composés phénoliques similaire pour l'ensemble des échantillons, mais différente d'un point de vue quantitatif. La composition de la fraction phénolique présente un intérêt dans la caractérisation variétale des huiles d'olive vierges [49].

Ce profil en composés phénoliques (Tableau V) est analogue à celui observé par d'autres auteurs pour les variétés espagnoles [50], les variétés turques [51], les variétés françaises [52] et les variétés algériennes [17].

En comparant aux taux des polyphénols totaux, pour toutes nos variétés étudiées, les secoiridoides représentent la classe majoritaire des polyphénols totaux, leurs distributions varient d'une variété à une autre ce qui est en accord avec les résultats de Torre-Carbot *et al*. [53], Gómez-Rico *et al*. [54] et ceux de Dabbou *et al*. [35]. C'est la variété *Neb Djemel* qui enregistre le pourcentage le plus élevé 83,61% et le pourcentage minimal est obtenu pour l'huile de la variété *Chemlal Tazmalt* (43,19%). Un coefficient de corrélation positif est enregistré r=0,98 entre le taux des polyphenols totaux et les dérivés d'oleuropéine et de ligstroside.

Les variétés *Neb Djemel* et *Bouricha* se caractérisent par les teneurs les plus élevées en dérivés d'oleuropéines (276 et 235 mg/kg) et dérivés de ligstroside (173 et 150 mg/kg), suivie des variétés *Chemlal, Ferkani, Blanquette de Guelma* et *Tabelout* dont les teneurs oscillent entre 119 et 103 mg/kg (dérivés d'oleuropéine) et entre 91 et 124 mg/kg (dérivés de ligstroside). Les teneurs les plus faibles sont relevées pour les variétés *Chemlal Tazmalt*, *Limli*, *Akerma* et *Aghenfas* dont les valeurs varient entre 36 et 61 mg/kg (dérivés d'oleuropéine) et entre 26 et 62 mg/kg (dérivés de ligstroside).

D'après Servili *et al*. [55] et Dabbou *et al*. [35], les

Tableau V - Teneurs en composés phénoliques (mg/Kg) des huiles d'olive de variétés algériennes par HPLC

	Aghenfas	*Akerma*	*Blanquette de Guelma*	*Bouricha*	*Chemlal*	*Chemlal Tazmalt*	*Ferkani*	*Limli*	*Neb Djemel*	*Tabelout*
Dérivés d'oleuropéine (mg/kg)	50	36	203	235	133	61	119	60	276	120
➢ Hydroxytyrosol	0,5	0,9	5	5	3	2	1	3	4	4
Dérivés de ligstroside (mg/kg)	26	44	115	150	101	50	91	62	173	124
➢ Tyrosol	3	4	28	26	3	3	4	26	5	11
Flavonoïdes (mg/kg)	44	49	18	25	23	34	14	10	33	13
➢ Apigénine ➢ Lutéoline	12 32	11 38	6 12	7 18	8 15	14 20	4 10	3 7	13 20	7 6
Lignanes (mg/kg)	16	38	33	51	38	41	42	25	21	29
Polyphénols oxydés (mg/kg)	14	15	51	55	54	67	13	42	19	22
Polyphénols totaux (HPLC) (mg/kg)	169	207	430	522	354	257	287	208	537	316

dérivés secoiridoides sous forme dialdehydique de l'acide élénolique liées à l'hydroxytyrosol (3,4-DHPEA-EDA) ou liés au tyrosol (*p*-HPEA-EDA) et les isomères d'oleuropéine aglycone (3,4-DHPEA-EA) sont les secoiridoides les plus abondants dans l'huile d'olive. Ils sont issus de l'hydrolyse des secoiridoides glucosides présents dans le fruit d'olive par activation de l'enzyme, β-glucosidase au cours de l'extraction [56, 57].

L'hydroxytyrosol (3,4-DHPEA) et le tyrosol (*p*-HPEA) sont les principaux alcools phénoliques présents dans les huiles des variétés étudiées ce qui est en accord avec les résultats obtenus par Servili *et al*. [55] et Ocakoglu *et al*. [34]. Toutes les variétés d'huiles étudiées présentent des teneurs en tyrosol plus élevées que celles en hydroxytyrosol, les valeurs oscillent entre 3 et 28 mg/kg pour le tyrosol, et entre 0,5 et 5 mg/mg pour l'hydroxytyrosol. Les variétés *Blanquette de Guelma* et *Bouricha* enregistrent les valeurs les plus élevées en tyrosol (28 et 26 mg/kg) et hydroxytyrosol (5 mg/kg), tandis que la valeur la plus faible en tyrosol est enregistrée pour les variétés *Aghenfas, Chemlal* et *Chemlal Tazmalt* (3 mg/kg) et la plus faible en hydroxytyrosol pour la variété *Aghenfas* (0,5 mg/kg).

Ce que l'on peut relever également est que malgré que la variété *Limli* se révèle pauvre en polyphenols totaux, elle enregistre un taux plus élevé en tyrosol que la variété *Neb Djemel* qui est considérée comme étant la variété la plus riche en polyphénols totaux. Les mêmes résultats sont obtenus par Dabbou *et al*. [35] sur la variété *Ascolana Tenera* introduite en Tunisie.

Nos variétés présentent des teneurs en alcool phénoliques (hydroxytyrosol et tyrosol) proches de celles des variétés espagnoles étudiées par Gómez-Rico *et al*. [54], pour lesquelles les teneurs en tyrosol sont comprises entre 1,2 et 29,8 mg/kg, celles en hydroxytyrosol entre 0,4 et 5 mg/kg; supérieures aux teneurs des variétés tunisiennes qui présentent des teneurs en tyrosol comprises entre 0,65 et 5,36 mg/kg et l'hydroxytyrosol comprises entre 0,05 et 1,59 mg/kg [36], mais inferieures aux teneurs des huiles commerciales siciliennes analysées par Saitta *et al*. [58] présentant des teneurs qui varient de 10,5 à 32,3 mg/Kg (tyrosol) et de 12,8 à 36,1 mg/Kg (hydroxytyrosol).

Brenes *et al*. [59] et Baiano *et al*. [43] ont observé que les teneurs en tyrosol et hydroxytyrosol sont généralement faibles dans l'huile nouvellement extraite mais augmentent graduellement au cours du stockage parallèlement à une diminution des formes phénoliques complexes telle que 3,4-DHPEA-EDA et *p*-HPEA-EDA ce qui permet de déduire que la dégradation de ces derniers génère les phénols simples telles que l'hydroxytyrosol et le tyrosol.

Qualitativement parlant, pour toutes nos huiles analysées, les lignanes viennent en 2ème position après les secoiridoides à l'exception des huiles des variétés *Aghenfas*, *Akerma* et *Neb Djemel* dont le taux des flavonoïdes sont supérieurs à ceux des lignanes, les mêmes résultats sont observés par García-Villalba *et al*. [50] sur les variétés espagnoles où les variétés *Picual* enregistrent des teneurs plus élevées en flavonoïdes par rapport aux lignanes, alors que pour la variété *Arbequina,* ils ont relevé des teneurs plus importantes en lignanes. Les pourcentages en lignanes de nos huiles

varient entre 3,91% (*Neb Djemel*) et 15,95% (*Chemlal Tazmalt*) par rapport aux polyphénols totaux.
Toutes les variétés d'huiles étudiées présentent des teneurs appréciables en lignanes, la variété *Bouricha* se caractérise par la teneur la plus élevée (51 mg/kg) suivie des variétés *Ferkani* et *Chemlal Tazmalt* avec des teneurs de 42 et 41 mg/kg respectivement, la valeur la plus faible est relevée pour la variété *Aghenfas*, le reste des variétés présentent des teneurs comprises dans l'intervalle de 21 et 38 mg/kg.
Bien que la variété *Neb Djemel* se révèle être riche en polyphénols totaux (537 mg/kg), elle ne renferme que 21 mg/kg en lignanes par contre la variété *Chemlal Tazmalt* qui est moins riche en polyphénols totaux (257 mg/kg) montre une teneur en lignanes de 41 mg/kg. En plus du facteur variété, ceci peut être attribué à l'origine géographique [60]. Nos résultats sont inferieurs à ceux obtenus par Criado *et al*. [61] sur la variété espagnole *Arbequina* cultivée à Tarragona et Jaén dont les teneurs sont de 65 mg/kg et 61 mg/kg respectivement, et largement inferieurs à ceux des variétés étudiées par Brenes *et al*. [59] dont les teneurs sont supérieures à 100 mg/kg, mais supérieurs aux teneurs des variétés *Picual* et *Hojiblanca* analysées par García-Villalba *et al*. [50] et les variétés tunisiennes analysées par Haddada *et al*. [33] dont les teneurs oscillent entre 3,15 et 9,35 mg/kg.
Toutes les variétés d'huiles étudiées présentent des teneurs appréciables en flavonoïdes, les variétés *Akerma* et *Aghenfas* se caractérisent par les teneurs les plus élevées 49 et 44 mg/kg respectivement, alors que les variétés *Limli*, *Tabelout* et *Ferkani* enregistrent les teneurs les plus faibles (10, 13 et 14 mg/kg respectivement). Les teneurs du reste des variétés oscillent entre 18 et 41 mg/kg. Ces résultats sont largement supérieurs à ceux obtenus par Criado *et al*. [61] et ceux de Ilyasoglu *et al*. [51].
Les flavonoïdes représentent 26,03% des polyphénols totaux pour la variété *Aghenfas* alors qu'elle enregistre la teneur la plus faible en polyphenols totaux, tandis que les variétés *Blanquette de Guelma* et *Tabelout* enregistrent les taux les plus faibles (4,18% et 4,11%).
D'après les résultats obtenus, l'apigénine et la lutéoline sont les flavonoïdes présents dans toutes les huiles analysées, avec une prédominance de la lutéoline à l'exception de la variété *Tabelout*. Les variétés étudiées par Andjelkovic *et al*. [11] et García-Villalba *et al*. [50] présentent un taux plus élevé en lutéoline alors que les variétés turques récoltées en 2006 révèlent une dominance de l'apigénine [34]. La variété *Akerma* se caractérise par la teneur la plus élevée en lutéoline (38 mg/kg), cette teneur est proche de celles des variétés françaises obtenues par Andjelkovic *et al*. [52]. C'est la variété *Chemlal Tazmalt* qui enregistre la teneur la plus importante en apigénine (14 mg/Kg) avec aussi une teneur assez élevée en lutéoline (20 mg/kg). On observe également que les variétés *Limli* et *Tabelout* se distinguent par leurs teneurs les plus faibles en apigénine (3 mg/kg) et lutéoline (6 mg/kg) respectivement, ces teneurs sont proches de celles rapportées par Andjelkovic *et al*. [11]. Pour le reste des variétés, les valeurs varient entre 7 et 32 mg/kg (lutéoline) et 4 et 13 mg/kg (apigénine).
D'après Brenes *et al*. [62], la concentration de la lutéoline diminue avec la maturation du fruit d'olive par contre celle de l'apigénine ne subit aucun changement ce qui pourrait expliquer les faible teneurs en lutéoline chez la variété *Tabelout* qui présente un indice de maturité élevé (6,97).

4. CONCLUSION

Cette étude a permis de donner une première appréciation de la qualité et de la composition des huiles d'olive de variétés algériennes. Les résultats de l'évaluation des paramètres de qualité ont montré que les dix variétés étudiés appartiennent toutes à la catégorie de l'huile d'olive vierge ''extra''.
Concernant la composition chimique des huiles en acides gras et en composés phénoliques, des variations cultivars-dépendantes ont été relevées. Pour les acides gras, toutes les huiles contiennent des teneurs supérieures à 60% en acide oléique, toutefois, les variétés *Aghenfas* et *Tablout* ont enregistré les plus grandes teneurs (> 70%) et les plus faibles teneurs en acide linoléique. La variété *Blanquette de Guelma* se distingue par le taux le plus élevé en acide linoléique (21,67%) et la variété *Akerma* a enregistré la plus faible teneur en acide gadoléique.
Les résultats de ce travail peuvent être considérés comme une caractérisation préliminaire des huiles d'olive algériennes en composés phénoliques. Le profil en composés phénoliques individuels nous permet de faire une distinction variétale, les variétés *Neb Djemel* et *Bouricha* se caractérisent par les teneurs les plus élevées en dérivés d'oleuropéine (276 et 235 mg/kg respectivement) et en dérivés ligstroside (173 et 150 mg/kg respectivement). Tandis que, les variétés *Blanquette de Guelma* et *Bouricha* enregistrent les taux les plus élevés en tyrosol (28 et 26 mg/kg) et hydroxytyrosol (5 mg/kg), la variété *Limli* se distingue des autres variétés par son taux élevé en tyrosol (26 mg/kg) alors qu'elle a enregistré une teneur faible en polyphénols totaux. La variété *Bouricha* se caractérise par la teneur la plus élevée en lignanes (51 mg/kg), tandis que les variétés *Akerma* et *Aghenfas* enregistre les taux les plus faibles en polyphénols totaux mais elles se caractérisent par les teneurs les plus élevées en fla-

vonoïdes 49 et 44 mg/kg respectivement.
Enfin, il est évident que la fraction phénolique peut fournir un outil précieux dans la caractérisation variétale des huiles d'olive.

REMERCIEMENTS

Nos remerciements les plus vifs s'adressent aux directeurs et au personnel des institutions suivantes: ITAFV de Takarietz (Bejaia), la ferme pilote A/Mira de Tazmalt (Bejaia) et le laboratoire central de Cevital à Bejaia.

REFERENCES BIBLIOGRAPHIQUES

[1] Conseil Oléicole International (novembre 2008). Huiles d'olive. Production, 1 p.
[2] Conseil Oléicole International. L'oléiculture en Algérie. ALGERIE E108/Doc n° 4, 22p
[3] P. Inglese, L'influence de la variété sur les caractéristiques qualitatives de l'huile d'olive. Olivae (54), 42-44 (1994)
[4] A. Koutsaftakis, F. Kotsifaki, E. Christopoulou, A. Cert, Etude triennale sur les variations de plusieurs caractéristiques chimiques et de divers composants mineurs des huiles d'olive vierges obtenues à partir d'olives cueillies à différents degrés de maturité. Olivae (80), 22-27 (2000).
[5] L. Abaza, M. Msalem, D. Douad, M. Zarrouk, Caractérisation des huiles de sept variétés d'olivier tunisiennes. Oléagineux Corps Gras Lipides *9* (2), 9-174 (2002)
[6] R. Aparicio, G. Luna, Characterization of monovarietal virgin olive oils. European Journal of Lipids Science and Technology 104, 1-12 (2002)
[7] A. El Antari, A. El Moudni, H. Ajana, Evolution comparative de la qualité et de la composition acidique de l'huile d'olive chez quelques variétés méditerranéennes cultivées au Maroc. Olivae (95), 26-31 (2003)
[8] G. Dugo, V. Lo Turco, D. Pollicino, E. Mavrogeni, F. Pipitone, Caractérisation d'huiles d'olive vierges siciliennes. Variation qualitative des huiles des fruits des cultivars « *Biancolilla* , *Nocellara del Belice* , *Cerasuola* , *Tonda Iblea* et *Crastu*» en fonction des techniques et de l'époque de récolte des olives. Olivae (101), 44-52 (2004)
[9] M. Paz Aguilera, G. Beltran, D. Ortega, A. Fernandez , A. Jimenez, M. Uceda, Characterization of virgin olive oil of Italian olive cultivars: Frantoio and Leccino, grown in Andalusia. Food Chemistry *89*, 387-391 (2005)
[10] L. Cerretani, A. Bendini, A. Del Caro, A. Piga, V. Vacca, M.F. Caboni, T. G. Toschi, Preliminary characterization of virgin olive oils obtained from different cultivars in Sardinia. European Food Research and Technology *222*, 354-36 (2006)
[11] M. Andjelkovic, S. Acun, V. Van Hoed, R. Verhe, J. Van Camp, Chemical composition of Turkish olive oil-Ayvalik. Journal of the American Oil Chemists' Society *86*, 135-140 (2009)
[12] M. Talantikite, H. Ait Amar, Composition acidique des huiles d'olives des trois cultivars d'Algérie. Olivae *5* (23), 29-31 (1988)
[13] R. Boussenadji, Contribution à la connaissance analytique de l'huile d'olive algérienne. Olivae (57), 50-53 (1995)
[14] A. Tamendjari, F. Angerosa, M.M. Bellal, Influence of *Bactrocera oleae* infestation on olive oil quality during ripening of Chemlal olives. Ital. J. Food Sci. *16* (3), 343-354 (2004a)
[15] A. Tamendjari, M.M. Bellal, R. Laribi, F. Angerosa, Impact de l'attaque de *Bactrocera oleae* et du stockage des olives de la variété *Chemlal* sur la qualité de l'huile. Riv. Ital. Sostanze Grasse *81* (1), 23-27 (2004b)
[16] M. Douzane, M.M. Bellal, Contribution à la caractérisation des huiles de quelques variétés populations d'olive algériennes : étude de quelques composés mineurs de la fraction insaponifiable. Olivae (103), 33-41 (2005)
[17] R. Laribi, P. Rovellini, L. Deflaoui, A. Aidli, S. Metouchi, L. Arrar, A. Tamendjari, Evolution of biophenolic compounds in virgin olive oil during olive ripening of Algerian cultivars. Riv. Ital. Sostanze Grasse *86* (3), 151-161 (2009)
[18] J.L. Perrin, Les composés mineurs et les antioxygènes naturels de l´olive et de son huile. Rev. Franç. Corps Gras *39*, 25-32 (1992)
[19] M. Uceda, P. Frias, Epocas de recoleccion. Evolucion del contenido graso del fruto y de la composicion del aceite. Proceedings of II Seminorio Oleicolo Internacional Olive Oil Council. Cordoba Spain (1975)
[20] Union Internationale de la Chimie Pure et Appliquée (U.I.C.P.A.), Méthode d'analalyses des matières grasses et dérivés. 6ème édition. Edition ETIG Paris (1979)
[21] Conseil Oléicole International (C.I.O.), Analyse spectrophotométrique dans l'ultraviolet. COI/T20/Doc6 N° 19 du 6 Juin 1996, Madrid, Espagne
[22] E.C. Regulation n° 796 of May 2006 changes EC-Regulation. 2568/91. Official J.L. 128/815/05/02. 2002. Bruxelles (Belgium) (2002)
[23] M. Tsimidou, G. Papadopolus, D. Boskou, Phenolic compounds and stability of virgin

olive oil part 1. Food Chem. (45), 141-144 (1992)
[24] A. Bendini, M. Bonoli, L. Cerretoni, B. Biggu-zi., G. Lercker., T.G. Toschi Liquidliquid and solid-phase extractions of phenols from vir-gin olives oil and their separation by chroma-tographic and electrophoretic methods. Journal of Chromatography (985), 425-433, (2003).
[25] NGD C89-2007. Determination of biophe-nols in olive oil by HPLC. Natural and oxidi-zed oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids.
[26] C.O.I, Norme commerciale applicable aux huiles d'olive et aux huiles de grignons d'oli-ve. COI/T.15/NC n° 3/Rév. 4. Conseil Oléico-le International (2009)
[27] K. Yousfi, R.M. Cert, J.M. Garcia, Changes in phenolic compounds of virgin olive oils during objectively described fruit maturation. European Food Research Technology *223*, 117-124 (2006)
[28] M.D. Salvador, F. Aranda. G. Fregapane, Influence of fruit ripening on 'Cornicabra' vir-gin olive oil quality: A study of four successi-ve crop seasons. Food Chemistry *73*, 45-53 (2001)
[29] N. Ben Youssef, W. Zarrouk, A. Carrasco-Pancorbo, Y. Ouni, A. Segura-Carretero, A. Fernandez-Gutierrez, D. Daoud, M. Zarrouk, Effect of olive ripeness on chemical proper-ties and phenolic composition of chétoui vir-gin olive oil. Journal of the Science of Food and Agriculture *90*, 199-204 (2010).
[30] W. Zarrouk, F.M. Haddada, B. Baccouri, I. Oueslati, W. Taamalli, Z. Fernandez, L. Lizza-ni-Cuvelier, D. Daoud, M. Zarrouk, Characte-rization of virgin olive oil from Southern Tuni-sia. European Journal of Lipids Science and Technology *110*, 81-88 (2008)
[31] J.E. Pardo, M.A. Cuesta, A. Alvarruiz, Eva-luation of potential and real quality of virgin olive oil from the designation of origin "Acei-te Campo de Montiel"(Ciudad Real, Spain). Food Chemistry *100*, 977-984 (2007)
[32] S. Dabbou, I. Rjiba, A. Nakbi, N. Gazzah, M. Issaoui, M Hammami, Compositional quality of virgin olive oils from cultivars introduced in Tunisian arid zones in comparison to Chem-lali cultivars. Scientia Horticulturae *124*, 122-127 (2010)
[33] F. M Haddada, D. Krichène, H. Manai, I. Oueslati, D. Daoud, M. Zarrouk, Analytical evaluation of six monovarietal virgin olive oils from Northern Tunisia. European Journal of Lipid Science and Technology *110*, 905-913 (2008)
[34] D. Ocakoglu, F. Tokatli, B. Ozen, F. Korel, Distribution of simple phenols, phenolic acids and flavonoids in Turkish monovarietal extra virgin olive oils for two harvest years. Food Chemistry *113*, 401-410 (2009)
[35] S. Dabbou, M. Issaoui, M. Servili, A. Taticchi, S. Sifi, G.F. Montedoro, M. Hammami, Cha-racterisation of virgin olive oils from Europe-an olive cultivars introduced in Tunisia. Euro-pean Journal of Lipids and Science Techno-logy *111*, 392-401 (2009)
[36] F.M. Haddada, H. Manai, I. Oueslati, D. Daoud, J. Sanchez, E. Osorio, M. Zarrouk, Fatty acid, triacylglycerol, and phytosterol composition in six Tunisian olive varieties. Journal of Agricultural and Food Chemistry *55*, 10941-10946 (2007)
[37] G. Gurdeniz, B. Ozen, F. Tokatli, Classifica-tion of Turkish olive oils with respect to culti-var, geographic origin and harvest year, using fatty acid profile and mid-IR spectro-scopy. European Food Research & Techno-logy *227*, 1275-1281(2008)
[38] M. Guerfel, Y. Ouni, A. Taamalli, D. Boujnah, E. Stefanoudaki, M. Zarrouk, Effect of location on virgin olive oils of the two main Tunisian olive cultivars. European Journal of Lipid Science and Technology *111*, 926-932 (2009)
[39] A. Allalout, D. Krichène, K. Methenni, A. Taa-malli, I. Oueslati, D. Daoud, M. Zarrouk, Cha-racterization of virgin olive oil from super intensive Spanish and Greek varieties grown in northern Tunisia. Scientia Horticulturae *120*, 77-83 (2009)
[40] I. Oueslati, C. Anniva, D. Daoud, M.Z. Tsimi-dou, M. Zarrouk, Virgin olive oil (VOO) pro-duction in Tunisia: The commercial potential of the major olive varieties from the arid Tata-ouine zone. Food Chemistry *112*, 733-741(2009)
[41] G. Di Bella, R. Maisano, L. La Pera, V. Lo Turco, F. Salvo, G. Dugo, Statistical characte-rization of Sicilian Olive oils from the pelori-tana and maghrebian zones according to the fatty acid profile. Journal of Agricultural and Food Chemistry *55* (16), 6568-6574 (2007)
[42] M. Tsimidou, Polyphenols and quality of vir-gin olive oil in retrospect. Journal of Food Science *10* (2), 99-112 (1998)
[43] A. Baiano, G. Gambacorta, C. Terracone, M.A. Previtali, C. Lamacchia, E. La Notte, Changes in phenolic content and antioxidant activity of Italian extra-virgin olive oils during storage. Journal of Food Science *74* (2), 177-183 (2009)
[44] L. Cerretani, M.J. Motilva, M.P. Romero, A. Bendini, G. Lercker, Pigment profile and chromatic parameters of monovarietal virgin olive oils from different Italian cultivars. Euro-

pean food research & technology *226*, 1251-1258 (2008)
[45] L.N. Ceci, A.A. Carelli, Characterization of monovarietal Argentinian olive oils from new productive zones. Journal of American Oil Chemist's Society *84*, 1125-1136 (2007)
[46] M.J. Tovar, M. Paz Romero, J. Girona, M.J. Motilva, L-Phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (*Olea europaea*_L cv *Arbequina*) fruit grown under different irrigation regimes. Journal of Science of Food and Agriculture *82*, 892-898 (2002)
[47] L. Di Giovacchino, N. Costantini, A. Serraiocco, G. Surricchio, C. Basti, Natural antioxidants and volatile compounds of virgin olive oils obtained by two or three-phases centrifugal decanters. European journal of lipid science and technology *103*, 279-285 (2001)
[48] F. Gutiérrez, M.J. Villafranca, M.J Castellano, Changes in the Main Components and Quality Indices of Virgin Olive Oil During Oxidation. Journal of the American Oil Chemists' Society *79* (7), 669-676 (2002)
[49] W. Dhifi, M. Ben Khedher, M. Elyes Kechouk, B. Marzouk, Etude qualitative et quantitative des arômes et des polyphénols de quelques huiles d'olive de Tunisie. Olivae (105), 36-40 (2006)
[50] R. Garcia-Villalba, A. Carrasco-Pancorbo, C. Oliveras-Ferraros, A. Vazquez-Martin, J.A. Menendez, A. Segura-Carretero, A. Fernandez-Gutierrez, Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method. Journal of Pharmaceutical and Biomedical Analysis *51*, 416-429 (2010)
[51] H. Ilyasoglu, B. Ozcelik, V.V. Hoed, R. Verhe, Characterization of Aegean Olive Oils by Their Minor Compounds. Journal of the American Oil Chemists' Society, DOI 10.1007/s11746-009-1538-5 (2010)
[52] M. Andjelkovic, C.J. Van, M. Pedra, K. Renders, C. Socaciu, R. Verhé, Correlations of the phenolic compounds and the phenolic content in some Spanish and French olive oils. Journal of Agricultural and Food Chemistry *56* (13), 5181-5187 (2008)
[53] D. De La Torre-Carbot, O. Jauregui, E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventos, M.C. Lopez-Sabater, Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. Journal of Agricultural and Food Chemistry *53* (11), 4331-4340 (2005)
[54] A. Gómez-Rico, G. Fregapane, M.D. Salvador, Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. Food Research International *41*, 433–440 (2008)
[55] M. Servili, R. Selvaggini, S. Esposto, A. Taticchi, G. Montedoro, G. Morozzi, Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. Journal of Chromatography *1054*, 113-127 (2004)
[56] M. Servili, G. Montedoro, Contribution of phenolic compounds to virgin olive oil quality. European Journal of Lipid Science and Technology *104*, 602-613 (2002)
[57] L.S. Artajo, M.P. Romero, M. Suarez, M.J. Motilva, Partition of phenolic compounds during the virgin olive oil industrial extraction process. European Food Research and Technology *225*, 617-625 (2007)
[58] M. Saitta, F. Salvo, G. Di Bella, G. Dugo, G.L. La Torre, Minor compounds in the phenolic fraction of virgin olive oils. Food Chemistry *112*, 525-532 (2009)
[59] M. Brenes, A. Garcia, P. Garcia, A. Garrido, Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. Journal of Agricultural and Food Chemistry *49* (11), 5609-5614 (2001)
[60] J. Tovar, M.J. Motilva, P.M. Romero, Changes in the phenolic composition of virgin olive oil from young trees (*Olea europaea* L. cv. *Arbequina*) grown under linear irrigation strategies. Journal of Agricultural Food Chemistry *49* (11), 5502-5508 (2001)
[61] M.N. Criado, J.R. Morello, M.J. Motilva, M.P. Romero, Effect of growing area on pigment and phenolic fractions of virgin olive oils of the Arbequina variety in Spain. Journal of American Oil Chemists Society *81*, 633-640 (2004)
[62] M. Brenes, A. Garcia, P. Garcia, J.J. Rios, A. Garrido, Phenolic compounds in spanish olive oils. Journal of Agricultural Food Chemistry *47* (9), 3535-3539 (1999)

Received June 8, 2010
Accepted September 22, 2010

Published on: 1st June 2011

TRADITIONAL MEDICINE IN NORTH SAHARA: THE "DEFFI"

KHADRA MAIZA, VICTORIA HAMMICHE[1] AND FADILA MAIZA-BENABDESSELAM [2]

[1]Laboratoire de Botanique Médicale, Faculté de Médecine, Université d'Alger, 18 Avenue Pasteur, 16000 Alger

[2]Laboratoire de Biotechnologies Végétales -FSNV- UAMB- Béjaia –Terga Ouzemour -06000- *Béjaia*

fadilamaiza@yahoo.fr

ABSTRACT:

The "deffi" is a drink very appreciated by the Saharan nomads of the Châamba ethnos group. Its composition, exclusively vegetable, utilizes forty plants which are majority spontaneous species. Some of them were the subject of studies which highlighted their chemical compounds and biological activities. A library search enables us to confirm the validity of their uses in traditional medicinal practices.

KEY WORDS: Ethnopharmacology,Folk medicine, *Deffi*, Châamba, Sahara, Algeria

INTRODUCTION:

The "Deffi", is a slightly fermented soft, tonic and reconstituting drink, very appreciated by the Châamba nomads of the septentrional Sahara of the area of El Goléa/El Meniâa (Fig. I).It is used during the "Ramadhan"(Fast period), for its beneficial effects on the body and the spirit. The "deffi" is drunk from the "ftour" (fast rupture) to the "shour" (fast resumption), pure or mixed with water or milk. Due to the increased risks of hemorrhages, it is strongly misadvised for the young girls, women during their menstrual period and for pregnant women. Its exclusively vegetable composition utilizes forty plants; the majorities are spontaneous species, some, which are considered to be essentials, come from the close areas like the High plateaus; they are cultivated or brought from herbalist.

STUDY AREA:

'Balh valley' of district Mandi in Himachal Pradesh (H.P.) is one of the most fertile areas situated on national highway (NH) 21. The district is situated 76˚37′- 77˚23′ East longitudes and 31˚13′- 32˚04′North latitudes. It is situated between Bilaspur-Mandi on national highway (NH) 21. This

region is famous for growing vegetables and other cash crops (3). The flora of this region is unique and diverse.

MATERIEL AND MÉTHODS:

Vernacular names and therapeutic indications allotted to each species were raised during detailed surveys conducted near the Saharan populations in order to obtain informations about the folklore uses, knowledge of local people and traditional healers about these popular plants (Maiza et al 1990; Maiza *et a.l* 1995; Maiza, 2008.). Investigations concerning the "Deffi" were carried out near Châamba nomads during the period preceding the month of "Ramadhan". We thus attended with the gathering of the spontaneous plants, their drying and the purchase of the imported plants from the High plateaus. The vernacular names were compared with those reported by others authors during works about north Africa species (Boulos, 1983; Trabut, 1935).Species were identified by consultation of available flora (Ozenda, 1983; Quezel & Santa, 1962-63), of Maire work (1933) and by direct comparison with samples of the URZA (Arid Zones Research Unite, Algiers) and the INA (National Agronomic Institute, Algiers) herbaria.

Drink Preparation:

Each dried plant was conditioned in gauze, in the form of small purse or "soura" -, from where the name given to the "deffi": "the forty purses drink". The purses were introduced into an intermediate size wineskin of goatskin named "chekoua" , similar to that used to beat milk. The wineskin was initially cleaned carefully with "ghatrân", tar obtained by calcination of the branches of *Juniperus phoenicea*. To the "forty purses" were added broad beans, lenses, amount of "ghers" (specie of dates), salt and a small volume of water and the mix was vigorously shaked. After 24 hours of contact the filtrate obtained constitutes the "Deffi". Supplementations each day by a dried apricot - "fermes" -, dates - "ghers" - and water were also carried out.

RESULTS:

On table 1 we reported the scientific and local names (Arabic and Tamazight) of the plants used in the "Deffi" composition, their traditional uses according to the results of the survey, and their known composition and proprieties reported in library. The plants used are essentially spontaneous species ; generally aerial parts of them are employed: *Ammodaucus leucotrichus, Artemisia herba alba*, etc.., but sometimes the flower like cornflowers of *Brocchia cinerea, Matricaria pubescens*, the fruit in case of *Rhus tripartita* for example or the seeds in case of *Peganum harmala, Stipagrostis pungens*.

Plants of close areas (●) provided by herbalists or imported by the nomads are also used: *Ajuga iva, Artemisia campestris, Juniperus phoenicea, Myrtus communis, Rosmarinus officinalis.*

Lastly, the "Deff" contains also crop plants (*) in its composition like fenugrec (*Trigonella foenum-graecum*) corn and barney seeds, broad beans, lenses, dates, rose petals, thyme, dry apricots. It contains also the "three cumins"(cumin, nigelle and caraway),cloves and various non identified resins. This traditional and familial formulation, obtained by maceration and continuous exhaustation contains plants with digestive and hepatic tropism, and tonic depuratives and aromatics ones. These activities justify its use during the period of "Ramadhan" when there is both foods fast in the day and excesses in the night. We can observe also the presence of species respectively well known as cardiotonics, antidiabetics or indicated in depressive states.

The emmanogog plants contained in the "Deffi" explain its prohibition for the young girls and the pregnant women. In spite of their known toxicity *Peganum harmala, Retama reteam* and *Ruta tuberculata* are used in the "Deffi" preparation they are added in very small amounts, or perhaps the other components from the other plants interact as antagonists by complexing or inhibiting toxic component. Further work is needed to answer to this question. Several species were investigated and the results of their studies highlighted chemical compounds and biological activities which explain the benefits of this traditional drink prepared from medicinal indigenous plants.

CONCLUSION:

The « *Deffi* » is a good digestive and tonic drink which makes hydratation possible during the difficult period of fast ("Ramadhan"). Prepared formerly in each house in the district of El Goléa/El Meniâa, two or three families still prepare it nowadays. However we can wonder whether there remains always the "drink with the forty purses" because with the climatic risks and the sedentarisation of the nomads it became increasingly difficult to join together the spontaneous plants which enter in its composition. The "Deffi" remains however a drink very required by the "Châamba"s, poeple who are still attached to the traditions and the plants of the Sahara.

REFERENCES:

Boulos, L. (1983): Medicinal Plants of North Africa. *Référence Publications Inc.*, USA. 286p

Maire, R. (1933): Etudes sur la flore et la végétation du Sahara central. Mémoires de la Société d'Histoire Naturelle de l'Afrique du Nord, n°3, mission du Hoggar II. *La Typo-Litho. Alger,* 272p.

Maiza ,K. (2008): Pharmacopée traditionnelle saharienne : Sahara algérien. *Thèse de Doctorat en Sciences Médicales*. Université d'Alger, Faculté de Médecine, Département de Pharmacie. 386p.

Maiza, K., Brac de la Perrière RA, Bounaga N, Hammiche V. (1990): Usages traditionnels des plantes spontanées d'El Goléa. *Actes du Colloque de l'Association*. Française pour la Conservation des Espèces Végétales. Mulhouse.

Maiza, K., Brac de la Perrière, R.A., Hammiche, V., (1995): Pharmacopée traditionnelle saharienne : Sahara septentrional. *Revue de Médecines et Pharmacopées Africaines.* **9**, n°1. pp 71-75.

Ozenda, P. (1983).: Flore du Sahara; *C.N.R.S.*, Paris. 622 p.

Quezel, P. and Santa, S. (1962-63): Nouvelle flore d'Algérie et des régions désertiques méridionales, *C.N.R.S.*, Paris. Vol 1,2. 1170p.

Trabut, L. (1935): Flore du Nord de l'Afrique : répertoire des noms indigènes des plantes spontanées, cultivées et utilisées dans le Nord de l'Afrique. *La typo-litho*. Alger. 355 p.

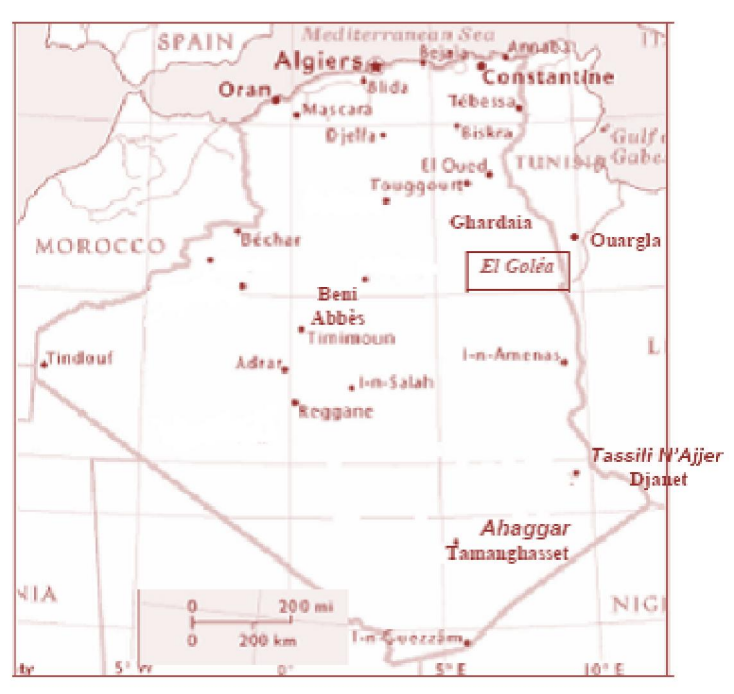


Fig 1 : situation map of the survey's area

Table 1 : The most important plants in the « *Deffi* » (main indication on bold)

Scientific name / Familly	**Arabic**	*Berber / Tamahak* [T]	**Traditionnal uses**	**Constituents / Properties**
Ajuga iva (L.) Schreb Lamiaceae Hv678 ●	Chendgoura	*Tuf tolba*	**Stomach and liver diseases (jaundice)** **Blood-cleansing** Diabetes, Vascular hypertension, rheumatisms, wounds Female sterility	Iridoid-glycosids Ecdysons Anti-inflammatory Hypoglycemic Hypotensive
Ammodaucus leucotrichus Coss. & Dur. Umbellifereae / Hv 684	Oum draiga	*Akâaman* [T]	**Stomach diseases : vomit, abdominal pains,** meteorism **Liver diseases** Cutaneous allergies Genital disorders: emmenagogue, abortive aphrodisiac *aromate and mixtures	Sesquiterpene-lactones Perillaldehyde Antimicrobial Cytotoxic Anti fungal
Anvillea radiata Coss. & Dur. Compositeae / Hv708	Nougd- l'hoor	*Akadkad* */ Tehetit* [T]	**Stomach and liver diseases** Diabetes Post partum care: analeptic, antiseptic	Flavonoids Sesquiterpene-lactones Antimicrobial, Cytotoxic
Artemisia campestris L. Compositeae / Hv650 ●	Degouft / Alala	*Tedjok* [T]	**Post partum care**: emmenagogue, analeptic **Helminthiases** Stomach and liver diseases, blood- cleansing Chills, aches Eruptive fevers (measle) Sores (healing) Mycosis, tinea *plant for mixtures	Flavonic compounds Antioxidant Hepatoprotective
Artemisia herba alba Asso. Compositeae / Hv657	Chih	*Zizri* [T] */ Zézéré* [T]	**Panacea: Helminthiases** Stomach diseases (vomit, diarrhoea, meteorism) **Hepatic insufficiency.** Diabetes	Flavonoids Sesquiterpene-lactones (santonine) Essential oil

			Genital disorders : **emmenagogue, abortive** **Post partum care:** meconium evacuation, stimulant of lactation Nervous disorders: headaches, emotions Skin diseases: wounds, mycosis *aromate * plant for mixtures * magic	Antimicrobial Cytotoxic Hypoglycemic Anthelminthic Insecticide
Asphodelus tenuifolius Cav. Asphodelaceae / Hv660	Achb l'ibel / Tazia	*Izian*	**Stomach diseases: constipation,** indigestion **New born :** urinary decrease and constipation Measle, fever Tonic Myalgia, aches *plant for mixtures*(local butter)	Triterpenic compounds Anthraquinonic compounds Lipids Laxative Anti-inflammatory Diuretic
Astericus graveolens (Forssk.) Less. Compositeae / Hv693	Nougd el h'bil	*Amayout* [T]	**Digestive and liver diseases,** headaches **Respiratory diseases** **Rheumatisms, aches, myalgia** **New born pathology:** colics, vomit Genital disorders: female sterility, gonorrhoea, difficult delivery Diabetes	Flavonoids Sesquiterpene-lactones Antimicrobial Cytotoxic Anti cancer
Brocchia cinerea Viss. Compositeae /Hv643	Guertoufa el beida / Chouihiya	*Takkelt* [T]	**Digestive diseases :** nausea, vomit, colics, constipation, indigestion **Serious respiratory diseases**: pleurisy and pneumonia **Pediatry** : fever Rheumatisms Female sterility Toothache * scorpion sting * aromate (tea, tobacco)	Flavonoids Sesquiterpene-lactones Sesquiterpene-coumarins Camphor Antimicrobial Expectorant Antiseptic Molluscicidal

			* plant for mixtures	
Coriandrum sativum L. Ombelliferae *	Kosbar	/	**Stimulate and régularizes gastric secretions,** meteorism, spasms Diabetes *condiment *plant for mixtures	Essential oil (linalol) Stomachic Aromatic
Cymbopogon schoenanthus (L.) Spreng. Poaceae / Hv677	Lemmad	*Tibérimt* [T]	**Digestive diseases :** meteorism, colics, **Rheumatisms, aching bones and joints** **Post partum care**: urinary decrease , drink for new mother after childbirth, analeptic Toothache, bad breath * aromate* plant for mixtures	Sesquiterpenes , Sterols Essential oil (pipéritone) Antimicrobial, Cytotoxic Anti cancer Analgesic Insecticide, Molluscicidal
Gymnocarpos decandrus Forssk. Caryophyllaceae / Hv 581	Djefna	/	**Psychosomatic diseases** Break down Evil eye, bad spirit * plant for mixtures *magic	No data
Haplophyllum *tuberculatum* (Forssk.) A.Juss. Rutaceae / Hv700	Fidjel	*Touf ichkan* [T]	Chills, **aching bones and joints,** **muscular pains, aches** Liver and intestinal diseases Genital disorders: dysmenorrhea, female sterility, difficult delivery, **emmenagogue** **Child convulsions** , headaches**, dizziness** *plant for mixtures	Alkaloids Flavonoids Sesquiterpene-lactones Essential oil Antimicrobial, Antifungal Anti cancer Antiplasmodiale * toxic
Helianthemum *eriocephalum* Pomel Cistaceae / Hv588	Semh'ari / Regig	*Tahaoua t* [T]	Strengthening	 * dromedary grazing
Juniperus phoenicea L. Cupressaceae / Hv 574 ●	Arâar	*Aïfz*	**Lung diseases** (**disinfectant**) Skin diseases, tumours, malignant wounds Kidney diseases : urinary	Essential oil (pinene) Diuretic

			decrease (diuretic) Helminthiases	Antiarthrosical
Matricaria pubescens Sch. Bip. Compositeae / Hv644	Ouazouaza / Guertoufa	*Aynasnis* [T]	**Rheumatisms, aches, bones, joints and muscular pains** Pediatry : measle, toothache, **fever** Skin diseases : dermatosis, itching, wounds	Flavonoids Essential oil
			Genital disorders: dysmenorrhea, post partum care : tonic Cough Conjonctivitis * scorpion sting * aromate *condiment (local butter)	Anti-inflammatory Antimicrobial Cytotoxic
Myrtus communis L. Myrtaceae / Hv589 ●	Rihan	*Tarihant*	**Intestinal diseases :** diarrhoea	Essential oil (terpenes)
			Diabetes Vascular hypertension Respiratory diseases, cold, cough Fever Dermatosis : mycosis, hair care	Antimicrobial Respiratory &genito urinary antiseptic Antidiarrheal Haemostatic Balsamic
Peganum harmala L. Zygophyllaceae / Hv649	Harmel	*Alora / Wa n'téfriwe n* [T]	**Rheumatisms, back pains** Genital disorders: **emmenagogue,** just maried sexual weakness Nervous disorders: **break down,** anxiety Diabetes. Jaundice	Alkaloids
			Helminthiases Fever, lips sore, herpes Eczema, tumours, wounds Parasites (louse) * blooming = mascot * plant for mixtures, *magic	Antimicrobial Antiparasitic Abortive Anti cancer * toxic
Retama raetam (Forssk.) Webb Leguminoseae / Hv	R'tem	*Tillouggit / Telit* [T]	**Anthelminthic**, purgative, abortive **Hepatitis** **Emmenagogue, abortive** Diabetes	Flavonoids Hypoglycemic

538			Cardiotonic **Rheumatisms (ignipuncture)**	* toxic
Rhetinolepis lonadioides Coss. Compositeae / Hv710	Kemoun - l'achar	*Tichert* [T]	Pulmonary infections: chills Stomach diseases **Reconstituant, tonic** *plant for mixtures * local butter	No data
Rhus tripartita (Ucria) Grande Anacardiaceae / Hv647	Djedâri	*Tahounaq* [T]	**Digestive diseases :** colics, diarrhoea Rheumatisms, aches, myalgia Tonic Enuresis Toothache * snake bite & * scorpion sting	Proanthocyanidins Antioxidant
Rosmarinus officinalis L. Lamiaceae / Hv707 ● .	Klil / Aklil	*Iazir*	**Liver** and stomach **diseases** , spasms Respiratory diseases : cough, asthma Rheumatisms Diabetes Parasites (louse) Traditional beauty care, hair care *aromate * snake bite	Flavonoids, polyphenols Essential oil Camphor Antioxidant Cholagogue and choleretic Hepatic stimulant Cicatrizant
Stipagrostis pungens (Desf.) De Winter Poaceae / Hv683	Drinn	*Toulloult* [T]	**Tonic, reconstituant** **Rheumatisms, aching joints and** muscular pains Infected wounds, abcesses **(drain-pipe)**	No data * dromedary grazing
Traganum nudatum Delile Amaranthaceae / Hv659	Domran	*Térahit* [T]	Intestinal disorders : **constipation** Rheumatisms, aching bones, joints and muscular pains *plant for mixtures	No data * dromedary grazing
Trigonella foenum-graecum L. *	Helba	*Ibedlioun* [T]	**Tonic, reconstituant :** anorexia, anémia, weakness Stomach diseases : nausea, blood	Flavonoids, Amino acids Steroidal saponins Mucilages

Leguminoseae / Hv669			cleansing Diabetes. Jaundice Genital disorders: aphrodisiac Skin diseases: **boils** Hair loss**,** dandruff *condiment *plant for mixtures	Hypoglycemic Hypocholesteremic Anti-inflammatory Immunostimulative Emollient Neuro-muscular stimulant

● Plants of the close areas

* Crop Plants

Name [T:] *local name in tamahaq (touareg lungage)*

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 12615–12624 12615
DOI:10.1021/jf1029509

Analysis by High-Performance Liquid Chromatography Diode Array Detection Mass Spectrometry of Phenolic Compounds in Fruit of *Eucalyptus globulus* Cultivated in Algeria

Lila Boulekbache-Makhlouf,[†] Emmanuelle Meudec,*[,‡] Mohamed Chibane,[†] Jean-Paul Mazauric,[‡] Sakina Slimani,[†] Max Henry,[§] Veronique Cheynier,[‡] and Khodir Madani[†]

[†]Faculty of Nature and Life Sciences, 3bs Laboratory, A. Mira University, Bejaia 06000, Algeria, [‡]INRA, UMR1083 Sciences pour l'œnologie, Montpellier, France, and [§]Structure et réactivité des systèmes moléculaires complexes (SRSMC), UMR 7565, Nancy-Université, CNRS, 5 rue Albert Lebrun, B.P. 80403, 540001 Nancy cedex, France

A method based on high-performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) following fractionation by chromatography on a Sephadex LH-20 column has been developed to determine the phenolic composition of fruit of *Eucalyptus globulus* growing in Algeria. The presence of 18 gallotannins, 26 ellagitannins, and 2 flavonols was established. Tentative identification is provided for these compounds on the basis of UV−visible spectra and mass spectrometry data. Most compounds described in this study have not previously detected in fruit of *E. globulus*. Moreover, this is the first report of methyl digalloyl diglucose, 3,3′-*O*-dimethylellagic acid 4-*O*-β-glucopyranoside, ellagic acid hexose, methyl ellagic acid pentose, methyltetragalloylglucose, and valoneic acid isomers (sanguisorbic, flavogallic acid dilactone) in the genus *Eucalyptus*. Quantitatively, ellagic acid and its derivatives, including ellagitannins, are largely predominant.

KEYWORDS: *Eucalyptus globulus* fruit; phenolic compounds; Sephadex LH-20 column; HPLC-DAD; HPLC-ESI-MS

INTRODUCTION

The genus *Eucalyptus* is indigenous to Australia, comprises more than 523 species and 138 varieties, and new species and varieties are still being described (*1*). Among all of these species, *Eucalyptus globulus* is the most widely cultivated in subtropical and Mediterranean regions (*2*). It was introduced in Algeria in 1854 by Ramel. There are extensive eucalypt plantations in Algeria: 30000 ha in 1990 and 39000 ha in 1995 (*3*). Its leaves, roots, and fruit have been used as traditional remedies for treatment of various diseases such as pulmonary tuberculosis (*4*), influenza (*5*, *6*), asthma (*7*), and diabetes (*8*−*10*). *E. globulus* is well-known for the volatile terpenoid constituents of its essential oil (*5*, *7*, *11*−*13*), and the phenolic compounds in its leaves and wood have been widely studied (*6*, *8*, *11*, *14*−*17*). According to the published works, the major phenolic compounds of this plant are hydroxybenzoic acids (vanillic, gallic, protocatechuic, ellagic, and gentisic acids) (*14*−*16*, *18*−*20*); hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric, and chlorogenic acids) (*18*, *16*, *20*); flavonols (kaempferol, quercetin, quercetin 3-*O*-rhamnoside, and quercetin 3-*O*-rutinoside) (*14*−*16*); a methyl flavone (naringenin) (*7*, *15*, *11*); a flavan-3-ol ((+)-catechin) (*5*); flavones (luteolin and apigenin) (*15*); hydrolyzable tannins (tellimagrandin, eucalbanin C, eucaglobulin, and pentagalloylglucose) (*5*, *14*, *15*); and condensed tannins (proanthocyanidins) (*14*, *19*−*21*). Phytochemical analysis has established that *E. globulus* contains monoterpene glycosides conjugated with gallic acid (*6*, *5*) and phloroglucinol−sesquiterpene-coupled compounds (*5*). Up to now, little has been done on the phenolic composition of the fruit of this plant. It has been reported to contain gallic acid (*22*, *23*), cypellocarpin C, macrocarpal A, macrocarpal B (*23*), and other ellagic acid derivatives (3,4,3′-*O*-trimethylellagic acid, 3-*O*-methylellagic acid 4′-*O*-(2″-*O*-acetyl)-α-L-rhamnopyranoside, 3-*O*-methylellagic acid, 4′-*O*-α-L-rhamnopyranoside, and 3-*O*-methylellagic acid) (*23*).

Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with diode array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) is frequently used for the separation, detection, and characterization of phenolic compounds from plants (*24*). UV−visible spectra allow identification of the class of compound, whereas ESI-MS data are useful for the structural determination of phenolic compounds (*24*). ESI is a gentle ionization method in MS, generating mainly deprotonated molecules $[M - H]^-$ of the compounds analyzed, when used in the negative ion mode (*25*). Further structural confirmation can be accomplished by tandem mass spectrometry carried out with an ion-trap (IT) mass analyzer, through analysis of the characteristic fragmentation patterns obtained by successive fragmentations of the parent and daughter ions (*24*).

Although a number of studies have been devoted to the identification of eucalypt phenolics, as described above, all of

*Corresponding author (e-mail meudec@supagro.inra.fr; phone 04-99-61-25-84; fax 04-99-61-28-57).

Published on Web 12/01/2010

them have focused only on specific families of compounds. To our knowledge, no exhaustive profiling has been published about eucalypt fruits.

The recovery of the fruits of this plant to obtain compounds with pharmaceutical or chemical applications, such as phenolic acids, flavonoids, and tannins, could be commercially interesting. The purpose of this study is to enhance knowledge of the phytochemical extract of the fruits of this plant and to highlight specific tracers for the future use of this extract in the industry as was the case for its leaves, which are used as a natural food additive in Japan (*26*). Therefore, we have developed a methodology that consists of combining fractionation using low-pressure chromatography on Sephadex LH-20 with HPLC-DAD-ESI-IT-MS analysis for comprehensive qualitative characterization of the phenolic composition of *E. globulus* fruits. Chromatography on a column filled with Sephadex LH-20 has been widely used for the fractionation of plant extracts (*24*, *27*). Phenolic compounds are separated by gel permeation (molecule sieve effect of the gel) or adsorption (hydrogen bonding), depending on the type of solvent used (*24*). Using column chromatography on Sephadex LH-20 in adsorption mode, nonpolymeric phenols, including phenolic acids, lower molecular weight gallotannins (*27*, *28*), flavonoids (*29*−*32*), and procyanidin oligomers (*24*) are eluted from the gel by ethanol and methanol, whereas hydrolyzable tannins with higher molecular weight are recovered by aqueous acetone (60−70%) (*33*, *34*). Using this approach, in total, 55 compounds have been identified or tentatively identified in *E. globulus* fruits, including phenolic acids, ellagitannins, and flavonols. Among them, 21 compounds had been identified earlier in eucalypt, but had not been described in the fruits, and 7 had never been detected in eucalypt species. Quantitative data on the major families are also provided.

MATERIALS AND METHODS

Chemicals. *E. globulus* fruits, Myrtaceae family, were obtained from their natural habitats. They were collected from the arboretum of Darguinah, Bejaia, in northeastern Algeria, in February 2008. The plant was identified on the basis of its morphological characteristics, and a specimen has been deposited in the 3bs Laboratory (University of Bejaia). Formic acid, tannic acid, and ellagic acid were from Sigma-Aldrich (St. Louis, MO). Gallic acid and isoquercitrin (quercetin 3-*O*-glucoside) were from Extrasynthese (Genay, France). Methanol, ethanol, acetone, hexane, and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Chlorhydric acid (HCl) and ellagic acid were purchased from Fluka (Buchs, Switzerland). Sephadex LH-20 was purchased from GE Heathcare (Uppsala, Sweden). Pure Milli-Q water was delivered by a water purification system from Millipore (Bedford, MA).

Sample Preparation. The sample was cleaned with tap water, dried in the drying oven at 40 °C during 5 days, and reduced to thin powder; 1 g was extracted with 100 mL of acetone/water (70:30, v/v) containing 0.5% acetic acid to prevent oxidation. The process of extraction continued for a week at room temperature in a dark place, using a magnetic blender. The extract was filtered through Whatman filter paper no. 4 and concentrated to dryness under reduced pressure by rotary evaporation at 40 °C. The obtained residue was defatted with hexane (25 mL × 3) to remove lipids, concentrated under reduced pressure, and lyophilized to obtain *E. globulus* fruit extract (EGFE).

Column Chromatographic Fractionation of Crude Extract. Sephadex LH-20 gel was used for fractionation by column chromatography. Crude extract was dissolved in aqueous ethanol (75%); after sonication during 20 min, the mixture was applied to a column filled with Sephadex LH-20 (length = 30 cm, internal diameter = 1.6 cm) and fractionated by consecutive elution with ethanol, methanol, and aqueous acetone (60%), at a flow rate of 1.7 mL/min. Five fractions (5 mL each) were eluted with ethanol, two fractions (10 mL each) were eluted with methanol, and eight fractions (10 mL each) were collected after elution by aqueous acetone (60%). After evaporation of solvents under vacuum at 40 °C, all fractions were reconstituted with ethanol, and then their absorbance was measured at 280 nm using an Agilent spectrophotometer. On the basis of the absorbance data, subfractions were pooled in three fractions (F1−F3). Solvent was evaporated to dryness under vacuum at 40 °C. Dried fractions were dissolved in 1 mL of ethanol/water/formic acid (75:24.5:0.5, v/v/v) and analyzed by HPLC-DAD-MS.

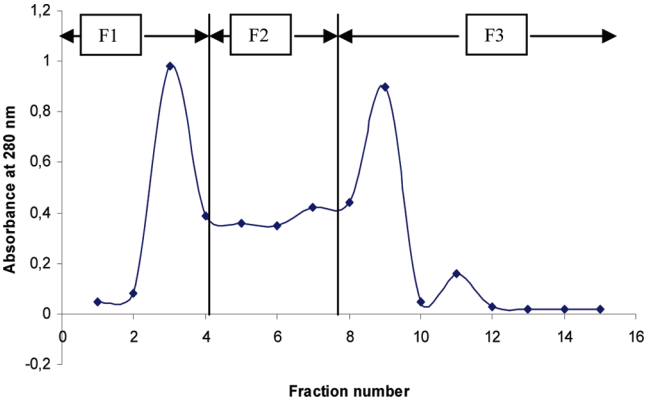


Figure 1. Sephadex LH-20 column chromatography profile of EGFE.

HPLC-DAD and ESI-MS Analysis. The HPLC analysis was carried out on a Waters 2690 HPLC system equipped with a Waters 996 DAD (Waters Corp., Milford, MA) and Empower software (Waters). The separation was performed at 30 °C using a 1.6 cm diameter, 30 cm long column. The solvents were 2% aqueous formic acid (solvent A) and acetonitrile/water/formic acid (80:18:2; solvent B). Gradient conditions were as follows: from 0 to 2% B in 3 min, from 2 to 3% B in 10 min, from 3 to 8% B in 10 min, from 8 to 20% B in 15 min, from 20 to 25% B in 5 min, from 25 to 65% B in 30 min, and then to 80% B in 7 min at a flow rate of 0.25 mL/min. The injection volume was 10 μL, and detection was carried out between 210 and 650 nm. After passing through the flow cell of the DAD, the column eluate was directed to an LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). Experiments were performed in negative ion mode. Scan range was 100−2000. The desolvation temperature was 300 °C. High spray voltage was set at 5000 V. Nitrogen was used as the dry gas at a flow rate of 75 mL/min. MS was carried out using helium as target gas, and collision energy was set at 30%. Identifications were achieved on the basis of the molecular ion mass, fragmentation, UV−visible spectra, and relative retention times or co-injection with standards.

Quantities were evaluated from peak areas in the HPLC profile, using external calibration curves established with gallic acid (at 280 nm), for gallic acid and gallotannins, ellagic acid (at 253 nm) for ellagic acid and its derivatives, and quercetin 3-*O*-glucoside (at 360 nm) for flavonols. Quantities are thus expressed as gallic acid, ellagic acid, and quercetin 3-*O*-glucoside equivalents for each class of compounds in milligrams per gram of powder of initial extract. For gallotannins and ellagitannins, quantities were estimated by calculating the concentration in moles of gallic acid (or ellagic acid) equivalent, dividing by the number of gallic (or ellagic) units in the molecule, and multiplying by the molecular weight.

RESULTS AND DISCUSSION

Sephadex LH-20 Column Chromatography of Crude Extract. Fractionation of EGFE was performed by column chromatography on Sephadex LH-20. Elution with ethanol, methanol, and aqueous acetone (60%), successively, yielded 15 fractions that were pooled in 3 fractions (labeled F1−F3), according to the absorbance readings at 280 nm (**Figure 1**).

Identification of Chromatographic Peaks. HPLC-DAD-MS analysis of each of these fractions showed a large number of compounds eluting throughout the chromatographic profiles. The profiles of the three fractions appeared to be quite different, and most of the compounds were recovered in only one of them (**Figure 2**). Moreover, some compounds eluting at the same retention time in the three fractions showed different UV−visible

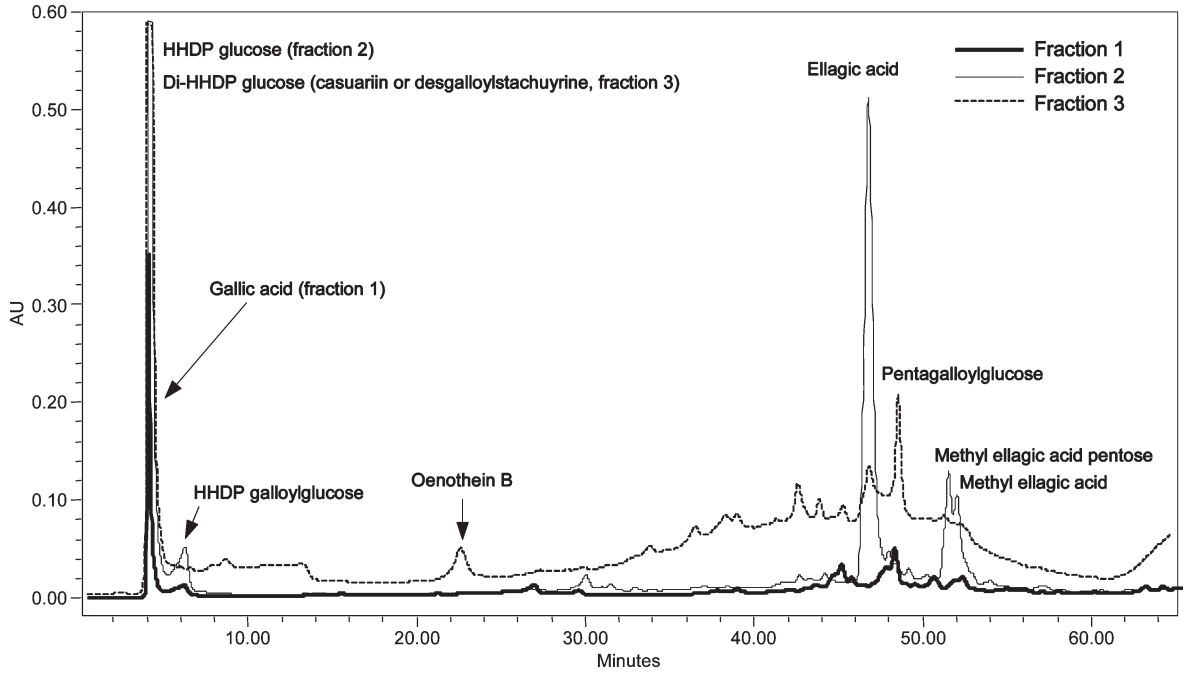


Figure 2. HPLC chromatogram at 280 nm of the three fractions recovered after Sephadex LH-20 chromatography of EGFE.

and MS spectra (e.g., peaks eluted at 5.89, 5.77, and 5.87 min, respectively, in F1, F2, and F3).

The UV−visible spectra, recorded with DAD, allowed three groups of compounds to be distinguished. The two major groups showed spectra resembling those of ellagic acid (with two absorbance maxima around 253 and 366 nm) and gallic acid (single maximum around 272 nm), respectively. The third group is minor and showed spectra that can correspond to those of flavonol derivatives (with two absorbance maxima 250−260 and 250−360 nm). These three groups were thus tentatively attributed to ellagitannins, gallotannins, and flavonol derivatives, respectively. Ellagitannins and gallotannins represent families of hydrolyzable tannins based on a sugar unit (usually D-glucose) multiply acylated with hexahydroxydiphenic acid (releasing ellagic acid (**1**) upon acid hydrolysis) or gallic acid (**2**) (releasing gallic acid upon acid hydrolysis), respectively (**Figure 3a**). Flavonols (e.g., quercetin (**3**)) are a group of flavonoid pigments, often found as glycosides. The λ_{max} values of flavonol derivatives and ellagitannins are the same, but the relative signal intensities at 250 and 360 nm are different, the second maximum being much less intense in the case of ellagitannins (*14*).

Each compound was further analyzed by mass spectrometry. Identifications were confirmed by comparison of the retention time and spectral data with those of reference compounds when available (i.e., for quercetin 3-*O*-glucoside, gallic acid, and ellagic acid). Data obtained for all peaks, including retention times, UV−visible spectra, molecular ion, and fragmentations obtained by MS^2 experiments, are given in **Table 1**. Several minor compounds could not be tentatively identified from their LC-DAD-MS data and are thus not presented.

MS^2 analysis provided $[M - H]^-$ molecular ions and fragmentation patterns that are characteristic of each group of compounds, namely, loss of galloyl groups (−152 amu) and galloyl groups plus water (−170 amu) for gallotannins and/or loss of hexahydroxydiphenoyl (HHDP) groups (−302 amu) for ellagitannins, as described earlier (*36*). Other characteristic fragmentations include loss of hexoside (−162 amu), pentoside (−132 amu) (*37*), rhamnoside (−145 amu) (*24*), and carboxylic function (−44 amu) (*36*). Tentative identifications of all compounds are provided below for each fraction.

Fraction 1. Eight compounds (F1-1−F1-8) were detected in fraction 1, which was eluted with ethanol from the Sephadex LH-20 column. The most polar one was gallic acid (**2**), identified from the comparison of its retention time, UV−visible spectrum, and MS signal (*m*/*z* 169) with those of an authentic standard. Gallic acid anion has been previously detected in fruits of *E. globulus* (*22*, *23*) and in its leaves and wood (*5*, *15*, *19*), in leaves of *E. camaldulensis* and *E. rudis* (*14*), and in bark and wood of *E. regnans* (*20*).

All other peaks were attributed to gallic acid derivatives on the basis of their UV−visible spectra and further characterized by mass spectrometry. Peaks F1-3 and F1-5 detected at *m*/*z* 499 and 535, respectively, were confirmed to be gallotannins on the basis of their characteristic fragment ions (313, 211, and 169). The ion at *m*/*z* 169 corresponds to gallic acid anion and the ion at *m*/*z* 313 to a galloylglucose group [M − H − 18, loss of water] (*37*). The daughter ion at *m*/*z* 211 has also been previously described among fragment ions formed from mono- through pentagalloylglucose (*24*). Mass spectrometric analysis of compound F1-7 revealed a molecular anion at *m*/*z* 659 that gave rise to monogalloyl diglucose (*m*/*z* 493) after loss of 166 amu, corresponding to a methylgalloyl group (*24*); this fragmentation is characteristic of a methyldigalloyldiglucose that, to our knowledge, has never been reported in *Eucalyptus*. Two compounds (F1-4 and F1-6) detected at *m*/*z* 497 provided fragment ions at 169 and 313 amu that can be interpreted as a gallic acid anion and a galloylglucose group, as explained above. They both gave rise to a neutral loss of 184 amu characteristic of an oleuropeic acid moiety (*6*). These MS spectra correspond to those of eucaglobulin (**Figure 3b**, **4**) (*5*, *6*) and globulusin B (**5**) (*6*), which have been already detected in leaves of *E. globulus* (*5*, *6*). This is the first report of these compounds in the fruits of *E. globulus*. These phenol glucosides could be differentiated by their UV absorbance maximum at λ_{max} 291 and 218−279 nm, for globulusin B (*6*) and eucaglobulin (*5*), respectively. The signal detected at *m*/*z* 519 (F1-8), showing the loss of an oleuropeic acid (M − 184), could be attributed to

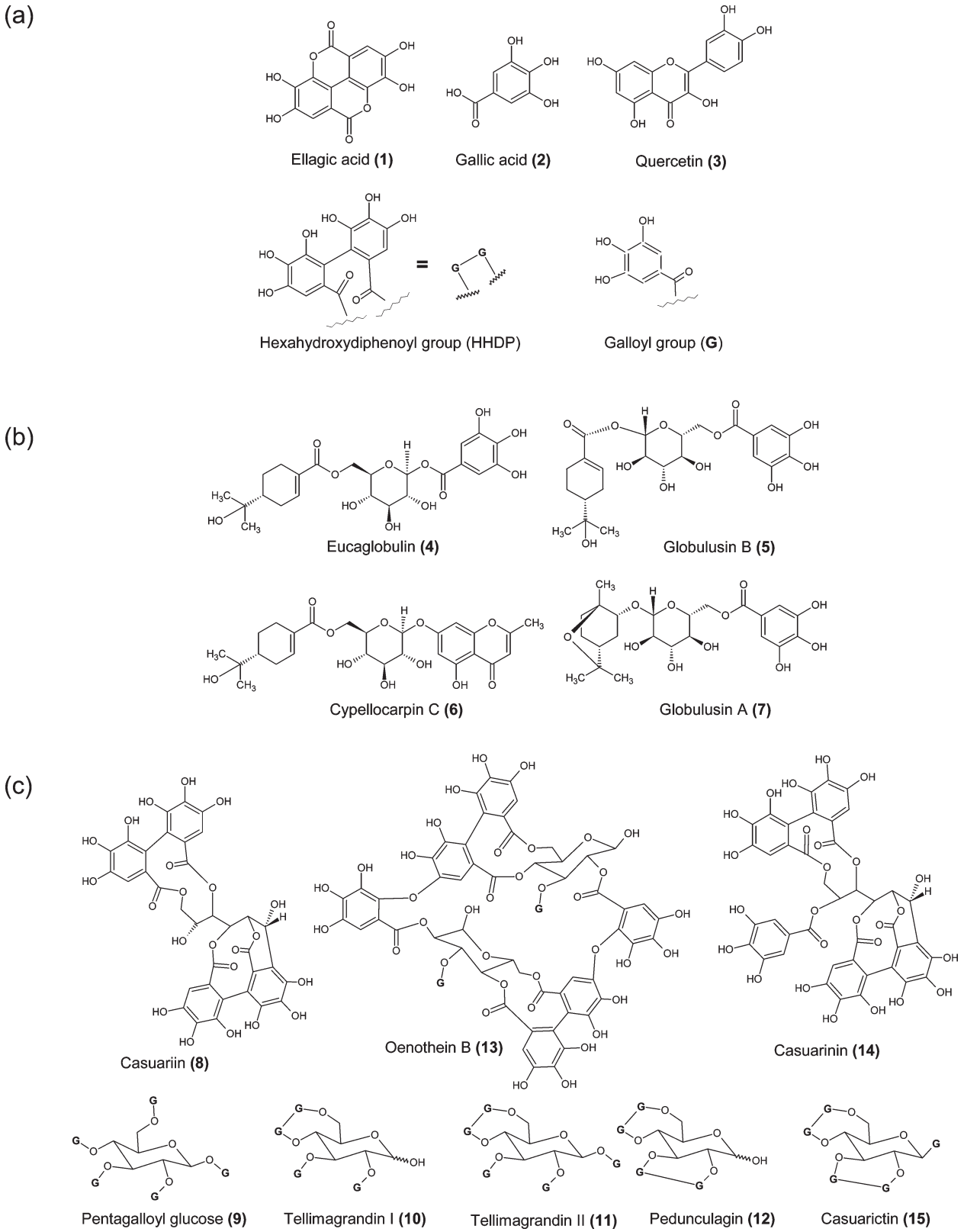


Figure 3. Chemical structures of phenolic compounds detected in EGFE.

cypellocarpin C (**6**), already reported in the leaves of *E. cypellocarpa* (*38*) and fruits of *E. globulus* (*22*).

The mass spectrum of the compound F1-2 showed a molecular anion at *m*/*z* 483, which may correspond to digalloylglucose. However, its UV spectrum (λ_{max} 220 and 276 nm) allowed this hypothesis to be ruled out. According to its UV spectrum and MS data, fragments at *m*/*z* 331 (M − 152, loss of gallic acid) and at *m*/*z* 313 (M − 170, loss of monoterpene moiety: hydroxyl-1,8-cineole or a galloyl group plus water)(*39*), this compound is tentatively identified as globulusin A (**7**, 2-hydroxy-1,8-cineole 2-*O*-*β*-D-(6′-galloyl)glucopyranoside), previously detected in leaves of *E. globulus* (*6*).

Fraction 2. Twenty-three different compounds were detected in fraction 2, eluted with ethanol and then methanol. They could be

Table 1. HPLC-DAD-ESI-MS Data for Phenolic Compounds in EGFE

peak	t_R (min)	λ (nm)	$[M-H]^-$/ $[M-2H]^{2-}$	MS^2 ions (*m*/*z*)	neutral loss	phenolic compound hypothesis (structure no.)	earlier reports in *Eucalyptus* species[a]
F1							
F1-1	5.89	232−272	169	ND[b]	ND	gallic acid (**2**) (co-injection)	E g F (*22*, *23*), E g L B W (*5*, *15*, *19*), E cam, rud L (*14*), E rgn B W (*20*)
F1-2	31.6	220−276	483	331−271−313−439	152−212−170−44	globulusin A (**7**)	E g L (*6*)
F1-3	45.76	282	499	439−313−211−169	60−126−288−330	gallotannin	
F1-4	47−48	218−279	497	169−211−313−437	328−286−184−60	eucaglobulin (**4**)	E g L (*5*, *6*)
F1-5	49.56	239−279	535	313−169	222−366	gallotannin	
F1-6	50.7	291	497	169−479−313−137	328−18−184−60	globulusin B (**5**)	E g L (*6*)
F1-7	60.45	229−279	659	493	166	methyl digalloyl diglucose	first report in genus E
F1-8	63.48	298−318sh	519	335−353−233	184−166−286	cypellocarpin C (**6**)	E g F (*6*), E cyp L (*38*)
F2							
F2-1	3.8	255−300	481	301−275	180−206	HHDP glucose	E nit W (*36*), E alb F (*41*)
F2-2	5.87	232−272	633	301- 463−257−275	170−376−358	HHDP galloylglucose	E nit W (*36*), con L (*40*)
F2-3	7.84	262	633	301−249−275-(481−463)	332−384−358−(152−170)	HHDP galloylglucose	E nit W (*36*), con L (*40*)
F2-4	12	265	633	301−339−421−481−249	332−294−212−152−384	HHDP galloylglucose	E nit W (*36*), E con L (*40*)
F2-5	27	268	633	301−463	332−170	HHDP galloylglucose	E nit W (*36*), E con L (*40*)
F2-6	33.1	269	483	271−331−313	212−152−170	digalloylglucose	E nit W (*36*), E con L (*40*)
F2-7	35	263	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-8	36. 75	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-9	37.17	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-10	38.56	269	635	483−465−423−248	152−170−212−387	trigalloylglucose	E nit W (*36*), E ros L (*14*), E con L (*40*)
F2-11	42.59	253−364	463	301	162	acid ellagic hexoside	first report in genus E
F2-12	43.16	255−355	463	301	162	quercetin 3-*O*-glucoside (co-injection)	E g, cam, rud L (*15*), E g, B (*44*), E gun H (*45*)
F2-13	44	251−364	477	315	162	methyl ellagic acid hexoside	E g F (*23*)
F2-14	44.2	279	787	617−635	170−152	tetragalloyl glucose	E nit W (*36*), E vim L (*40*)
F2-15	47	253−366	301	229	72	ellagic acid (**1**) (co-injection)	E sp (*15*, *26*), E g and E rgn B (*20*)
F2-16	47.1	253−355	477	415−301	62−176	quercetin 3-*O*-glucuronide	E sp L (*15*, *26*), E con L (*40*)
F2-17	48.98	246 −370	491	329−313	162−178	3,3′-*O*-dimethylellagic acid 4-*O*-β-glucopyranoside	first report in genus E
F2-18	50	223−242−255−366	629	477−315−301	152−314−328	galloyl ester of a methylellagic acid hexoside	E g L (*16*)
F2-19	51.7	251−364	447	315	132	methylellagic acid pentose	first report in genus E
F2-20	51.98	246−363	315	ND	ND	methylellagic acid	E g F (*23*)
F2-21	53.19	279	649	497−479	152−170	methyl trigalloylglucose	E nit W (*36*)
F2-22	54.33	239−279	649	479	170	methyl trigalloylglucose	E nit W (*36*)
F2-23	57	220−277	801	631−649	170−152	methyl tetragalloylglucose	first report in genus E
F3							
F3-1	3.77	265	783	301−481−275−765	482−302−508−18	casuariin (**8**)/ desgalloylstachyurin	E nit W (*36*), E con L (*40*)
F3-2	4.53	267	951	907−(783−301)	44−(168−650)	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-3	6.11	269	783	481−765−721−301	302−18−62−482	casuariin (**8**)/ desgalloylstachyurin	E nit W (*36*), E con L (*40*)
F3-4	8−13	265	783	301−481	482−302	pedunculagin (**12**)	E nit W (*36*), E con, vim L (*40*)
F3-5	8−13	265	783	301−481	482−302	pedunculagin anomer	E nit W (*36*), E con, vim L (*40*)
F3-6	14.2	267	951	907	44	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-7	20	267	951	907	44	trisgalloy HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-8	22.59	265	1567/783	765−935	802−632	oenothein B (**13**)	E sp (*40*, *26*), E alb F (*41*)
F3-9	25	267	951	907	44	trisgalloyl HHDP glucose isomer	E nit W (*36*), E vim L (*40*)
F3-10	27.4	265	935	917−633−783−301	18−302−152−634	casuarinin (**14**)	E nit W (*36*), E alb F (*41*)
F3-11	28.01	281.7	935	917−633−783−301	18−302−152−634	casuarinin anomer	
F3-12	34	270	785	301−483−615	484−302−170	tellimagrandin I (**10**)	E g L (*5*), E ros (*14*), E nit W (*36*), E alb F (*41*), E con, vim L (*40*)
F3-13	34.1	258−366	1085	765−633−473	320−452−612	cornusiin B (**17**) or eucalbanin A (**16**)	E alb F (*41*) or E nit W (*36*)
F3-14	35.7	265	785	ND	ND	HHDP digalloylglucose isomer	E nit W (*36*), E con L (*40*)
F3-15	36.65	258−366	1085	765−783−633	320−302−452	cornusiin B (**17**) or eucalbanin A (**16**)	E alb F (*41*), E nit W (*36*)
F3-16	37.8	253−371	469	425	44	valoneic acid dilactone (**18**) or one of its isomers	first report in genus E
F3-17	38.38	270	785	ND	ND	HHDP digalloylglucose isomer	E nit W (*36*)
F3-18	39.33	266	935	633−301−451	302−634−484	casuarictin (**15**)	E nit W (*36*)
F3-19	39.99	255−366	469	425	44	valoneic acid dilactone (**18**) or one of its isomers	first report in genus E
F3-20	43.5	280	937	767−(741)−465−301	170−(196)−472−636	tellimagrandin II (**11**)	E nit W (*36*), E vim L (*40*)
F3-21	44.21	279	787	617−635	170−152	tetragalloylglucose	E nit W (*36*), E vim L (*40*)
F3-22	45.6	280	787	617−635	170−152	tetragalloylglucose	E nit W (*36*), E vim L (*40*)
F3-23	47.7	239−279	939	769−787	170−152	pentagalloylglucose (**9**)	E g L (*5*), E nit W (*36*), E alb F (*41*)
F3-24	52.78	239−279	2182/1091	939−1285−1787−1935	244−898−396−248	ellagitannin	

[a] Key to simple nomenclature: E, eucalyptus; g, globulus; alb, alba; cam, camaldulensis; con, consideniana; gun, gunnii; nit, nitens; rud, rudis; ros, rostra; rgn, regnans; sp, species; vim, viminalis; cyp, cypellocarpa; L, leaves; , bark; F, fruit; W, wood; H, hook. [b] ND, not identified.

divided into three groups, corresponding respectively to gallotannins, ellagitannins, and flavonols, on the basis of their UV−visible spectra.

The first group contained 14 compounds, showing a λ_{max} around 270 nm and fragment ions characteristic of gallotannin derivatives. Among them, 9 were galloylglucose derivatives, whereas 5 also contained one or two HHDP groups. Compound F2-6 showed a λ_{max} at 269 nm and a molecular anion at *m*/*z* 483, yielding fragment ions corresponding to the loss of a galloyl group (−152), of a galloyl group plus a water molecule (−180), and of another fragment of 212 amu, characteristic of galloylglucose derivatives, as explained above. It could thus be assigned to digalloylglucose previously detected in *E. nitens* wood (*36*) and *E. consideniana* leaves (*40*). Similarly, the mass signals at *m*/*z* 635 (F2-7−F2-10) with their characteristic fragment ions at *m*/*z* 483 and 465 corresponded to trigalloylglucose isomers, detected earlier in *E. nitens* wood (*36*), leaves of *E. consideniana* (*40*), and *E. rostra* leaves (*14*). F2-14, present in a small amount in fraction 2, could be attributed to a tetragalloylglucose. This compound was more abundant in fraction 3 (F3-21) and will be described below along with the other tetragalloylglucose isomers present in this fraction.

The ions detected at *m*/*z* 801 and 649 (F2-21, F2-22, and F2-23) also showed fragments corresponding to losses of 152 and 170 amu, indicating gallotannin structures. The MS data (+ 14 amu compared to the molecular ions of tri- and tetragalloylglucose) suggest that these compounds are methylated tetragalloylglucose and trigalloylglucose, respectively. The latter has been previously detected in *E. nitens* wood (*36*).

The $[M - H]^-$ at *m*/*z* 481 (F2-1) with absorbance maxima 255−300 nm and fragment ions at *m*/*z* 301 (loss of 180, loss of a galloyl group plus water) has been assigned to an HHDP glucose, reported earlier in the wood of *E. nitens* (*36*) and identified as 2,3-(*S*)-HHDP-D-glucose in *E. alba* fruits (*41*).

The signals detected at *m*/*z* 633 (F2-2, F2-3, F2-4, and F2-5) were attributed to HHDP galloylglucose isomers on the basis of their molecular ion and fragment ions at *m*/*z* 301 (loss of 332, which indicated the presence of a galloylglucose unit) (*42*), corresponding to HHDP, and at 463 (loss of a galloyl group plus water). These compounds have been previously detected in *E. nitens* wood (one isomer) (*36*) and *E. consideniana* leaves (three isomers) (*40*).

Seven mass signals detected in fraction 2 could be attributed to ellagic acid and its derivatives. The presence of free ellagic acid (**1**) in fraction F2 was confirmed by its retention time (47 min) and MS data (*m*/*z* 301) (F2-16). The peak eluted at 51.98 min (F2-20) with *m*/*z* 315 and UV−visible spectrum (λ_{max} 246 and 363 nm), characteristic of ellagic acid, was tentatively identified as methylellagic acid, which has been previously detected in fruits of *E. globulus* and identified as 3-*O*-methylellagic acid (*23*). The two peaks F2-13 and F2-19, showing similar UV−visible spectra with two absorbance maxima at 251 and 364 nm, were also postulated to be ellagic acid derivatives. MS analysis of the former showed an intense molecular anion at *m*/*z* 477, which yielded a fragment ion at *m*/*z* 315 attributed to methylellagic acid, through loss of a hexosyl group (*m*/*z* 162). This compound is thus a methylellagic acid hexoside, presumably 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside identified earlier in *E. globulus* fruits (*23*). The peak at *m*/*z* 463 (F2-11) with a fragment at *m*/*z* 301 has been assigned to ellagic acid hexoside. The peak at *m*/*z* 447 (F2-19) yielded a fragment ion at *m*/*z* 315 through loss of 132 amu, corresponding to a pentose residue. It could thus be assigned to methylellagic acid pentoside as described in raspberry fruits (*37*). To our knowledge, this is the first report of these two compounds in the genus *Eucalyptus*. Another compound detected at *m*/*z* 629 (F2-18) showed fragments at *m*/*z* 477 (methylellagic acid hexoside, loss of 152 amu, corresponding to a galloyl group) and at *m*/*z* 315 (methyl ellagic acid, loss of 314 amu, corresponding to a galloylhexoside) and was thus identified as a galloyl ester of a methylellagic acid hexoside. This compound has been already detected in *E. globulus* leaves (*16*). Finally, a compound at *m*/*z* 491 (F2-17) and its fragment at *m*/*z* 329 $[M - H - 162]^-$, which corresponds to loss of a hexose, can be interpreted as dimethylellagic acid hexose (*43*). This is the first report of this compound in the genus *Eucalyptus*. Several methylellagic acid derivatives, namely, 3-*O*-methylellagic acid, 3,4,3′-*O*-trimethylellagic acid, 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside, and 3-*O*-methylellagic acid 4′-*O*-(2″-*O*-acetyl)-α-L-rhamnopyranoside, have been identified earlier in the fruits of *E. globulus* (*23*). Methylellagic acid derivatives have also been previously detected in eucalypt species such as leaves of *E. globulus*, *E. camaldulensis*, and *E. rudis* (*15*) and stem bark of *E. globulus* (*20*, *44*).

The last group of compounds detected in this fraction was flavonols, showing the same fragment ion at *m*/*z* 301, which could be attributed to quercetin. One of them, detected at *m*/*z* 463 (F2-12) (i.e., 301 + 162 amu, corresponding to a hexoside residue), can be identified as a quercetin hexoside, presumably quercetin 3-*O*-glucoside, the presence of which has been reported in the leaves of *E. globulus*, *E. camaldulensis*, and *E. rudis* (*14*−*16*, *26*), in the bark of *E. globulus*, and in the hook *E. gunnii* (*45*) and *E. consideniana* (*40*). Further argument in favor of this identification is provided by coelution with the corresponding standard. The other compound, detected at *m*/*z* 477 (F2-16) and yielding the aglycone fragment ion at *m*/*z* 301 through loss of a glucuronide substituent (−176 amu), was tentatively identified as quercetin 3-*O*-glucuronide, which has been already described in *Eucalyptus* species (*15*, *40*, *45*). This is the first report of flavonols in *E. globulus* fruits.

Fraction 3. Twenty-four compounds were detected in this fraction. They could be classified as gallotannins and ellagitannins on the basis of their UV spectra and characteristic mass fragmentations.

Compounds at *m*/*z* 787 (F3-21 and F3-22) are assigned to tetragalloylglucose isomers, which are characterized by fragment ions at *m*/*z* 635 and at *m*/*z* 617, corresponding to loss of a galloyl residue (M − H − 152) and of a gallic acid group (M − H − 170), respectively, as described by Barry and co-workers (*36*). These compounds have been previously detected in wood of *E. nitens* (*36*) and in *E. viminalis* leaves (*40*).

The compound at *m*/*z* 939 (F3-23) was identified according to its UV−visible and mass spectra data (loss of a galloyl residue [M − H − 152] and of gallic acid [M − H − 170]) as pentagalloylglucose (**9**), yielding fragment ions at *m*/*z* 787 and at *m*/*z* 769, respectively (*46*). Pentagalloylglucose has been described in wood of *E. nitens* (*36*) and in *E. alba* fruits (*41*).

Other compounds present in the fraction showed a loss of 302 amu, characteristic of ellagitannin structures (*36*). The mass signal detected at *m*/*z* 785 (F3-12, F3-14, and F3-17), with fragment ions at *m*/*z* 301 (loss of digalloylglucose) and *m*/*z* 483 (loss of HHDP), correspond to digalloylglucose, presumably tellimagrandin I (**10**), which has been previously identified in the leaves of *E. globulus* and the wood of *E. nitens* (*5*, *36*), in *E. rostra* leaves (*14*), in *E. alba* fruits (*41*), and in *E. consideniana* leaves (*40*). The signal at *m*/*z* 937 (F3-20), yielding fragment ions at *m*/*z* 767 (M − 170, loss of gallic acid) and *m*/*z* 465 (losses of HHDP and gallic acid groups) can correspond to tellimagrandin II (**11**), which has been detected earlier in *E. nitens* wood (*36*) and *E. viminalis* leaves (*40*). This compound differs from tellimagrandin I by the presence of one additional galloyl unit at the anomeric center of the glucopyranosyl core. Tellimagrandin II is produced by enzymatic transformation of pentagalloylglucose (*47*).

Another group of compounds with λ_{max} around 270 nm showed mass signals characteristic of HHDP glucose derivatives, with molecular ions detected at m/z 783 (F3-1, F3-3, F3-4, and F3-5), yielding a fragment ion at m/z 301, which corresponds to ellagic acid (M − 482, loss of HHDP glucose) as well as loss of ions at m/z 481, which correspond to deprotonated HHDP glucose (M − 302, loss of HHDP). These compounds can correspond to di-HHDP glucose. F3-1 and F3-3 yielded a fragment at m/z 765 (M − 18, loss of water). An abundant loss of 18 amu is characteristic of C-glycosidic ellagitanins (*36*), which are presumably casuariin (**Figure 3c**, **8**), and its anomer desgalloylstachyurin. Casuariin has been previously detected in *E. nitens* wood (*36*) and *E. alba* fruit (*41*). F3-4 and F3-5 can correspond to pedunculagin anomers (2,3;4,6-di-HHDP glucose, **12**) or pedunculagin isomers, as reported by Barry and co-workers (*36*). Three isomers of this compound have been previously detected in *E. nitens* wood (*36*), whereas only one was found in *E. consideniana* leaves (*40*).

Compound F3-8 yielded two ions at m/z 1567 and 783, corresponding to the monocharged and doubly charged ions of a compound with a molecular weight of 1568. Fragment ions at m/z 765, loss of 802 amu, which is reported to be ellagitannin (*42*), and 935 ($[M - H]^-$ ion of galloyl-bis-HHDP-glucopyranose), allowed assignment to a dimer of tellimagrandin I, linked by two valoneoyl groups, oenothein B (**13**) identified in leaves of eucalyptus species (*26*, *40*) and in fruits of *E. alba* (*41*).

On the basis of molecular weight and MS/MS data, the four peaks F3-2, F3-6, F3-7, and F3-9 with m/z 951 would appear to be ellagitannins. Loss of 44 amu from the $[M - 1]^-$ ion is consistent with a free carboxyl group. These compounds could be assigned to trigalloyl HDDP glucose isomers, which have been already detected in *E. nitens* wood (*36*). These consist of an HDDP glucose and a trisgalloyl group such as valoneic acid dilactone (**18**) (*46*) or one of its isomers (tergallic acid dilactone (**19**)), sanguisorbic acid, or flavogallic acid dilactone. Three peaks at m/z 935 (F3-10, F3-11, and F3-18) are also detected; they could be attributed to casuarinin (**14**) and its anomer and casuarictin (**15**); these two isomers could be distinguished by their retention times and also by their MS/MS spectra. The MS/MS spectra of casuarinin and of its anomer showed an ion resulting from a loss of water, owing to its open glucose ring structure. Casuarinin and casuarictin have been already detected in *E. nitens* wood (*36*). Compounds detected at m/z 1085 (F3-13 and F3-15) with fragment ion at 633 corresponding to the HHDP group (M − 452, loss of trigalloyl group) were assigned to eucalbanin A (**16**) or its isomer cornusiin B (**17**), which has been already detected in the fruit extract of *E. alba* (*41*).

The peak at m/z 469 (F3-16 and F3-19) with UV−visible absorption spectra similar to that of ellagic acid gives a fragment ion at m/z 425 (valoneic acid dilactone with loss of CO_2). This compound was tentatively identified as valoneic acid dilactone (**18**) (*46*) or one of its isomers (tergallic acid dilactone (**19**), sanguisorbic acid, or flavogallic acid dilactone). This is the first report of this compound in *Eucalyptus* species. Valoneic acid dilactone can be a product of hydrolysis of cornusiin B or of oenothein B and tergallic acid dilactone a product of its isomer eucalbanin A (*46*).

Co-occurrence of most compounds detected in this fraction can be explained by the proposed biosynthetic pathway for the formation of the hydrolyzable tannins in oak leaves (*48*). Consecutive galloylation steps of glucose lead to the formation of pentagalloylglucose. Oxidative coupling between galloyl groups at C-4 and C-6 produces tellimagrandin II, which yields casuarictin after oxidative coupling between galloyl groups at C-2 and C-3. Cleavage of the galloyl group at C-1 of this compound leads to the formation of casuarinin. Furthermore, it has been suggested that the C-glycosidic ellagitannins are formed from pedunculagin, through formation of casuarinin as intermediate.

HPLC-DAD-ESI-MS/MS enabled detection of a series of gallotannins and ellagitannins and of two flavonols in the fruits of *E. globulus*. Fractionation by low-pressure chromatography on Sephadex LH-20 proved to be efficient to separate lower and higher molecular weight compounds and enabled separation of compounds coeluted in the HPLC procedure.

The concentrations of the major compounds present in EFGE were evaluated from the HPLC profiles of each fraction (**Table 2**). It should be emphasized that these values are indicative, as they are calculated using external calibration with a limited number of standards. Moreover, some changes induced in the relative amounts of the various compounds may have occurred during the extraction and fractionation procedures, because hydrolyzable tannins can be modified by desiccation or thermal treatments and easily undergo partial hydrolysis under acidic conditions.

Most of the phenolic compounds were recovered in F3 (109.4 mg/g of EGFE) and F2 (97.5 mg/g of EGFE), whereas F1 contained only small amounts (7.3 mg/g of EGFE) (**Table 2**).

F1, eluted with ethanol, contained gallic acid, terpenyl derivatives of galloylglucose (tentatively identified to eucaglobulin, globulisin B) and of noreugenin glucoside (tentatively identified as cypellocarpin C), and derivatives of lower molecular weight gallotannins in small amounts (**Table 2**).

F2 contained high levels of ellagitannins (HHDP glucose, HHDP galloylglucose, trigalloylglucose, methyltrigalloylglucose, and methyltetragalloylglucose), ellagic acid, and ellagic acid derivatives (methylellagic acid, ellagic acid hexose, methylellagic acid hexose and pentose) (**Table 2**), which is in agreement with previous studies reporting that ellagic acid and its derivatives are the major phenolic class that characterizes *E. globulus* fruits (*22*, *23*). However, the large amount of ellagic acid and HHDP glucose could be due to hydrolytic reaction during the extraction procedure. Gallotannins and flavonols (presumably quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide) were present only as minor components in this fraction (**Table 2**).

F3, eluted with aqueous acetone, contains tetra- and pentagalloylglucose and higher molecular weight ellagitannins, as expected from the literature (*24*, *25*, *36*, *46*). Di-HHDP glucose (casuariin or desgalloylstachyurin) is particularly abundant in this fraction but can result from hydrolysis of ellagitannins during the extraction procedure.

Fractionation on Sephadex LH-20 followed by HPLC-DAD-MS analysis proved to be efficient for the identification of phenolic compounds in eucalypt fruit. Ellagic acid derivatives were the major phenolic compounds in the fruit extract, ellagic acid and its smaller molecular weight derivatives being recovered in fraction F2, whereas ellagitannins were distributed in fractions F2 and F3. Among the wide range of phenolic compounds (including structural isomers) detected in this study, gallic acid, ellagic acid derivatives, and cypellocarpin C had been previously identified in the fruits of *E. globulus* (*22*, *23*), whereas quercetin derivatives, globulusins A and B, and hydrolyzable tannins were described for the first time in this material. Among hydrolyzable tannins, methyltrigalloylglucose, di-HHDP glucose, HHDP glucose, HHDP galloylglucose, digalloylglucose, trigalloylglucose, tetragalloylglucose, trisgalloyl HHDP glucose, casuarinin, casuarictin, cornusiin B, eucalbanin A, and tellimagrandin II are reported here for the first time in *E. globulus* plants. These compounds have been detected earlier in the wood of *E. nitens* (*36*). Methyltetragalloylglucose, methyldigalloyldiglucose, methylellagic acid pentose, and valoneic acid dilactone are described for the first time in the genus *Eucalyptus*. It should be emphasized

Table 2. Contents of Phenolic Compounds in EGFE

	concn (mg/g)	correction factor[a]	corrected concn[b] (mg/g)	concn (mg/g) per family
compounds in F1				
gallic acid	5.24[c]	170[c]	5.24[c]	
globulusin A	0.01[c]	484[c]	0.01[c]	terpenyl derivatives: 1.75
eucaglobulin	0.16[c]	498[c]	0.16[c]	
globulusin B	1.24[c]	498[c]	1.24[c]	
cypellocarpin C	0.34[c]	520[c]	0.34[c]	
methyldigalloylglucose	0.06[c]	330[c]	0.03[c]	
total F1	**7.05**		**7.03**	
compounds in F2				
digalloylglucose	0.39[c]	318	0.19[c]	gallotannins: 0.7
trigalloylglucose	0.13[c]	212	0.04[c]	
trigalloylglucose	0.32[c]	212	0.11[c]	
trigalloylglucose	0.02[c]	212	0.01[c]	
trigalloylglucose	0.18[c]	212	0.06[c]	
tetragalloylglucose	0.09[c]	197	0.02[c]	
methyltetragalloylglucose	1.06[c]	200.5	0.27[c]	
HHDP glucose	95.99[c]	240.5	48.09[c]	ellagitannins: 50.6
HHDP galloylglucose	6.77[c]	211.33	2.26[c]	
HHDP galloylglucose	0.71[c]	211.33	0.24[c]	
ellagic acid hexose	0.24[d]	464	0.24[d]	ellagic acid and derivatives: 45.8
methylellagic acid hexose	0.41[d]	478	0.41[d]	
ellagic acid	32.35[d]	302	32.35[d]	
dimethylellagic glucopyranose	0.52[d]	492	0.52[d]	
galloyl ester of methylellagic acid	0.05[d]	630	0.05[d]	
methylellagic acid pentose	8.15[d]	448	8.15[d]	
methylellagic acid	4.05[d]	316	4.05[d]	
quercetin 3-*O*-glucoside	0.25[e]	464	0.25[e]	flavonols: 0.5
quercetin 3-*O*-glucuronide	0.21[e]	478	0.21[e]	
total F2	**149.69**		**96.80**	
compounds in F3				
tetragalloylglucose	1.39[c]	197	0.35[c]	gallotannins: 5.0
tetragalloylglucose	7.55[c]	197	1.89[c]	
pentagalloylglucose	14.02[c]	157.6	2.80[c]	
casuariin/desgalloylstachyurin	377.39[c]	196	94.35[c]	ellagitannins: 101.5
pedunculagin	1.22[c]	196	0.30[c]	
pedunculagin	2.44[c]	196	0.61[c]	
trisgalloyl HHDP glucose isomer	0.17[c]	190.4	0.03[c]	
trisgalloyl HHDP glucose isomer	0.26[c]	190.4	0.05[c]	
oenothein B	18.43[c]	196	2.30[c]	
casuarinin	0.65[c]	187.2	0.13[c]	
casuarinin	0.22[c]	187.2	0.04[c]	
tellimagrandin I	1.46[c]	196.5	0.36[c]	
HHDP digalloylglucose isomer	0.31[c]	196.5	0.08[c]	
HHDP digalloylglucose isomer	3.70[c]	196.5	0.92[c]	
casuarictin	0.33[c]	187.2	0.07[c]	
tellimagrandin II	3.00[c]	187.6	0.60[c]	
cornusiin or eucalbanin	0.49[d]	1086	0.49[d]	
cornusiin or eucalbanin	1.12[d]	1086	1.12[d]	
valoneic acid or isomer	0.11[d]	470	0.11[d]	ellagic acid and derivatives: 2.9
valoneic acid or isomer	0.07[d]	470	0.07[d]	
ellagic acid	2.73[d]	302	2.73[d]	
total F3	**437.06**		**109.42**	
total F1 + F2 + F3	**593.80**		**213.25**	

[a] Correction factor: molecular weight/number of gallic (ellagic, flavonol) unit in the molecule. [b] Calculated using the correction factor. [c] Gallic acid equivalent. [d] Ellagic acid equivalent. [e] Quercetin 3-*O*-glucoside equivalent.

that only tentative identification can be provided on the basis of UV−visible and MS spectra, even when associated with co-injection with standards. Further work should thus be performed to provide formal identification of phenolics tentatively characterized in this study.

ACKNOWLEDGMENT

We thank Houari Mohand Oussalem (Chef de service de la conservation forestière de Béjaia) for providing plant material and Dr. Chibane-Amalal Hayette (Université M'hamed Bouguerra de Boumerdès) for her support.

LITERATURE CITED

(1) Benyahia, S.; Benache, S.; Benayache, F.; Quintana, J.; Lopez, M.; Leon, F.; Hernandez, J. C.; Estévez, F.; Bermejo, J. Isolation from *Eucalyptus occidentalis* and identification of a new kaempferol derivative that induces apoptosis in human myeloid leukemia cells. *J. Nat. Prod.* **2004**, *67*, 527−531.

(2) Takahashi, T.; Kokubo, R.; Sakaino, M. Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*. *Lett. Appl. Microbiol.* **2004**, *39*, 60−64.

(3) Mahmoudi, Y. *La Thérapeutique par les Plantes les plus Communes en Algérie*; Palais du Livre : Blida, Algeria, 1988; Vol. 97, p 58.

(4) Sherry1, E.; Warnke, P. H. Successful use of an inhalation phytochemical to treat pulmonary tuberculosis. *Phytomedicine* **2004**, *11*, 95−97.

(5) Hou, A. J.; Liu, Y. Z.; Yang, H.; Lin, Z. W.; Sun, H. D. Hydrolysable tannins and related polyphenols from *Eucalyptus globulus*. *J. Asian Nat. Prod. Res.* **2000**, *2*, 205−212.

(6) Hasegawa, T.; Takano, F.; Takata, T.; Niiyama, M.; Ohta, T. Bioactive monoterpene glycosides conjugated with gallic acid from the leaves of *Eucalyptus globulus*. *Phytochemistry* **2008**, *69*, 747−753.

(7) Rombi, M. *100 Plantes Médicinales, Composition, Mode d'Action et Intérêt Thérapeutique*; Romart: Paris, France, 1991; p 101.

(8) Alison, M. G.; Peter, R. F. Antihyperglycemic actions of *Eucalyptus globulus* (eucalyptus) are associated with pancreatic extra-pancreatic effects in mice. *J. Nutr.* **1998**, *128*, 2319−2323.

(9) Gallagher, A. M.; Flatt, P. R.; Duffy, G.; Abdel-Wahab, Y. H. A. The effects of traditional antidiabetic plants on *in vitro* glucose diffusion. *Nutr. Res.* (*N.Y.*) **2003**, *23*, 413−424.

(10) Jouad, A.; Maghrani, M.; El Hassani, R. H.; Eddouks, M. Hypoglycemic activity of aqueous extract of *Eucalyptus globulus* in normal and streptozotocin-induced diabetic rats. *J. Herb, Spice Med. Plants* **2003**, *10*, 462−464.

(11) Bruneton, J. *Pharmacognosie, Phytochimie, Plantes Médicinales*; Lavoisier: Paris, France, 1993; p 916.

(12) Balage, T. Cineole-rich eucalyptus. *Int. J. Aroma.* **1997**, *8*, 15−21.

(13) Ikawati, Z.; Wahyuono, S.; Maeyama, K. Screening of several Indonesian medicinal plants for their inhibitory effect on histamine release from RBL-2H3 cells. *J. Ethnopharmacol.* **2001**, *75*, 249−256.

(14) Cadahia, E.; Conde, E.; Garcia-Vallejo, M. C.; Fernandez, R. de Simon High pressure liquid chromatography analysis of polyphenols in leaves of *Eucalyptus camaldulensis*, *E. globulus* and *E. rudis*: proanthocyanidins, ellagitannins and flavonol glycosides. *Phytochem. Anal.* **1997**, *8*, 78−83.

(15) Conde, E.; Cadahia, E.; Garcia-Vallejo, M. C. Low molecular weight polyphenols in leaves of *E. camadulensus*, *E. globulus*, *E. rudis*. *Phytochem. Anal.* **1997**, *8*, 186−193.

(16) Atoui, A. K.; Mansouri, A.; Boskou, G.; Kefalas, P. Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chem.* **2005**, *89*, 27−36.

(17) Close, D. C.; Beadle, C. L.; Battaglia, M. Foliar anthocyanin accumulation may be a useful indicator of hardiness in eucalyptus seedling. *For. Ecol. Manag.* **2004**, *198*, 169−181.

(18) Osawa, T.; Namiki, M. A novel type of antioxidant isolated from leaf wax of eucalyptus leaves. *Agric. Biol. Chem.* **1981**, *45*, 735−739.

(19) Cruz, J. M.; Dominguez, H.; Parajo, J. C. Anti-oxidant activity of isolates from acid hydrolysates of *Eucalyptus globulus* wood. *Food Chem.* **2005**, *90*, 503−511.

(20) Yazaki, Y.; Edwin Hillis, W. Polyphenols of *Eucalyptus globulus*, *E. regnans* and *E. deglupta*. *Phytochemistry* **1976**, *15*, 1180−1182.

(21) Cork, S. J.; Krockenberger, A. K. Methods and pitfalls of extracting condensed tannins and other phenolics from plants: insights from investigations on Eucalyptus leaves. *J. Chem. Ecol.* **1991**, *17*, 123−134.

(22) Guo, Q. M.; Yang, X. W. Cypellocarpin C and other compounds from the fruits of *Eucalyptus globulus* Labill. *Biochem. Syst. Ecol.* **2006**, *34*, 543−545.

(23) Yang, X. M.; Guo, Q. M. Studies on chemical constituents in fruits of *Eucalyptus globulus*. *Zhongguo Zhong Yao Za Zhi* **2007**, *32*, 496−500.

(24) Soong, Y. Y.; Barlow, P. J. Isolation and structure elucidation of phenolic compounds from longan (*Dimocarpus longan* Lour.) seed by high-performance liquid chromatography−electrospray ionization mass spectrometry. *J. Chromatogr., A* **2005**, *105*, 270−277.

(25) Salminen, J.-P.; Ossipov, V.; Loponen, J.; Haukioja, E.; Pihlaja, K. Characterisation of hydrolysable tannins from leaves of *Betula pubescens* by high-performance liquid chromatography−mass spectrometry. *J. Chromatogr., A* **1999**, *864*, 283−291.

(26) Amakura, Y.; Yoshimura, M.; Sugimoto, N.; Yamazaki, T.; Yoshida, T. Marker constituents of the natural antioxidant eucalyptus leaf extract for the evaluation of food additives. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1060−1065.

(27) Wettasinghe, M.; Shahidi, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primose meals. *Food Chem.* **2000**, *70*, 17−26.

(28) Yoshida, T.; Nakata, F.; Okuda, T. Tannins and related polyphenols of melastomataceous plants. VIII. Nobotanins L, M and N, trimeric hydrolyzable tannins from *Tibouchina semidecandra*. *Chem. Pharm. Bull.* **1999**, *47*, 824−827.

(29) Wanasundara, N. U.; Amarowicz, R.; Shahidi, F. Partial characterization of natural antioxidants in canola meal. *Food Res. Int.* **1996**, *28*, 525−530.

(30) Lohézic-Le-Dévéhat, F.; Tomasi, S.; Fontanel, D.; Boustie, J. Flavonols from *Scurrula ferruginea* Danser (Loranthaceae). *Z. Naturforsch.* **2002**, *57C*, 1092−1095.

(31) Lee, S. J.; Chung, H. Y.; Lee, I. K.; Oh, S. U.; Yoo, I. D. Phenolics with inhibitory activity on mouse brain monoamine oxidase (MAO) from whole parts of *Artemisia vulgaris* L. (mugwort). *Food Sci. Biotechnol.* **2000**, *9*, 179−182.

(32) Wolbis, M.; Nowak, S.; Kicel, A. Polyphenolic compounds in *Scopolia caucasica* Kolesn. ex Kreyer (*Solanacea*). *Acta Pol. Pharm.* **2007**, *6*, 241−245.

(33) Tahrouch, S.; Andary, C.; Raior, S.; Mondolot, L.; Gargadennec, A. Polyphenol investigation of *Argania spinosa* (Sapotaceae) endemic tree from Morocco. *Acta Bot. Gallica* **2000**, *147*, 225−232.

(34) McDougall, G.; Shpiro, F.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Different polyphenolic components of soft fruits inhibit α-glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760−2766.

(35) Tian, F.; Li, B.; Ji, B.; Zhang, G.; Luo, Y. Identification and structure−activity relationship of gallotannins separated from *Galla chinensis*. *Food Sci. Technol.* **2009**, *42*, 1289−1295.

(36) Barry, K. M.; Davies, N. W.; Mohammed, C. L. Identification of hydrolysable tannins in the reaction zone of *Eucalyptus nitens* wood by high performance liquid chromatography−electrospray ionisation mass spectrometry. *Phytochem. Anal.* **2001**, *12*, 120−127.

(37) Mertz, C.; Cheynier, V.; Gunata, Z.; Brat, P. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8616−8624.

(38) Ito, H.; Koreishi, M.; Tokuda, H.; Nishino, H.; Yoshida, T. Cypellocarpin A−C, phenol glycosides esterified with oleuropeic acid, from *Eucalyptus cypellocarpa*. *J. Nat. Prod.* **2000**, *63*, 1253−1257.

(39) Someya, Y.; Kobayashi, A.; Kikue, K. Isolation and identification of *trans*-2- and *trans*-3-hydroxy-1,8-cineole glucosides from *Alpinia galangal*. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 950−953.

(40) Santos, S. C.; Waterman, P. G. Polyphenols from *Eucalyptus consideniana* and *Eucalyptus viminalis*. *Fitoterapia* **2001**, *72*, 95−97.

(41) Yoshida, T.; Maruyama, T.; Nitta, A.; Okuda, T. Eucalbanins A, B and C, monomeric and dimeric hydrolysable tannins from *Eucalyptus alba* Reinw. *Chem. Pharm. Bull.* **1992**, *40*, 1750−1754.
(42) Lee, J. H.; Johnson, J. V.; Talcott, S. T. Identification of ellagic acid conjugates and other polyphenolics in Muscadine grapes by HPLC-ESI-MS. *J. Agric. Food Chem.* **2005**, *53*, 6003−6010.
(43) Grzegorz, P.; Jaromir, B. ellagic acid derivatives and naphtoquinones of *Dionaea Muscipula* from in vitro cultures. *Phytochemistry* **1996**, *41*, 775−778.
(44) Kim, J. P.; Lee, I. K.; Yun, B. S.; Chung, S. H.; Shim, G. S.; Koshino, H.; Yoo, I. D. Ellagic acid rhamnosides from the steam bark of *Eucalyptus globulus*. *Phytochemistry* **2001**, *57*, 587−591.
(45) Curir, P.; VanSumere, C. F.; Termini, A.; Barthe, P.; Marchesini, A.; Dolci., M. Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunnii* hook micropropagated through axillary bud stimulation. *Plant Physiol.* **1990**, *92*, 1148−1153.
(46) Cantos, E.; Espin, J. C.; Lopez-Bote, C.; De La Hoz, L.; Ordonez, J. A.; Tomas-Barberan, F. A. Phenolic compounds and fatty acids from acorns (*Quercus* spp.), the main dietary constituent of free-range Iberian pigs. *J. Agric. Food Chem.* **2003**, *51*, 6248−6255.
(47) Niemetz, R.; Gross, G. G. Oxidation of pentagalloylglucose to the ellagitannin, tellimagrandin II, by a phenol oxidase from *Tellima grandiflora* leaves. *Phytochemistry* **2003**, *62*, 301−306.
(48) Salminen, J. P.; Roslin, T.; Karonen, M.; Sinkkonen, J.; Pihlaja, K.; Pulkkinen, P. Seasonal variation in the content of hydrolyzable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. *J. Chem. Ecol.* **2004**, *30*, 1693−1711.

Received for review July 29, 2010. Revised manuscript received November 3, 2010. Accepted November 9, 2010.

E F
F E *Entomologie faunistique – Faunistic Entomology* 2011 (2010) **63** (3), 109-113

Contribution à l'analyse de la diversité entomologique des milieux insulaires de la région de Jijel (Algérie)

Riadh Moulaï* & Lyes Aissat

Faculté des Sciences de la Nature et de la Vie, Université de Béjaia, 06000 Béjaia, Algérie. * Auteur pour correspondance: E-mail: moulai741@hotmail.com

L'analyse de la diversité entomologique de trois milieux insulaires, situés à l'ouest de Jijel (Algérie) montre l'existence de 140 espèces. L'île Petit Cavallo, s'est révélé être la plus riche avec 133 espèces, elle est suivi par l'île Grand Cavallo (91 espèces) et l'îlot Grand Cavallo (25 espèces). Huit ordres d'insectes sont recensés aussi bien sur l'île Petit Cavallo, que sur l'île Grand Cavallo. L'îlot Grand Cavallo, ne renferme que trois ordres. Du point de vue spécifique, les Coléoptères sont les plus diversifiés sur l'île Petit Cavallo et sur l'île Grand Cavallo. Sur l'îlot Grand Cavallo, ce sont les Hyménoptères qui sont les plus riches en espèces. En termes d'abondance, les Coléoptères sont les plus abondants sur l'île Petit Cavallo (35,77 %). Sur l'île Grand Cavallo, ce sont les Hémiptères qui sont les plus nombreux (27,54 %). Sur l'îlot Grand Cavallo, les Hyménoptères sont les plus abondants (55,32 %). Du point de vue de la similarité entomologique les îles Grand et Petit Cavallo paraissent assez proches (82 %) et semblent se différencier de l'îlot Grand Cavallo (39 % pour les deux combinaisons).
Mots-clés: Milieux insulaires, insectes, diversité, abondance, similarité, Jijel.

Analysis of the entomological diversity of three islands located west of Jijel (Algeria) shows the existence of 140 species. The Petit Cavallo island, proved to be the richest with 133 species, it is followed by the Grand Cavallo Island (91 species) and the Grand Cavallo islet (25 species). Eight insect orders are listed on both the Petit Cavallo Island, on the island of Grand Cavallo. The Grand Cavallo islet contains only three orders. Coleoptera are the most diversified both on island Petit Cavallo and on Cavallo Grand Island. Hymenoptera are the most diverse on the islet Grand Cavallo. In terms of abundance, the beetles are most abundant on the island Petit Cavallo (35.77 %). On the island of Grand Cavallo, the Hemiptera are the most abundant (27.54 %). On the islet Grand Cavallo, the Hymenoptera are the most abundant (55.32 %). From the point of view of the similarity Islands Grand and Petit Cavallo seem fairly similar (82 %) and appear to be differentiated from the islet Grand Cavallo.
Keywords: Insular environment, insects, diversity, abundance, similarity, Jijel.

1. INTRODUCTION

La diversité biologique dans les milieux insulaires a été bien étudiée dans la partie nord de la Méditerranée (Cheylan, 1984; Dajoz, 1987; Blondel, 1995; Vidal, 1998; Medail & Vidal, 1998), ce qui n'est pas le cas de la rive sud de la Méditerranée, notamment en Algérie, où il est vrai que malgré une côte de 1200 km de long, le nombre d'îles et d'îlots est très limité. Les travaux sur l'évaluation de la diversité faunistique des îles et îlots de l'Algérie sont quasi inexistants, sauf de rares études s'intéressants à des taxons bien définis à l'exemple des oiseaux marins nicheurs (Jacob & Courbet, 1980; Boukhalfa, 1990; Moulai, 2005; Moulai, 2006; Bougaham, 2008). Peu de choses sont connues sur les insectes qui peuplent les îles et îlots d'Algérie. Seules les fourmis des îles Habibas semblent être répertoriées (Bernard, 1958). C'est dans ce cadre que s'insère notre démarche qui vise à identifier la richesse entomologique de trois îlots situés à l'ouest de la côte de Jijel.

2. MATERIEL ET METHODE

2.1. Présentation de la région d'étude

L'étude de la diversité des insectes insulaires, s'est déroulée sur trois îlots localisés sur le littoral occidental de la ville de Jijel (36°49' Nord, 5°45' Est). Il s'agit de l'île Grand Cavallo, l'îlot Grand Cavallo et de l'île Petit Cavallo (Figure 1).
L'île Grand Cavallo, est située à 20 km de Jijel dans la localité d'El Aouana. Elle est séparée du

continent de 950 m et sa superficie est de 6 ha. Son relief est assez accidenté. Sa face Ouest comprend une falaise avec une altitude maximale de 50 m. Le substrat, est de type magmatique, composé de feldspath blanc de grande taille et de mica blanc à texture grenue. Le couvert végétal est principalement de type matorral haut, pouvant atteindre quatre mètres de hauteur. Il est formé principalement de Pistachier lentisque (*Pistacia lentiscus*), de Filaire, (*Phillyrea angustifolia*) et d'Olivier sauvage (*Olea europea oleaster*).

L'îlot Grand Cavallo est également situé dans la région d'El Aouana. Il est séparé du rivage par une distance de 50 m. Sa superficie est de 0,15 ha avec une altitude maximale de 30 m. La nature du substrat est de type magmatique à feldspath blanc et micas noirs (Biotite), d'une texture microgrenue. Le site a une structure rocheuse et dénudée. Le couvert végétal est localisé essentiellement au sommet de l'îlot. On peut trouver *P. lentiscus*, *P. angustifolia* et quelques sujets de Palmier doum (*Chamaerops humilis*).

L'île Petit Cavallo est localisée à 13 Km de Jijel dans la région d'Andreu. Elle est séparée du continent par une distance de 750 m. Elle s'étend sur une superficie de 4 ha. Le relief est assez plat avec une altitude maximale de 10 m. Les roches sédimentaires prédominent, sous forme de Grès moyen à ciment dolomitique (Grès numidien). Dans l'ensemble, le site est recouvert d'un matorral dégradé, composé essentiellement de *Pistacia lentiscus* et de *Phillyrea angustifolia*.

2.2. Méthodologie

L'inventaire de la faune entomologique s'est déroulé durant la période printanière et estivale, entre mars et juillet 2009. Nous avons échantillonné la plupart des sites occupés par l'entomofaune. Pour les insectes évoluant au niveau du sol (terricoles) à l'exemple des Coléoptères, des Dermaptères, des Diptères et des Hyménoptères, une série de 10 pièges trappes (pots barber) ont été déposés par île. Pour ceux qui fréquentent la strate herbacée (Orthoptères, Coléoptères, Lépidoptères, Hémiptères,...), ils ont été récoltés par fauchage à l'aide d'un filet fauchoir. Les insectes qui fréquentent la strate arbustive ont été récoltés par fauchage et à l'aide d'un parapluie japonais. L'échantillonnage est complété par la chasse à vue des insectes. On note aussi l'utilisation des pièges colorés, mais ces derniers ont été rendu inutilisables par les Goéland leucophées (*Larus michahellis*) qui renversaient les pièges à chaque fois. L'identification des spécimens récoltés a été rendu possible grâce à des ouvrages et des clés spécialisés. Des collections comportant des insectes récoltés dans le milieu continental proche ont été aussi consultées.

La fréquence des prélèvements est aléatoire, car les sorties sur site sont assez irrégulières, ils dépendent largement de l'état de la mer.

3. RESULTATS ET DISCUSSION

Les résultats des récoltes d'insectes réalisées sur les milieux insulaires de Jijel, durant le printemps et l'été 2009, ont révélé l'existence de 140 espèces. L'île Petit Cavallo semble être la plus riche avec 133 espèces. Elle est suivie par l'île Grand Cavallo avec 91 espèces (Tableau 1). Le site le plus pauvre en insectes est l'îlot Grand Cavollo avec seulement 25 espèces.

En terme de richesse moyenne, exprimée en nombre moyen d'individus par espèce, c'est l'île Grand Cavallo qui enregistre la valeur la plus élevée avec 5,95 individus par espèce, il est suivi par l'île Petit Cavallo avec 4,86 et enfin l'îlot Grand Cavallo avec 4,24 (Tableau 1). Les différences constatées entre les trois sites sont liées à plusieurs paramètres, on peut citer par ordre d'importance, la nature et la richesse du couvert végétale, la superficie des îlots, la distance par rapport au continent et enfin l'intensité des perturbations exogènes, qu'elles soient d'origines humaine ou relatives à la présence de colonies d'oiseaux marins (Mac Arthur & Wilson, 1967; Cheylan, 1984; Vidal, 1998; Ponel & Andrieu-Ponel, 1998). Pour notre cas, la diversité du couvert végétal semble être déterminante dans la structuration des populations d'insectes. Dans ce cadre, les inventaires floristiques réalisés sur les mêmes sites, montrent l'existence de 77 espèces végétales sur l'île Petit Cavallo, 66 espèces sur l'île Grand Cavallo et 17 espèces végétales sur l'îlot Grand Cavallo (Hanifi-Benhamiche *et al*., 2010). Le nombre d'espèces d'insectes sur les îles de Jijel est fonction de la diversité floristique.

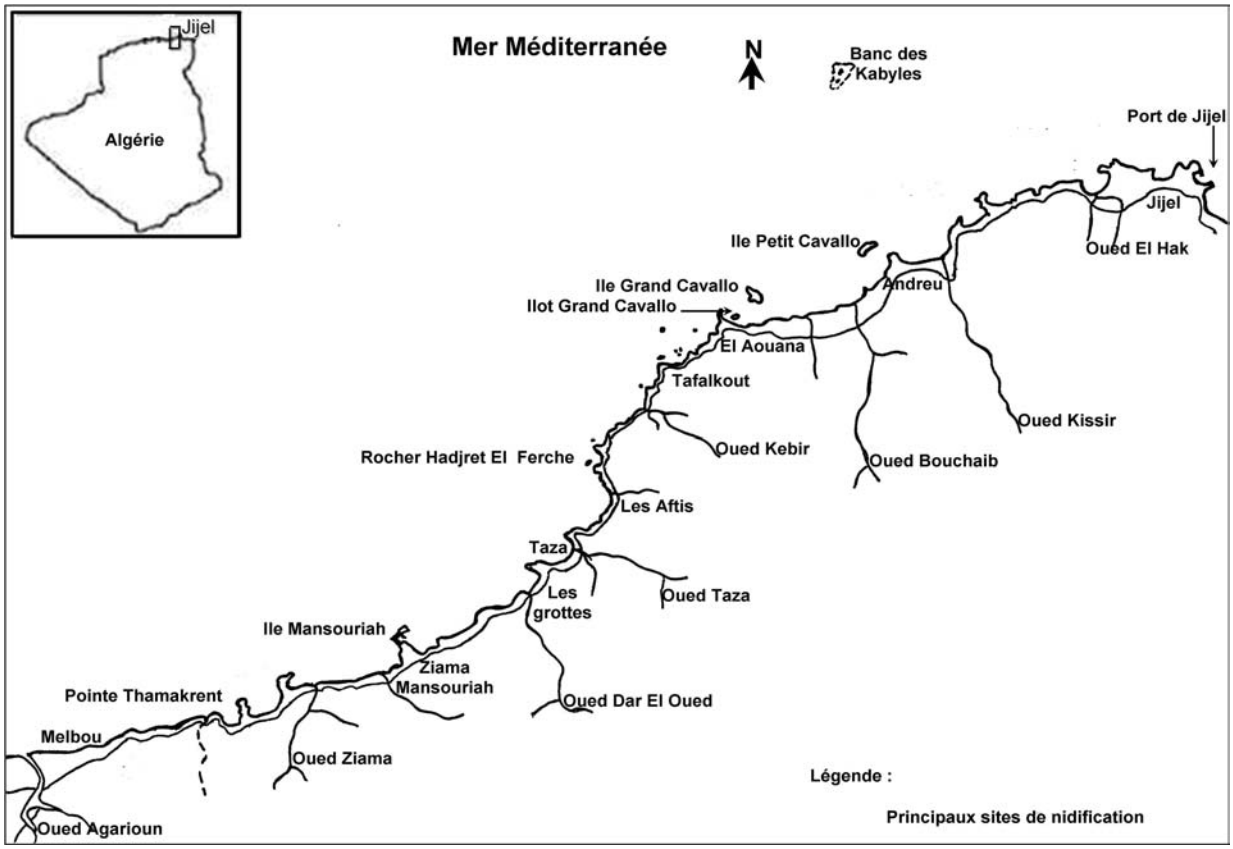


Figure 1: Localisation géographique des principaux milieux insulaires à l'ouest de Jijel (Echelle : 1/120 000)

Huit ordres d'insectes sont observés aussi bien sur l'île Petit Cavallo, que sur l'île Grand Cavallo. Tandis que sur l'îlot Grand Cavallo, on ne retrouve que les Coléoptères, les Hyménoptères et les Diptères (Tableau 1). En terme d'espèces, les Coléoptères sont les plus rependus au niveau des deux plus grandes îles (43 espèces sur l'île Petit Cavallo, 31 espèces sur l'île Grand Cavallo). Sur l'îlot Grand Cavallo, ce sont les Hyménoptères qui sont les plus diversifiés, avec 10 espèces. Il est intéressant de constater que sur l'île Petit Cavallo, les punaises (Hémiptères) et les Hyménoptères sont les plus diversifiés après les Coléoptères, avec 25 espèces pour chacun des ordres. Pour l'île Grand Cavallo, ce sont les Hyménoptères qui viennent en deuxième position avec 20 espèces (Tableau 1). En termes d'abondance les données paraissent un peu différentes. Sur l'île Petit Cavallo, ce sont les Coléoptères qui sont les plus abondants (35,77 %) suivi par les Hémiptères (26,84 %). Sur l'île Grand Cavallo, ce sont les Hémiptères qui sont les plus nombreux (27,54 %). Les Coléoptères suivent, avec 25,27 %. Sur l'îlot Grand Cavallo, les Hyménoptères, notamment les Formicidés, sont les plus abondants (55,32 %) (Tableau 1).

Il est intéressant de noter la présence, non négligeable des Diptères sur les trois sites (11,55 % sur l'île Petit Cavallo, 12,82 % sur l'île Grand Cavallo et 20,37 % sur l'îlot Grand Cavallo) (Tableau 1). Parmi les Diptères ce sont les Calliphoridés qui dominent, ces derniers sont certainement favorisés par les déjections et les cadavres des Goélands leucophées qui nichent sur les îles de Jijel (Bougaham & Moulai, 2008).

L'utilisation du coefficient de similarité de Sorensen (Maguran, 1988) entre les trois stations, montre que ce sont les îles Petit Cavallo et Grand Cavallo qui présentent la faune entomologique la plus proche, avec un degré de similarité de 82 % (Tableau 2). La similarité est beaucoup plus faible entres ces deux derniers sites et l'îlot Grand Cavallo; elle n'excède pas les 40 % pour les deux combinaisons (Tableau 2). La similarité entre les îles de Jijel semble être en relation avec celle de la flore. Dans ce sens Hanifi-Benhamiche *et al*. (2010) notent une similarité végétale de 61 % entre l'île Petit Cavallo et l'île Grand Cavallo, 38 % entre l'île Grand Cavallo et l'îlot Grand Cavallo et 27 % entre l'île Petit Cavallo et l'îlot Grand Cavallo.

Tableau 1: Nombre d'espèces (Nb Esp.), nombre d'individus (Nb Indi.) et fréquences centésimales (F %) par ordre d'insectes identifiés sur trois milieux insulaires à Jijel

Milieux insulaires	Île Petit Cavallo			Île Grand Cavallo			Îlot Grand Cavallo		
Ordres	Nb Esp.	Nb Indi.	F %	Nb Esp.	Nb Indi.	F %	Nb Esp.	Nb Indi.	F %
Orthoptera	5	21	3,75	1	66	12,13	-	-	-
Dermaptera	2	6	0,91	2	6	1,1	-	-	-
Hemiptera	25	174	26,84	11	148	27,54	-	-	-
Coleoptera	43	232	35,77	32	138	25,27	9	25	24,25
Neuroptera	1	2	0,3	1	2	0,36	-	-	-
Hymenoptera	25	124	18,91	20	93	17,04	10	57	55,32
Lepidoptera	8	14	2,87	7	21	3,83	-	-	-
Diptera	24	74	11,55	17	68	12,82	6	24	20,37
Total	133	647	100	91	542	100	25	106	100
Richesse moyenne		4,86			5,95			4,24	

Tableau 2: Coefficient de similarité de Sorensen, appliqué aux espèces d'insectes de trois milieux insulaires de Jijel

	Ile Petit Cavallo	Ile Grand Cavallo	Ilot Grand Cavallo
Ile Petit Cavallo	100	82	39
Ile Grand Cavallo	-	100	39
Ilot Grand Cavallo	-	-	100

Bibliographie

Bernard F. (1958). Les fourmis des îles Pélagie. Comparaison avec d'autres faunes insulaires. *Esttrato dalla Rivista di Biologia Coloniale **XVI***, p. 67-79

Bougaham A. & Moulai R. (2008). Effectifs et dynamique démographique du Goéland leucophée, *Larus michahellis* dans la région de Jijel (Algérie). *1er séminaire national sur les milieux naturels, biodiversité et éco- développement*, Jijel, le 25 et 26 novembre 2008.

Bougaham A. (2008). *Contribution à l'étude de la biologie et de l'écologie des oiseaux de la côte à l'ouest de Jijel*. Mémoire de Magister en Biologie de la Conservation et Ecodéveloppement, Univ. Béjaia, 103 p.

Boukhalfa D. (1990). Observations de quelques espèces d'oiseaux de mer nicheurs sur la côte d'Oran (Algérie). *L'oiseau et la Revue Française d'Ornithologie* **60**(3), p. 248-251.

Cheylan G. (1984). Les mammifères des îles provençales. *Travaux Scientifiques du parc national de port- Cros* **10,** p. 13-25.

Dajoz R. (1987). Les peuplements de Coléoptères Ténébrionidés des îles grecques. *Bulletin de la Société Zoologique de France* **112**(1-2), p. 212-231.

Hanifi-Benhamiche S., Bouyahmed H., Bougaham A.F. & Moulai R. (2010). Qualitative study of the vascular flora of three insular environments concerning the west coast of Jijel (Island Grand Cavallo, Island Petit Cavallo and Islet Grand Cavallo) (Algeria). *International conference on the conservation and sustainable use of wild plant diversity,* 4-8 May 2010, Kolympari, Chania, Crete (Greece). Orthodox academy of Crete (OAC), Institute of theology and ecology (ITHE).

Jacob J.P. & Courbet B. (1980). Oiseaux de mer nicheurs sur la côte en Algérie. *Le Gerfaut* **70,** p. 385-401.

Mac Arthur R.H. & Wilson E.O. (1967). *The theory of island biogeography*. Princeton University Press, Princeton, 244 p.

Maguran A.E. (1988) *Ecological diversity and its measurement.* University Press, Cambridge, 177 p.

Medail F. & Vidal E. (1998). Rôle des goélands leucophées dans l'implantation et l'expansion d'espèces végétales allochtones sur l'archipel de Riou (Marseille, France). *Biocosme Mésogéen* **15**(1), p. 123-140.

Moulai R. (2005). Contribution à l'évaluation de la diversité biologique des îlots de la côte occidentale de Béjaia (Algérie). *Actes du 1er Séminaire International sur l'environnement et ses problèmes connexes,* Bejaia du 05 au 07 Juin 2005.

- (2006). *Bioécologie de l'avifaune terrestre et marine du Parc National de Gouraya (Béjaia), cas particulier du Goéland leucophée,* Laru michahellis *Naumann*, *1840*. Thèse Doctorat d'état, Sciences agronomiques, Institut national d'Agronomie, El Harrach, 185 p.

Ponel P. & Andrieu-Ponel V. (1998). Eléments pour un inventaire des arthropodes des îles satellites du Parc national de Port-Cros: Bagaud, Gabinière, Rascas *Travaux Scientifiques du parc national de Port-Cros* **17,** p. 81-90

Vidal E. (1998). *Organisation des phytocénoses en milieu insulaire méditerranéen perturbé. Analyse des inters relations entre les colonies de Goélands leucophées et la végétation des îles de Marseille*. Thèse doctorat, Biologie des populations et écosystèmes – Aix-Marseille III, 243 p.

(15 réf.)

PLACE DES DECHETS MENAGERS DANS L'ALIMENTATION DU GOELAND LEUCOPHEE, *LARUS MICHAHELLIS* DE LA REGION DE JIJEL (ALGERIE)

Riadh MOULAÏ et Abdellazize Franck BOUGAHAM

Université de Béjaia - Faculté des sciences de la nature et de la vie, Université de Béjaia-06000 Béjaia, Algérie
www.univ-bejaia.dz

SUMMARY

Place household waste in the diet of Yellow-legged Gull, Larus michahellis of Jijel (Algeria)

The diet of adult Yellow-legged Gull of different breeding sites in the region of Jijel (Algeria) is dominated by waste coming from the discharges (82, 45 % in average). Other foraging habitat (terrestrial and marine) appear to be less crowded.

Keywords: Yellow-legged gulls, diet, household waste, Jijel

1. Introduction

Dans le bassin Méditerranéen, le Goéland leucophée connaît une forte expansion démographique depuis une quarantaine d'années (Yessou & Beaubrun, 1995 ; Thibault *et al.*, 1996 ; Sadoul, 1998).Cette espèce, très plastique du point de vue de son habitat de reproduction se rencontre aussi bien en milieu lagunaire qu'en bordure des fleuves, sur des îlots rocheux, voire en milieu urbain littoral (Goutner, 1992). Du fait de sa grande taille, de son comportement colonial et territorial, de son agressivité, de son opportunisme, et de son abondance, le Goéland leucophée est maintenant accusé de provoquer de multiples problèmes environnementaux, et de nombreuses voix s'élèvent pour réclamer la régulation et le contrôle de ses populations (Vidal *et al.*, 1998). De fait, cette espèce est maintenant généralement considérée comme surabondante du fait de ses impacts sur la biodiversité animale et végétale et de ses interférences nombreuses avec différents intérêts humains (Salathe, 1983 ; Vincent, 1987 ; Beaubrun, 1988 ; Oro & Martinez-Vilalta, 1994 ; Walmsley, 1995 ; Bosch, 1996 ; Cadiou, 1997 ; Clergeau, 1997 ; Vidal *et al.*, 1997 ; Vidal *et al.*, 1998 ; Bonnet *et al.*, 1999). Cette surabondance est généralement attribué à la conjonction de deux facteurs : (1) la mise à disposition par l'homme de ressources alimentaires abondantes, facile d'accès et régulièrement renouvelées (essentiellement les ordures ménagères déposées dans des sites à ciel ouvert, mais également les rebuts de la pêche industrielle, jetés à la mer) et (2) la protection légale de l'espèce et des sites favorables à la nidification.

Email : moulai741@hotmail.com

L'écologie alimentaire du Goéland leucophée est relativement bien connue pour les populations de la rive nord occidentale de la Méditerranée (Bosch *et al.*, 1994 et 2000 ; Gonzalez-Solis *et al.*, 1997; Gonzalez-Solis, 2003 ; Duhem, 2004). En Afrique du Nord, les travaux relatifs à l'alimentation de l'espèce sont moins nombreux. On peut citer les études de Beaubrun (1988) et de Gonzalez-Solis (2003) pour le Maroc ou encore la contribution de Moulaï *et al.* (2005 et 2008) sur le régime alimentaire des goélands dans la région de Béjaia en Algérie.
Notre étude qui a pour cadre la région de Jijel (Nord-est de l'Algérie), vise à quantifier la part des déchets ménagers dans le régime alimentaire des Goélands leucophées de la région et ainsi essayer de déterminer les causes de l'essor démographique de cette espèce au niveau de la côte Jijelienne.

2. Présentation de la région d'étude

L'étude du régime alimentaire des Goélands leucophées de la région de Jijel à lieu au niveau de quatre sites de nidifications, localisées à l'Ouest de la ville de Jijel (au Nord-Est de la capitale Alger) (Fig.1). Il s'agit d'Est en Ouest, des colonies de l'île Petit Cavallo, de l'île Grand Cavallo, de l'îlot Grand Cavallo et des falaises de la Pointe Thamakrent. (Fig.1).

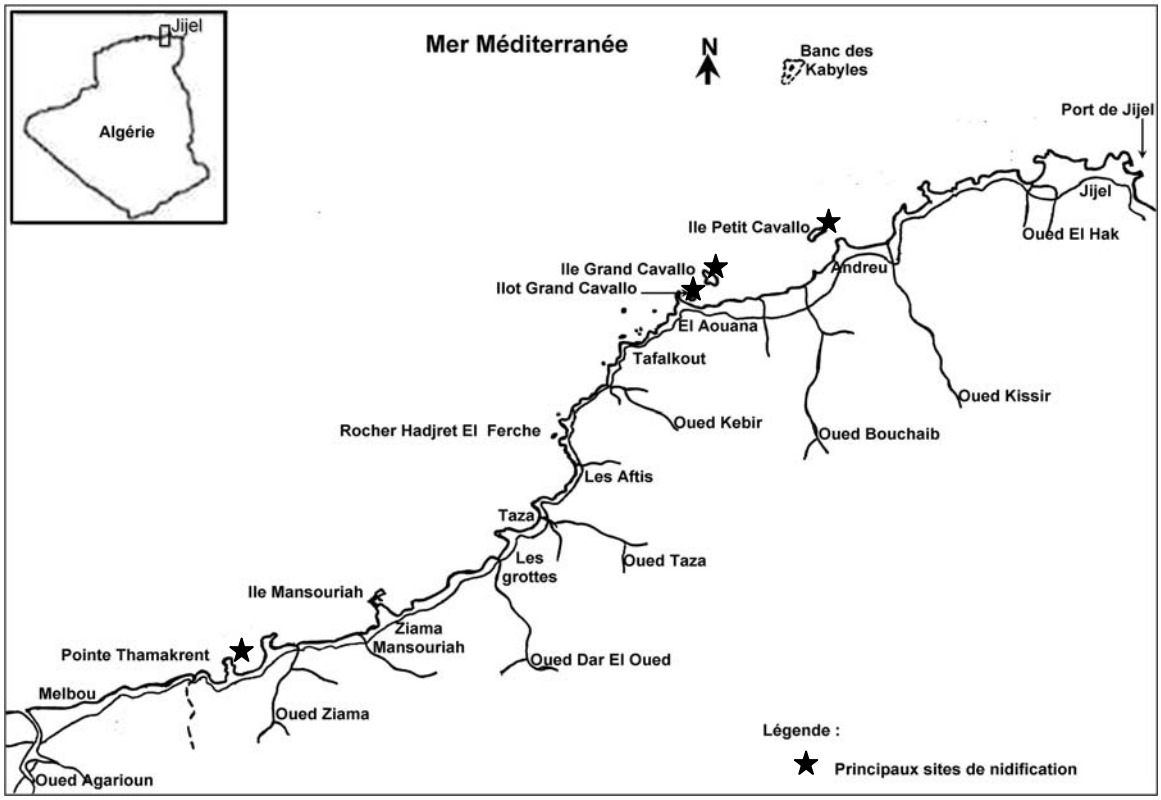


Fig. 1 : Localisation géographique des principaux sites de nidification de *Larus michahellis* dans la région de Jijel (Echelle : 1/ 120 000)

L'île Petit Cavallo est localisée à 13 Km de Jijel dans la région d'Andreu. Elle est séparée de l'abord continental par une distance de 750 m. Elle s'étend sur une superficie de 4 ha. Le relief est assez plat avec une altitude maximale de 10 m. Les roches sédimentaires prédominent, sous forme de Grès moyen à ciment dolomitique (Grès numidien) (Hassissene comm. perso.). Dans l'ensemble, le site est recouvert d'un mattoral plus au moins haut, composé essentiellement de *Pistacia lentiscus* et *Phillyrea angustifolia*. En outre, il abrite une importante flore, présentée principalement par

Dactylis glomerata, *Daucus sp* et *Lavatera cretica*. En effet, l'inventaire des végétaux vasculaires réalisé au niveau de ce site, nous montre l'existence de 77 espèces. L'île Grand Cavallo est située à 20 Km de Jijel dans la localité d'El Aouana. Elle est séparée du continent de 950 m, sa superficie est de 6 ha. Son relief est assez accidenté. Sa face Ouest comprend une falaise avec une altitude maximale de 50 m. Le substrat, est de type magmatique, composé de Feldspath blanc de grande taille et de Mica blanc, à texture grenue (Hassissene comm. perso.). Généralement, le couvert végétal est de type mattoral haut, il peut atteindre 4 mètres de hauteur. Il est formé principalement de *Pistacia lentiscus* et de *Phillyrea angustifolia*, et en partie, d'une flore nitratophile, à l'exemple de, *Lavatera cretica, Urtica membranacea, Chenopodium murale.* L'îlot Grand Cavallo est aussi situé dans la région d'El Aouana. Il est séparé du rivage par une distance de 50 m. Il possède une superficie de 0,15 ha avec une altitude maximale de 30 m. La nature du substrat est de type magmatique à Feldspath blanc et Micas noirs (Biotite), d'une texture microgrenue (Hassissene comm. perso.). Le site a en grande partie, une structure rocheuse et dénudée. Le couvert végétal, est sous forme de touffes localisées au sommet de l'îlot. On trouve *Pistacia lentiscus*, *Phillyrea angustifolia* et quelques sujets de *Chamaerops humilis*. On note aussi, quelques plantes herbacées. Les falaises de la Pointe Thamakrent, sont localisées à la limite côtière entre Jijel et Bejaia (Tiksert) et à 10 Km environs de l'embouchure de l'oued Agarioun (Souk-El-Tenine). Elles font partie d'adrar Djemaa N'sia. La colonie de Goéland leucophée est installée sur une Roche mère de nature calcaire, gris massif du passé Jurassique (Dogger) (Hassissene comm. perso.). La falaise est haute d'environ 50 m. Du point de vue végétal, le site est caractérisée par une végétation herbacée dominante, nous citons l'exemple, d'*Inula crithmoïdes* et de *Capparis spinosa*.

3. Méthodologie

Le régime alimentaire de *Larus michahellis* de la région est déterminé, grâce à l'analyse des pelotes de régurgitations des adultes. La collecte des pelotes fraîches s'est déroulée du 24 avril au 1er juin 2007. Un échantillon de 26, 33, 19 et 19 pelotes a été récolté respectivement sur l'île Grand Cavallo, l'île Petit Cavallo, l'îlot Grand Cavallo et sur les falaises de la Pointe Thamakrent. Les pelotes de rejection sont récupérées dans des cornets en papier portant le numéro, la date et le lieu de récolte, ensuite transportées et conservées dans un endroit sec au laboratoire. Une fois au laboratoire, la pelote est placée dans une boite de pétri. Elle est diluée dans de l'eau, afin de faciliter sa manipulation. Ensuite la pelote est décortiquée, à l'aide de deux paires de pince entomologiques. Sous la loupe binoculaire, les fragments qui constituent la pelote, sont séparés et triés. Les fragments détachés, sont récupérés et arrangés dans une autre boite de pétri tapissée de papier quadrillé portant le numéro, la date et le lieu de la collecte. Enfin, nous avons procédé à la détermination et à la quantification des espèces proies. L'identification des proies est réalisée grâce à des collections de références et des ouvrages spécialisés. L'identification des contenus des pelotes de régurgitations, nous a permis de déterminer trois catégories de restes alimentaires, à savoir la catégorie d'éléments d'origine animale qui renferme les vertébrés terrestres et marins ainsi que les invertébrés terrestres et marins, la catégorie d'éléments d'origine végétale qui comprend les graines, les épicarpes de fruits et divers fragments végétaux et la catégorie qui comprend les déchets organiques et inorganiques comme le verre, le plastique, les coquilles d'œufs, le liège… etc. Ces différentes catégories sont ensuite regroupées selon les habitats de leurs provenances, les habitats terrestres naturels, les habitats marins et les décharges. Bien évidemment, les coquilles d'œufs, les os de boucherie et les éléments inorganiques se rapportent aux décharges publiques (Duhem *et al.*, 2003 ; Moulai, 2006). Les habitats agricoles ou naturels, seront par la suite appelés ‹‹ habitats terrestres ››, qui regroupe les fragments d'insectes (mandibules, élytres et cerques), petits vertébrés et escargots. L'habitat marin, correspond entre autres aux écailles de poissons, aux os ou otolithes, aux invertébrés marins et aux carapaces de crustacés (Duhem *et al.*, 2003).

Email : moulai741@hotmail.com

Pour comparer la composition du régime alimentaire entre les différents sites de reproduction, nous avons utilisé le coefficient de similarité de Soerensen (C_s) (Maguran, 1988) :

$$C_s = 2\ J \times 100 / a + b$$

Avec a = nombre d'espèces présentes dans le site a ; b = nombre d'espèces présentes dans le site b et J = nombre d'espèces communes aux sites a et b. Cet indice varie de 0 à 100. Il est égal à 0 lorsque les aliments consommés sur les deux sites sont totalement différents et égal à 100 dans le cas contraire.

4. Résultats

L'analyse d'un total de 97 pelotes de régurgitation, dans la région de Jijel a révélé l'existence de 108 types d'aliments (Annexe 1). La richesse totale la plus élevée est enregistrée sur l'île Grand Cavallo, avec 62 types d'aliments. La richesse la plus faible est notée quant à elle, sur les falaises de la Pointe Thamakrent avec 42 types d'aliments (Tab. 1). Pour la richesse moyenne par pelote, la valeur la plus élevée est observée sur l'îlot Grand Cavallo, avec un chiffre de 9,3, suivie de l'île Grand Cavallo avec 9,2. La aussi la richesse moyenne par pelote est moins élevée sur les falaises de la Pointe Thamakrent, avec 7,4 types d'aliments par pelote.

Tableau 1 : Fréquences des catégories alimentaires identifiés dans les pelotes de régurgitations de *Larus michahellis* à Jijel, exprimées en fréquence centésimale et en fréquenced'occurrence

Colonies / Catégories alimentaires	Ile Grand Cavallo		Ile Petit Cavallo		Ilot Grand Cavallo		Pointe Thamakrent	
	Fc %	***Fo %***	***Fc %***	***Fo %***	***Fc %***	***Fo %***	***Fc %***	***Fo %***
Invertébrés terrestres	9,31	46,15	10,84	42,42	19,56	36,84	0,45	5,26
Vertébrés terrestres	4,65	50	3,01	30,30	1,44	31,57	1,35	15,78
Vertébrés marins (*Pisces*)	5,37	61,53	4,51	42,42	3,14	68,42	5,40	52,63
Invertébrés marins	0,35	3,84	00	00	00	00	00	00
Végétaux naturels	00	00	00	00	0,24	5,26	0,45	5,26
Déchets carnés	2,50	26,92	2,10	21,21	0,72	15,78	2,70	31,57
Déchets végétaux	23,29	92,30	28,31	84,84	15,45	84,21	21,17	68,42
Autres déchets	54,48	100	51,20	93,93	59,42	100	68,46	100
Richesse totale (S)	62		60		51		42	
Richesse moyenne (S')	9,19		8,60		9,26		7,36	

• *Fc %* : Fréquence centésimale
• *Fo %* : Fréquence d'occurrence (Fréquence d'apparition dans les pelotes)
• Richesse totale (S) : Nombre total de types d'aliments
• Richesse moyenne (S') : Nombre moyen de types d'aliments par pelotes

Les différents types d'aliments identifiés à Jijel, sont répartis en 8 catégories alimentaires, il s'agit des invertébrés terrestres et marins, des vertébrés terrestres et marins, des végétaux naturels et des déchets (carnés, végétaux et autres déchets) (Annexe 1 ; Tab. 1). Il ressort du Tab. 1 que la catégorie "autres déchets" est la mieux représentée dans le régime alimentaire des goélands leucophées adultes de Jijel, elle est présente en moyenne avec 58,3 %, elle est suivie par la catégorie des déchets végétaux, avec 22 % (Tab. 1). Ensuite ce sont les invertébrés terrestres qui arrivent en troisième position, notamment sur l'île et l'îlot Grand Cavallo et sur l'île Petit Cavallo. En effet, au

Email : moulai741@hotmail.com

niveau de l'îlot Grand Cavallo, la part des invertébrés terrestres est de 19,5 %. Sur les falaises de la Pointe Thamakrent, la part des poissons n'est pas négligeable, ils arrivent en troisième position, avec 5,4 %. On note que, la catégorie déchets carnés est faiblement représentée dans le régime alimentaire de *Larus michahellis* de Jijel (Tab. 1).
La composition des pelotes de régurgitation des goélands leucophées de Jijel est dominée par les déchets inorganiques et les déchets végétaux, avec une fréquence d'occurrence qui va de 68,4 % à 100 % selon les colonies (Tab. 1). Les poissons sont eux aussi fortement représentés avec des fréquences qui vont de 42,4 % à 68,4 %. En ce qui concerne les invertébrés terrestres, ils sont assez bien représentés à l'îlot de Grand Cavallo et à l'île Petit Cavallo, avec respectivement 36,8 % et 42,4 %. Á l'île Grand Cavallo, les vertébrés terrestres (essentiellement des poussins de *Larus michahellis*) arrivent juste après les déchets inorganiques et végétaux et les vertébrés marins avec une présence de 50 % dans les pelotes (Tab. 1 et Annexe 1).

En termes d'habitat d'alimentation, les goélands leucophées de la région de Jijel paraissent assez dépendants des décharges d'ordures ménagères de la région, avec 82,5 % en moyenne. À la vue du Tab. 2, la distribution de fréquences des trois types d'habitats d'alimentation est généralement homogène. La proportion des aliments provenant des décharges qui entre dans le régime alimentaire de *Larus michahellis* dépasse les 75 % au niveau des quatre sites de reproduction (Tab. 2). En générale, l'habitat terrestre suit les décharges, avec en moyenne 12,6 %, excepté au niveau de la colonie installée sur les falaises de la Pointe Thamakrent où l'habitat marin vient en deuxième position, avec 5,8 % (Tab. 2).

Tableau 2 : Régime alimentaire des adultes du Goéland leucophée en fonction du type d'habitat d'alimentation (% catégories alimentaires)

	Habitats d'alimentation		
Colonies	**Terrestre**	**Marin**	**Décharges**
Ile Grand Cavallo	13,96	5,72	80,72
Ile Petit Cavallo	13,85	4,51	81,61
Ilot Grand Cavallo	21,00	3,38	75,59
Pointe Thamakrent	1,80	5,85	92,27

En règle générale, le régime alimentaire de Goélands leucophées paraît similaire entre les quatre colonies considérées (Fig. 2).

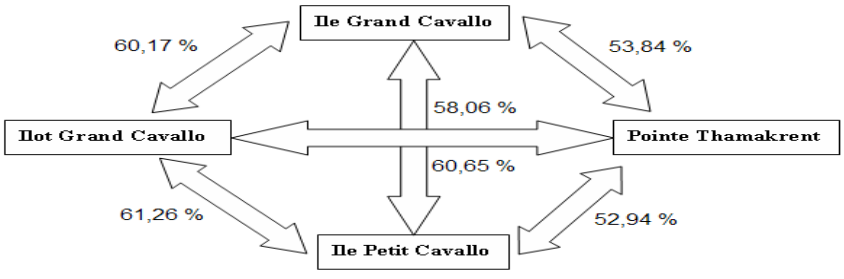


Fig. 1 : Affinité coenotique stationnelle appliquée au régime alimentaire de *Larus michahellis* des colonies de Jijel

Les colonies de l'île Petit Cavallo et celle de l'îlot Grand Cavallo semblent les plus proches, avec une similarité de 61,2 % (Fig. 2). La plus faible similarité est enregistrée entre la colonie de l'île Petit Cavallo et la colonie des falaises de la Pointe Thamakrent, avec 52,9 %. En regroupant les colonies de l'île Grand et Petit Cavallo et de l'îlot de Grand Cavallo, l'application du coefficient de similarité de Soerensen montre des valeurs très proches, elles varient entre 60,1 et 61,2 % et elles diffèrent sensiblement de celle notée sur les falaises de la Pointe Thamakrent (Fig. 2).

5. Discussions

L'analyse des pelotes des différents sites de reproduction, montre que le menu trophique des goélands leucophées de la région est très diversifié (Annexe 1). Les goélands leucophées de Jijel présentent une assez large niche trophique, comprenant à la fois les insectes, les oiseaux, les poissons et les divers déchets. Cette analyse confirme la forte variabilité du régime alimentaire de l'espèce (Isenmann, 1976 ; Beaubrun, 1988 ; Borgo & Spano, 1994 ; Gonzales-Solis *et al*., 1997 ; Duhem *et al*., 2003). Des cas de cannibalisme sont aussi à noter (Annexe 1). La diversité alimentaire des goélands adultes de l'île Grand et Petit Cavallo, indique que ces reproducteurs font plus d'effort pour nourrir leurs poussins, que leurs congénères des autres colonies (Moulai, 2006). De ce fait, les poussins sur l'île Grand et Petit Cavallo restent probablement plus longtemps sans protection de la part d'un adulte ce qui augmente les risques de prédation (Bosch & Sol, 1998). Ces résultats peuvent expliquer les faibles productivités notés sur ces derniers sites. L'alimentation des goélands adultes de l'ensemble des colonies de Jijel repose assez fortement sur les déchets provenant des décharges. Les autres habitats d'alimentation, sont plus ou moins assez bien fréquentés par ces oiseaux. En termes d'occurrence, les différents types de déchets, composent de fait la grande majorité des restes retrouvés dans les pelotes. Ces tendances sont en conformité avec les données de Moulai *et al*. (2005), de Moulai (2007) et Moulai *et al*. (2008), ce qui indique que l'habitat d'alimentation principale de *Larus michahellis* de la côte occidentale de Jijel semble être les décharges d'ordures ménagères. Les goélands leucophées de la région fréquentent en deuxième lieu l'habitat marin. Il est à signaler qu'un moratoire de la pêche au chalut a eu lieu à la fin du mois d'avril. Au-delà, les activités de pêche se limitent au niveau des pêcheurs amateurs de la région dont les rebuts de pêche sont faiblement présents dans le menu des goélands leucophées de la région. Par conséquent, les goélands leucophées seraient attirés par les différents dépotoirs de déchets ménagers de la région pour palier à ce manque. Les décharges d'ordures ménagères offrent en effet une nourriture abondante, facile d'accès et largement disponible en toute saison (Bosch *et al*. 1994 ; Sol *et al*., 1995 ; Duhem, 2004). La décharge municipale de la ville de Jijel, décharge non contrôlée située dans la région du "Mezighitane" qui reçoit quelques 160 tonnes de déchets par jour (A.p.c.J., 2007), semble être la principale source de nourriture pour les goélands des colonies de la côte occidentale de Jijel. Il en est de même pour la colonie de la Pointe Thamakrent ; elle est située à 10 km de la décharge inter-communale de Souk-El-Tenine, ou encore à près de 10 km de la décharge du village Mansouriah (Aït Saad Allah) qui reçoit 2,5 tonnes par jour (A.p.c.Z-M., 2007). Apparemment, les colonies de goélands leucophées de la région se trouvent dans les mêmes situations d'accessibilité aux ressources d'origines anthropiques. Néanmoins, le régime alimentaire des goélands adultes de la Pointe Thamakrent est moins diversifié par rapport aux autres colonies. Á ce titre, Duhem (2004) indique que lorsque l'accessibilité aux décharges est faible, la fraction des autres ressources alimentaires, déterminée par la proportion de pelotes contenant des restes alimentaires provenant de trois habitats d'alimentation, et notamment des restes du milieu marin, et la diversité alimentaire tendent à augmenter. Ainsi, une forte accessibilité aux décharges conduit à un régime alimentaire faiblement diversifié, dominé par les restes provenant des décharges, et une faible accessibilité aux décharges induit une diversification du régime alimentaire. La prédominance

des déchets de décharge dans le régime alimentaire du Goéland leucophée est également observée dans d'autres zones de sa répartition géographique (Bosch *et al*., 1994 ; Sol *et al*., 1995 ; Duhem, 2004 ; Moulai *et al*.,2008) et correspond au patron communément trouvé pour d'autres espèces de grands goélands comme le Goéland argenté, *Larus argentatus* (Hunt, 1972 ; Belant *et al*., 1993 ; Pons, 1994) ou le Goéland à bec cerclé, *Larus delawarensis* (Brousseau *et al*., 1996). La capacité des grands goélands à utiliser efficacement les décharges comme habitat d'alimentation est considérée comme la principale cause responsable de l'expansion de leurs populations (Bosch *et al*., 1994 ; Thibault *et al*., 1996).
Le régime alimentaire global des goélands leucophées de la région de Jijel n'est pas très différent. La grande similitude dans la composition du régime alimentaire des goélands adultes des colonies de l'île Grand Cavallo, de l'île Petit Cavallo et de l'îlot Grand Cavallo, montre en effet que, ces oiseaux ont une accessibilité potentielle aux mêmes ressources alimentaires, notamment aux décharges d'ordures ménagères.

6. Références bibliographiques

[1] A.p.c.J., 2007 - *La collecte des déchets ménagers de la commune de Jijel.* Rapport interne, Service nettoiement, Assemblée populaire communale de Jijel.

[2] A.p.c.Z-M., 2007- *La collecte des déchets ménagers de la commune de Ziama Mansouriah.* Rapport interne, Service nettoiement, Assemblée populaire communale de Ziama-Mansouriah.

[3] Beaubrun P.-C., 1988 - *Le Goéland leucophée (Larus cachinnans michahellis) au, Maroc. Reproduction, alimentation, répartition et déplacements en relation avec les activités de pêche.* Thèse Doctorat d'état, Univ. Montpellier, 448 p.

[4] Belant J.-L., Seamans T.-W., Gabrey S.-W. & Ickes S.-K., 1993- Importance of landfills to nesting Herring Gulls. *The Condor,* 95: 817 – 830.

[5] Bonnet V., Vidal E., Medail F. & Tatoni T., 1999 – Analyse diachronique des changements floristiques sur un archipel Méditerranéen périurbain (îles du Frioul, Marseille). *Rev. Ecol. (Terre et vie),* Vol. 54 (1) : 3 - 18.

[6] Borgo E. & Spano S., 1994 – Primi dati sull Gabbiano reale *Larus cachinnans* all'isola Gallinara (Liguria occidentale). *Boll. Mus. Ist. biol. Univ. Genova,*(58 – 59) : 215 – 226.

[7] Bosch M., 1996. The effects of culling on attacks by Yellow-legged Gulls *Larus cachinnans)* upon three species of Herons. *Colonial Waterbirds* 19 (2): 248 – 252.

[8] Bosch M. & Sol D., 1998 – Habitat selection and breeding success in Yellow-legged Gulls *Larus cachinnans. Ibis,* 140: 415 - 421.

[9] Bosch M., Oro D. & Ruiz X., 1994 – Dependence of Yellow-legged Gulls *(Larus cachinnans)* on food from human activity in two Western Mediterranean colonies. *Avocetta* 18: 135 – 139.

[10] Bosch M., Oro D., Cantos F.-J. & Zabala M., 2000 – Short-term effects of culling on the ecology and population dynamics of the yellow-legged gull. *J. Appl. Ecol.,* 37: 369 - 385.

[11 Brousseau P., Lefebvre J. & Giroux J.-F., 1996 - Diet of Ring-billed Gull chicks in urban and non- urban colonies in Quebec. *Colonial Waterbirds,* 19: 22 – 30.

[12] Cadiou B., 1997 – La reproduction des goélands en milieu urbain: Historique et situation actuelle en France. *Alauda,* 65 (3) : 209 – 227.

[13] Clergeau P., 1997 – *Oiseaux à risques en ville et en campagne. Vers une gestion intégrée des populations.* Ed. Inst. nati. rech. agro. (I.N.R.A.), Versailles, 374 p.

[14] Duhem C., 2004 - *Goélands surabondants et ressources alimentaires anthropiques : Cas des colonies insulaires de Goélands leucop hé es du littoral provençal.* Thèse Doctorat, Univ. Paul Cézanne (Aix Marseille III), 180 p.

[15] Duhem C., Vidal E., Legrand J. & Tatoni T., 2003 - Opportunistic feeding responses of the Yellow-legged Gull *Larus michahellis* to accessibility of refuse dumps. *Bird Study,* 50: 61 - 67.

[16] Gonzales-Solis J., Ruiz X. & Jover L., 1997 – Influence of food availability on interactions between *Larus cachinnans* and *L. audouinii Cana. J. Zool.*, 75: 719- 724.

[17] Gonzales-Solis J., 2003 – Impact of fisheries on activity, diet and predatory interactions between yellow-legged and Audouin's gulls breeding at the Chafarinas Islands. *Sci. Mar*., 67 (Suppl. 2): 83-88.

[18] Goutner V., 1992 – Habitat use in Yellow-legged Gull *(Larus cachinnans michahelis)* in coastal wetland colonies of North-East Greece. *Avocetta,* 16: 81 - 85.

[19] Hunt G.-L. Jr., 1972 – Influence of food distribution and human disturbance on the reproductive success of Herring Gulls. *Ecology, 53*: 1051 - 1061.

[20] Isenmann P., 1976 – Contribution à l'étude de la biologie du Goéland argenté à pieds Jaunes *(Larus argentatus michahellis)* en Camargue. *Rev. Ecol. (Terre et vie)* 30 (4): 551- 563.

[21] Maguran A.-E., 1988 - Ecological diversity and its measurement. Cambridge University Press, Cambridge, 177 p.

[22] Moulai R., Sadoul N. & Doumandji S., 2005 - Nidification urbaine et à l'intérieur des terres du Goéland leucophée *Larus michahellis* en Algérie. *Alauda* 73 (3) : 195-200.

[23] Moulai R., 2006 - *Bioécologie de l'avifaune terrestre et marine du Parc National de Gouraya (Béjaia), cas particulier du Goéland leucophée, Larus michahellis Naumann*, 1840. Thèse Doctorat d'état, Sci. Agro., Inst. Nat. Agro. El Harrach, 185 p.

[24] Moulai R., 2007 – Fluctuation saisonnière des oiseaux de la décharge publique de la ville de Béjaia (Algérie), cas particulier du goéland leucophée (*Larus michahellis*). *Ostrich*, 78 (2) : 527 – 531.

[25] Moulai R., DOUMANDJI S. & SADOUL N., (2008) - Impact des décharges d'ordures ménagères sur le régime alimentaire du Goéland leucophée *Larus michahellis* dans la région de Béjaia (Algérie). *Rev. Ecol.* (*Terre Vie*). *Vol.* 63: 239-250.

[26] Oro D. & Martinez-Vilalta A., 1994 – Factors affecting kleptoparasitim and predation rates upon colony of Audouins Gull *(Larus audouinii)* by Yellow-legged Gulls *(Larus cachinnans)* in Spain. *Colonial Waterbirds 17* (1): 35 - 41.

[27] Pons J.-M., 1994 – Feeding strategies of male and female Herring Gulls during the breeding season under various feeding conditions. *Ethology Ecology and Evolution,* 6 : 1 - 12.

[28] Sadoul N., 1998 – Expansion des Laridés en Camargue : populations en bonne santé ou dysfonctionnement. *Actes du* 36*éme Colloq. Interrég.Ornith., Neuchâtel,* 1996. *Nos oiseaux* 45, Suppl. 2 : 83 - 86.

[29] Salathé T., 1983 – Prédation du Flamant rose *Phonicopterus ruber roseus* par le Goéland leucophée *Larus cachinnans* en Camargue. *Rev. Ecol. (Terre et vie), Vol.* 37: 43 - 52.

[30] Sol D., Arcos J.-M. & Senar J.-C., 1995 – The influence of refuse tips on the winter distribution of Yellow-legged Gulls *Larus cachinnans. Bird Study, 42*: 216 - 221.

[31] Thibault J.-C., Zotier R., Guyot I. & Bretagnolle V., 1996 – Recent trends in breeding marine birds of the Mediterranean region with special reference to Corsica. *Colonial Waterbirds,* 19: 31-40.

[32] Vidal E., Medail F., Tatoni T. & Bonnet V., 1997 – Impact du Goéland Leucophée *Larus cachinnans michahellis* sur les milieux naturels provençaux. *Faune de Provence (C.E.E.P.),* 18 : 47 - 53.

[33] Vidal E., Médail F. & Tatoni T., 1998 – Is the Yellow-legged Gull a super abundant bird in Mediterranean? Impact on fauna and flora, conservation measures and research priorities. *Biodiversity and Conservation,* 7: 1013 – 1026.

[34] Vincent T., 1987 – La nidification urbaine des Goélands argentés *(Larus argentatus* et *Larus cachinnans) :* une généralisation du phénomène en France ? *L 'oiseau et R.F.O.,* 57 (1) : 47 – 48.

[35] Walmsley J., 1995 – Le Goéland leucophée en tant que ''Super prédateur'' dans le milieu salé (Salin industriels). *Le Guêpier,* 6 : 21 - 26.

[36] Yésou P. & Beaubrun P.-C., 1995 – *Le Goéland leucophée Larus cachinnans.* pp. 328-329 cités par Nouvel atlas des oiseaux nicheurs de France 1985-1989, D.YEATMAN-BERTHELOT et G. JARRY. *Soc. Etud. Ornith. France, Paris.*

Annexe 1 : Nature et fréquence des items alimentaires identifiés dans les pelotes de régurgitations de *Larus michahellis* des colonies de Jijel (ni : nombre d'items, Fc% : fréquence centésimal de l'item)

Catégories	Groupes	Items	Ile Grand Cavallo		Ile Petit Cavallo		Ilot Grand Cavallo		Pointe Thamakrent	
			ni	Fc %	ni	Fc %	ni	Fc %	ni	Fc %
Invertébrés terrestres	Gasteropoda	*Euparipha pisana*	3	1,07	-	-	-	-	-	-
	Aranea	Arachnida sp. Ind.	-	-	1	0,30	-	-	-	-
		Araneide sp. ind.	1	0,35	-	-	-	-	-	-
	Coleoptera	Tenebrionidae sp_1 ind.	3	1,07	1	0,30	1	0,24	-	-
		Tenebrionidae sp_2 ind.	1	0,35	-	-	-	-	-	-
		Pterostichidae *sp*. Ind.	1	0,35	-	-	-	-	-	-
		Ocypus olens	-	-	9	2,71	25	6,03	-	-
		Chrysomelidae *sp*. Ind.	1	0,35	-	-	-	-	-	-
		Apion sp. ind.	1	0,35	-	-	-	-	-	-
		Carabidae *sp*. ind.	-	-	-	-	2	0,48	-	-
		Bubas sp. ind.	-	-	-	-	2	0,48	-	-
		Pachychila sp. ind.	-	-	1	0,30	-	-	-	-
		Oxytherea funesta	-	-	2	0,60	-	-	-	-
		Poecilus sp_1 ind.	-	-	2	0,60	-	-	-	-
		Poecilus sp_2 ind.	-	-	2	0,60	-	-	-	-
		Tropinota sp. ind.	-	-	1	0,30	-	-	-	-
		Lixus sp. ind.	-	-	1	0,30	-	-	-	-
	Hymenoptera	*Pheidole pallidula*	1	0,35	3	0,90	-	-	-	-
		Cataglyphis bicolor	3	1,07	4	1,20	-	-	-	-
		Messor barbara	5	1,79	6	1,81	3	0,72	-	-
		Tetramorium biskrensis	1	0,35	-	-	-	-	-	-
		Monomorium salomonis	3	1,07	-	-	-	-	-	-
		Hymenoptera *sp*. ind.	-	-	1	0,30	-	-	-	-
		Camponotus sp. ind.	-	-	1	0,30	-	-	-	-
	Dermaptera	*Forficula auricularia*	1	0,35	1	0,30	48	11,59	-	-
		Anisolabis mauritanicus	1	0,35	-	-	-	-	-	-
	Heteroptera	Coreidae *sp*. ind.	-	-	-	-	-	-	1	0,45
Invertébrés Marins	Mollusca	Lamellibranchia *sp*. ind.	1	0,35	-	-	-	-	-	-
Vertébrés terrestres	Laridae	*Larus michahellis*	5	1,79	6	1,81	2	0,48	-	-
	Turdidae	*Luscinia megarynchos*	-	-	1	0,30	-	-	-	-
	Sylviidae	Sylviidae *sp*. ind.	-	-	-	-	-	-	1	0,45
	Fringillidae	Fringillidae *sp*. ind.	-	-	-	-	-	-	1	0,45
		Aves sp_1 ind.	1	0,35	1	0,30	1	0,24	-	-
		Aves sp_2 ind.	3	1,07	2	0,60	1	0,24	-	-
		Aves sp_3 ind.	3	1,07	-	-	-	-	1	0,45
		Aves sp_4 ind.	1	0,35	-	-	1	0,24	-	-
	Rodentia	*Rattus sp*. ind.	-	-	-	-	1	0,24	-	-
Végétaux naturels	Algua	*Ulva sp*. ind.	-	-	-	-	1	0,24	1	0,45
Vértébrés marins (Poisson)	Pisces	Pisces sp_1 ind.	5	1,79	5	1,51	2	0,48	2	0,90
		Pisces sp_2 ind.	3	1,07	4	1,20	3	0,72	4	1,80
		Pisces sp_3 ind.	3	1,07	4	1,20	4	0,96	3	1,35
		Clupeidae sp_1 ind.	2	0,71	-	-	1	0,24	-	-
		Clupeidae sp_2 ind.	-	-	2	0,60	-	-	1	0,45
		Triglidae sp_1 ind.	1	0,35	-	-	-	-	-	-
		Triglidae sp_2 ind.	1	0,35	-	-	-	-	-	-
		Boops boops	-	-	-	-	2	0,48	-	-
		Pagellus acarne	-	-	-	-	1	0,24	-	-
		Ophidion barbatum	-	-	-	-	-	-	1	0,45
		Spicara maena	-	-	-	-	-	-	1	0,45
Déchets	Gallinacae	*Gallus domesticus*	-	-	7	2,10	1	0,24	-	-
		Bos taurus	4	1,43	-	-	1	0,24	2	0,90

Email : moulai741@hotmail.com

carnés	Bovidae	Bovidae *sp*$_1$ ind.	1	0,35	-	-	1	0,24	3	1,35
		Bovidae *sp*$_2$ ind.	2	0,71	-	-	-	-	1	0,45
Déchets de Végétaux	Solanaceae	*Capsicum annum*	8	2,86	11	3,32	10	2,41	5	2,25
		Lycopersicum esculentum	3	1,07	16	4,83	1	0,24	5	2,25
		Solanaceae *sp*. ind.	-	-	-	-	1	0,24	-	-
	Liliaceae	*Allium sativum*	1	0,35	5	1,51	5	1,20	1	0,45
		Allium cepa	-	-	-	-	-	-	1	0,45
		Liliaceae *sp*. ind.	-	-	1	0,30	-	-	-	-
	Rutaceae	*Citrus sinensis*	1	0,35	12	3,62	8	1,93	3	1,35
		Citrus lemon	-	-	-	-	-	-	3	1,35
	Oleaceae	*Olea europea*	1	0,35	4	1,20	2	0,48	2	0,90
		Phillyrea angustifolia	-	-	1	0,30	3	0,72	-	-
		Oleaceae *sp*. ind.	-	-	-	-	1	0,24	-	-
	Rosaceae	*Malus pumila*	2	0,71	-	-	-	-	-	-
		Prunus persica	-	-	-	-	-	-	1	0,45
		Crataegus sp. ind.	2	0,71	1	0,30	-	-	-	-
	Poaceae	Poaceae *sp*. ind.	16	5,73	7	2,10	10	2,41	8	3,60
		Zostera *sp*. ind.	-	-	-	-	-	-	-	-
	Palmaceae	*Phoenix dactylifera*	2	0,71	-	-	-	-	-	-
	Compositae	*Cynara scolymus*	-	-	1	0,30	-	-	-	-
	Cucurbitaceae	Cucurbitaceae *sp.* ind.	-	-	-	-	1	0,24	-	-
	Fagaceae	*Quercus suber*	1	0,35	-	-	-	-	-	-
	Crassulaceae	Crassulaceae *sp*. ind.	-	-	-	-	1	0,24	-	-
	Brassicaceae	Brassicaceae *sp*. ind.	1	0,35	-	-	-	-	-	-
	Moraceae	*Ficus sp*. ind.	-	-	1	0,30	-	-	1	0,45
		Dicotyledone sp. ind.	4	1,43	6	1,81	-	-	1	0,45
		Fruit sp$_1$ ind.	8	2,86	10	3,02	9	2,17	6	2,70
		Fruit sp$_2$ ind.	1	0,35	1	0,30	1	0,24	-	-
		Fruit sp$_3$ ind.	8	2,86	9	2,71	7	1,69	6	2,70
		Fruit sp$_4$ ind.	4	1,43	2	0,60	3	0,72	-	-
		Fruit sp$_5$ ind.	1	0,35	-	-	-	-	-	-
		Fruit sp$_6$ ind.	1	0,35	-	-	-	-	-	-
		Fruit sp$_7$ ind.	-	-	-	-	1	0,24	3	1,35
		Fruit sp$_8$ ind.	-	-	3	0,90	-	-	-	-
		Fruit sp$_9$ ind.	-	-	1	0,30	-	-	-	-
		Fruit sp$_{10}$ ind.	-	-	2	0,60	-	-	-	-
		Monocotyledone sp. ind.	-	-	-	-	-	-	1	0,45
Autres déchets		Cailloux	61	21,86	46	13,89	185	44,68	90	40,54
		Bois	3	1,07	11	3,32	3	0,72	6	2,70
		Poils humains	13	4,65	12	3,62	9	2,17	5	2,25
		Fibre synthétique	7	2,50	5	1,51	5	1,20	2	0,90
		Verre	4	1,43	1	0,30	2	0,48	4	1,80
		Plastique	14	5,01	19	5,74	8	1,93	10	4,50
		Papier aluminium	2	0,71	2	0,60	2	0,48	1	0,45
		Liège	10	3,58	17	5,13	6	1,44	2	0,90
		Papier	13	4,65	19	5,74	10	2,41	10	4,50
		Goudron	11	3,94	10	3,02	3	0,72	6	2,70
		Frag. de peinture	1	0,35	-	-	-	-	-	-
		Coton	2	0,71	2	0,60	-	-	-	-
		Frag. de coquilles d'oeufs	9	3,22	16	4,83	10	2,41	14	6,30
		Mousse planche	1	0,35	4	1,20	-	-	1	0,45
		Laine	1	0,35	1	0,30	-	-	-	-
		Fer	-	-	3	0,90	1	0,24	1	0,45
		Caoutchouc	-	-	-	-	1	0,24	-	-
		Tissu	-	-	1	0,30	-	-	-	-
		Zinc	-	-	1	0,30	-	-	-	-
		Poils de *Rattus sp*	-	-	-	-	1	0,24	-	-
	Totaux	108	279	99,95	332	99,97	414	99,83	222	99,94

Food Chemistry 121 (2010) 561–568

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

HPLC sugar profiles of Algerian honeys

Salim Ouchemoukh [a,*], Paul Schweitzer [b], Mostapha Bachir Bey [a], Hafsa Djoudad-Kadji [a], Hayette Louaileche [c,*]

[a] Département de Biologie physico-chimique, Faculté des Sciences de la Nature et de la Vie, Université de Béjaia, Route de Targa-Ouzemour, 06000 Béjaia, Algeria
[b] CETAM lorraine, Laboratoire d'analyses et d'Ecologie Apicole, 1B, Rue Jeanne d'ARC, 57310 Guénange, France
[c] Département des Sciences Alimentaires, Faculté des Sciences de la Nature et de la Vie, Université de Béjaia, Route de Targa-Ouzemour, 06000 Béjaia, Algeria

ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form 15 April 2009
Accepted 13 December 2009

Keywords:
Honey types
Sugar profiles
Oligosaccharides
HPAEC-PAD
PCA

ABSTRACT

Sugar profiles of fifty honey samples from different regions of Algeria are analysed by HPLC with pulsed amperometric detection. These samples consisted of 25 multifloral and 25 unifloral honeys. Eleven sugars (two monosaccharides, nine oligosaccharides) are quantified. The mean values of fructose and glucose are in the range 35.99–42.57% and 24.63–35.06%, respectively. These monosaccharides are the main sugars of all honey samples. The sucrose, maltose, isomaltose, turanose and erlose are present nearly in all the samples, while raffinose and melezitose are detected in few samples. Furthermore, trehalose is present only in two samples and none of the samples contain melibiose. Low amounts of melezitose, raffinose and erlose are present in the range of 0.03–2.14%, 0.03–0.35% and 0.01–2.35%, respectively. PCA (Principal Component Analysis) showed that the cumulative variance was approximately 40% and Apiaceae honeys are correctly classified using FDA (Factorial Discriminant Analysis).

1. Introduction

Honey is produced by honeybees from nectar of different plants, as well as from honeydew. It is one of the most complex foods produced by nature, as the only sweetening agent that can be used by the humans without processing. Honey may be essentially described as a highly concentrated water solution of two sugars, fructose and glucose, with small amounts of various more complex sugars (Molan, 1996). In almost all honey types, fructose predominates, glucose being the second main sugar (Cavia et al., 2002). Many other substances such as acids, proteins, minerals, pigments, flavour and aroma substances, vitamins also occur in honey. These constituents are due to the maturation of the honey, some are added by the bees and some others are derived from the plant (Anklam, 1998).

Honey sugars are formed by the action of several enzymes on nectar sucrose. The result is a complex mixture made up of about 70% monosaccharides and 10–15% disaccharides composed of glucose and fructose with the glycosidic bond in different positions and configurations (De la Fuente, Sanz, Martinez-Castro, & Sanz, 2006). Sugars in honey are responsible for properties such as viscosity, hygroscopy, granulation and energy value. Honey is used as an ingredient in hundreds of manufactured foods (Cavia et al., 2002). Honey oligosaccharides present potential prebiotic activity (prebiotic index values between 3.38 and 4.24), increasing the populations of *Bifidobacteria* and *Lactobacilli* (Sanz, Polemis, et al., 2005). Sugars profiles of different types of honey have been reported by many scientists (Esti, Panfili, Marconi, & Carmela Trivisonno, 1997; Goodall, Dennis, Parker, & Sharman, 1995; Sanz, Gonzalez, Lorenzo, Sanz, & Martinez-Castro, 2005). Different chromatographic techniques such as HPLC (Cotte, Casabianca, Chardon, Lheritier, & Grenier-Loustalot, 2003; Devillers, Morlot, Pham-Delègue, & Doré, 2004) or GC–MS (Lazaridou, Biliaderis, Bacandritsos, & Sabatini, 2004; Sanz, Sanz, & Martinez-Castro, 2002, 2004) have been used for sugars analysis. These methods are validated by the International Honey Commission (Bogdanov, Ruoff, & Persano Oddo, 2004). HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection) is one of the most useful techniques for oligosaccharide determination (Morales, Corzo, & Sanz, 2008). Molan (1996) reported that 27 oligosaccharides can be found in honey. Using capillary gas chromatography, Low and Sporns (1988) found 16 sugars in honey, including eleven disaccharides (maltose, turanose, kojibiose, sucrose, palatinose, laminaribiose, gentiobiose, cellobiose, isomaltose, neotrehalose, nigerose) and 5 trisaccharides (erlose, isopanose, panose, theanderose, maltotriose). Sanz, Polemis, et al. (2005) reported also the presence of four tetrasaccharides, one pentasaccharide and one hexasaccharide in New Zealand honeydew honey.

Disaccharides content has been used to characterise honey type and origin. For examples, Mateo and Bosch-Reig (1997), Mateo and Bosch-Reig (1998) used the sugar profiles for classifying Spanish

* Corresponding authors. Tel./fax: +213 34 21 47 62 (S. Ouchemoukh).
E-mail addresses: ouchemoukhsalim@yahoo.fr (S. Ouchemoukh), haylouaileche@yahoo.fr (H. Louaileche).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.12.047

honeys; maltose, nigerose and turanose proved to be useful for the differentiation of Brazilian honeys from several geographical regions (Da Costa Leite et al., 2000). Sugar profiles have been used to differentiate floral honeys from honeydew honeys which present lower values of glucose and fructose and higher levels of oligosaccharides mainly melezitose or erlose (Weston & Brocklebank, 1999). Moreover, the relative amount of fructose and glucose is useful for the classification of unifloral honeys (Bogdanov et al., 2004).

There is a vast number of floral species characteristic of Algeria visited by the honeybees. However, reports of the chemical composition of Algerian honeys are scarces (Makhloufi et al., 2007; Ouchemoukh, Louaileche, & Schweitzer, 2007). The aims of this research can be summarised under two headings: to study the sugar profiles of Algerian honeys using HPAEC-PAD and to apply different statistical methods to characterise Algerian honey types.

2. Materials and methods

2.1. Honey samples

Fifty honey samples are collected from different regions of Algeria between the years 2004–2006 (Table 1). The most of the samples are harvested directly from beekeepers and the extracted honeys are attained by centrifugation. The samples are stored in a refrigerator in airtight plastic containers until analysis. The botanical origin of the honeys is studied according to Louveaux, Maurizio, and Vorwohl (1978). Pollen analysis showed 25 multifloral and 25 unifloral honeys.

2.2. Standard sugars

Eleven pure sugars (fructose, glucose, disaccharides, trisaccharides) are from ICN biomedicals (USA) and itemised as follows.

Table 1
Botanical and geographical origins of honey samples.

Sample	Botanical origin	Geographic origin (year of collect)
H01–H07	Multifloral	Bejaia (2006)
H08–H09		Tizi-Ouzou (2006)
H10		Blida (2006)
H11		Algiers (2006)
H12–H13		Blida (2006)
H14		Bejaia (2005)
H15		Blida (2005)
H16		Blida (2006)
H17		Mila (2006)
H18		Setif (2006)
H19		Bejaia (2006)
H20–H25		Bejaia (2004)
H26–H29	*Eucalyptus*	Bejaia (2006)
H30		Tizi-ouzou (2006)
H31		Boumerdes (2006)
H32		Blida (2006)
H33		Blida (2005)
H34		Tlemcen (2006)
H35		Bejaia (2006)
H36 – H39	*Erica arborea*	
H40 – H42	*Apiaceae*	Blida (2006)
		Bouira (2006)
		Setif (2006)
H43	*Capparis*	Bejaia (2006)
H44	*Citrus*	Blida (2006)
H45	*Myrtaceae*	Blida (2006)
H46	*Hedysarum*	Jijel (2006)
H47	*Lotus*	Setif (2006)
H48	*Trifolium*	
H49	*Annarrhinum*	Batna (2006)
H50	*Melilotus*	Bejaia (2004)

2.2.1. Disaccharides

Sucrose (α-D-glucopyranosyl β-D-fructofuranoside), trehalose (α-D-glucopyranosyl α-D-glucopyranoside), maltose (α-D-glucopyranosyl (1–4) D-glucopyranose), turanose (α-D-glucopyranosyl (1–3) D-fructose), isomaltose (α-D-glucopyranosyl (1–6) D-glucopyranose) and melibiose (α-D-galactopyranosyl (1–6) D-glucopyranose).

2.2.2. Trisaccharides

Erlose (α-D-glucopyranosyl (1–4) α-D-glucopyranosyl β-D-fructofuranoside), melezitose (α-D-glucopyranosyl (1–3) β-fructofuranosyl (2–1) α-D-glucopyranoside), raffinose (α-D-galactopyranosyl (1–6) α-D glucopyranosyl (1–2) β-D fructofuranoside).

2.3. Analysis by high-performance anion-exchange chromatography (HPAEC)-PAD

HPLC with pulsed amperometric detection was used to identify sugar profiles of honey samples. Sugars behave as very weak acids at high pH (12–14) and are partially or totally ionised. They are separated by an ion-exchange mechanism (Bogdanov, Martin, & Lüllmann, 1997).

2.3.1. Sample preparation

One gram of honey is dissolved in few millilitres of ultra-pure water and transferred to a 100 ml volumetric flask. Ultra-pure water is added to the mark, and the solution is well mixed. One millilitre of this solution is pipetted to 50 ml volumetric flask and ultra-pure water is added to the mark of the flask (Bogdanov et al., 1997).

2.3.2. Sample analysis

The sample analysis is done with harmonised methods of the european honey commission. The chromatography is performed with a Dionex GP 50 (USA) incorporating a Carbopac PA1 (4 × 250 mm) anion-exchange column and a PA1 (4 × 50 mm) guard column. 25 μl of prepared sample is injected into the loop of the chromatograph.

The flow rate is 0.5 ml/mn and sugars are detected by pulsed amperometric detection with a gold working electrode and a Ag/AgCl reference electrode. The separation is realised with a gradient of two mobile phases. Phase A is ultra-pure water and phase B is 0.2 M NaOH (HPLC grade, Fischer Scientific, France). The working electrode is maintained at the followings potentials and durations during the operation: $E_1 = 0.2$ V ($t_1 = 0.50$ s), $E_2 = 0.7$ V ($t_2 = 0.1$ s), $E_3 = -0.9$ V ($t_3 = 0.1$ s) (Bogdanov et al., 1997; Weston & Brocklebank, 1999).

2.4. Statistical analysis

Multivariate statistical treatments (Hierarchical Cluster Analysis (HCA), PCA, FDA) are carried out using statistica®. These statistical methods allow the obtaining of graphic representations which constitute the best possible summary of the information including in a large table of data. Also, they enable us to represent objects or variables on a graph in order to study the proximity of objects and to classify them.

3. Results and discussion

In this study, 10 sugars are identified and quantified, including two monosaccharides, five disaccharides and three trisaccharides (Fig. 1). Table 2 shows the means, standard deviations and ranges of sugars of honey samples. The results obtained confirm that the

monosaccharides glucose and fructose are the main sugars in all samples. Fructose is the major sugar in honey samples followed by glucose, maltose, saccharose, turanose, isomaltose, erlose, melezitose, raffinose and trehalose. There is a great difference between the amount of fructose and trehalose. This latter is only detected in 2 samples (H01, H33). The value of this sugar is very low with respect to other detected sugars.

In our knowledge, sugar composition of *Capparis* honey is not reported by scientists. The present study gives the sugar profile of this honey type (H43) in Table 2.

The sugars composition depends highly on the types of flowers used by the bees, as well as regional and climatic conditions (Mateo & Bosch-Reig, 1998).

The total sugars content of honey samples varies between 73.05 and 81.38%. Two honey types (multifloral, *Apiaceae*) have the same level of total sugars. The result of total sugars content (76%) of Algerian multifloral honeys is in agreement with those reported by Popek (2002).

Fructose is always the most important sugar quantitatively (range 35.99–42.57%), followed by glucose (range 24.63–35.06%) (Table 2). These results are in agreement with those of Pérez-Arquillué, Conchello, Arifio, Juan, and Herrera (1995). Molan (1996) reported that the proportions of fructose and glucose are determined by the composition of the plant secretion. Fig. 2a represents hierarchical cluster analysis of sugars of honey samples obtained by Euclid's distances and shows that monosaccharides form one group, because there is not a great difference between the levels of fructose and glucose, and other sugars constitute another group. The monosaccharide sugar content, with values ranging between 67.35% and 73.43%, are within the limits permitted by the Council of the European Union (2002) (>60%). The ratio of fructose to glucose is between 1.11 and 1.36, indicating the variety of floral

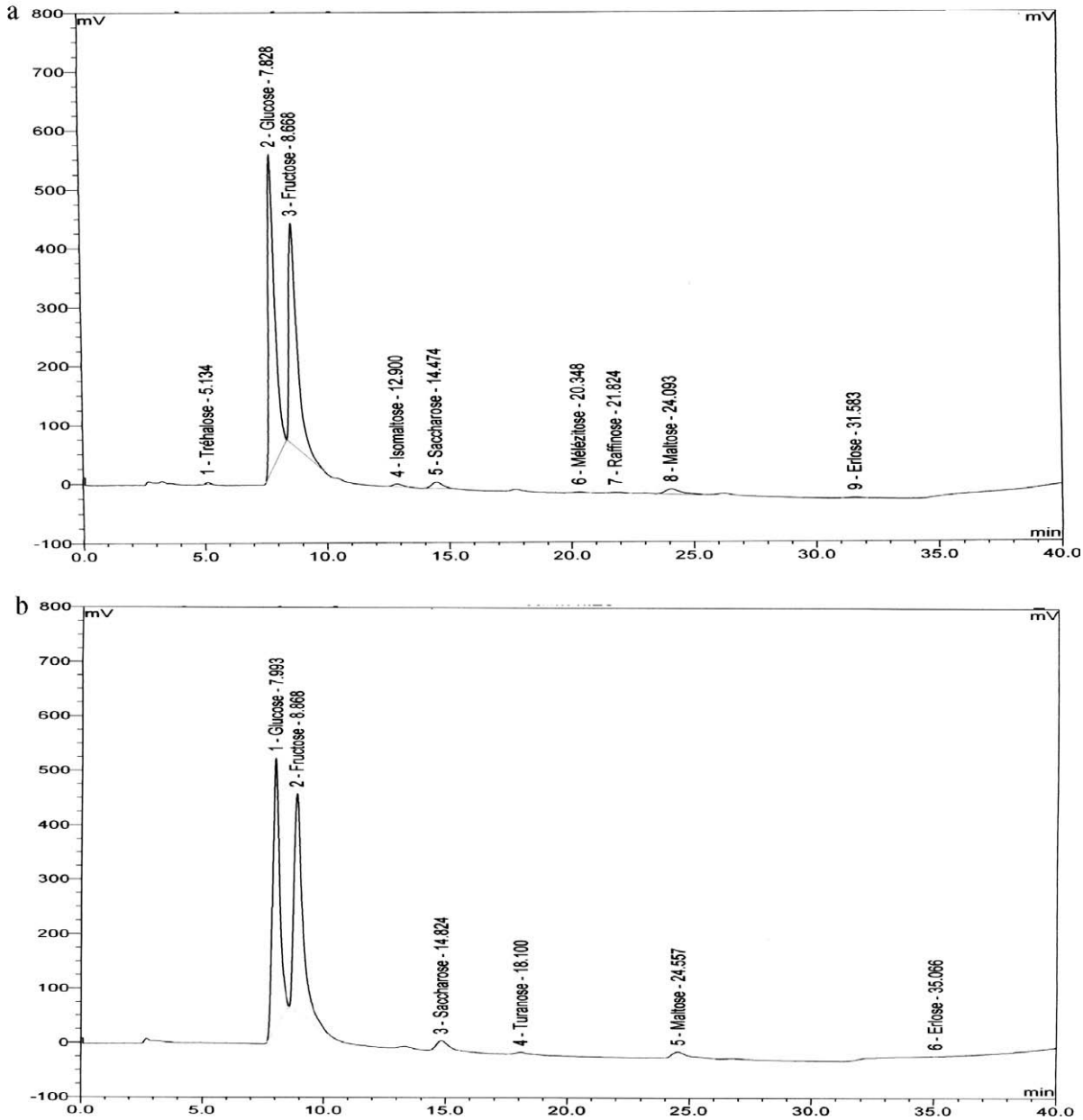


Fig. 1. HPAEC-PAD chromatographic profiles of sugars in 2 honey samples: (a) multifloral, H01; (b) *Citrus*, H44.

Table 2
Sugars values (g/100 g) of honey types.

Sugar (%)	Honey type (number of samples)											
	Multifloral (n = 25)	*Eucalyptus* (n = 10)	*Erica* (n = 4)	*Apiaceae* (n = 3)	*Capparis* (n = 1)	*Citrus* (n = 1)	*Myrtaceae* (n = 1)	*Hedysarum* (n = 1)	*Lotus* (n = 1)	*Anarrhinum* (n = 1)	*Melilotus* (n = 1)	*Trifolium* (n = 1)
Fructose												
Mean ± SD	38.93 ± 1.73	38.68 ± 1.47	38.27 ± 1.15	38.95 ± 1.27	38.37	40.81	40.19	36.70	37.68	39.13	38.45	39.56
Range	35.99–42.57	36.29–40.91	36.92–39.72	37.99–40.39								
Glucose												
Mean ± SD	31.65 ± 1.84	30.82 ± 2.29	33.89 ± 1.23	29.42 ± 5.22	30.81	31.96	33.24	30.65	32.37	32.53	34.67	29.10
Range	26.23–34.38	25.16–33.34	32.66–35.06	24.63–34.99								
M (F + G)[a]	70.59 ± 2.70	69.50 ± 3.32	72.15 0.98	68.37 ± 5.45	69.18	72.77	73.43	67.35	68.66	71.66	73.12	70.05
(F/G) ratio	1.23 ± 0.08	1.26 0.11	1.13 ± 0.07	1.32 ± 0.23	1.25	1.28	1.21	1.20	1.36	1.20	1.11	1.16
Maltose												
Mean ± SD	1.72 ± 0.64	1.67 ± 0.84	1.59 ± 0.54	1.55 ± 0.63	1.67	3.42	3.10	2.17	1.04	0.68	1.65	1.45
Range	0.47–3.30	0–2.70	0.97–2.25	0.91–2.18								
Sucrose												
Mean ± SD	1.35 ± 0.71	2.08 ± 1.06	1.02 ± 0.44	2.15 ± 0.97	1.37	2.87	2.78	5.26	1.05	1.50	0.81	2.10
Range	0–2.63	1.03–4.52	0.48–1.41	1.43–3.25								
Isomaltose												
Mean ± SD	0.79 ± 0.60	0.53 ± 0.70	0.41 ± 0.08	2.49 ± 1.9	0.00	0.00	0.39	0.40	0.61	1.13	0.00	1.61
Range	0.37–2.73	0.36–2.50	0.30–0.50	1.67–4.72								
Turanose												
Mean ± SD	0.96 ± 0.48	0.85 ± 0.49	0.45 ± 0.17	1.41 ± 0.47	1.26	1.15	0.90	0.74	0.00	1.63	1.17	2.02
Range	0–1.96	0–1.83	0.29–0.54	1.03–1.94								
Trehalose												
Mean ± SD	0.01 ± 0.03	0.01 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Range	0–0.14	0–0.14										
Melibiose	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Erlose												
Mean ± SD	0.35 ± 0.50	0.18 ± 0.17	0.08 ± 0.14	0.19 ± 0.26	0.50	0.01	0.63	1.14	0.30	0.03	0.04	0.95
Range	0–2.35	0.01–0.54	0–0.3	0.01–0.48								
Raffinose												
Mean ± SD	0.05 ± 0.09	0.04 ± 0.05	0.01 ± 0.02	0.19 ± 0.16	0.22	0.00	0.16	0.00	0.00	0.00	0.00	0.00
Range	0–0.35	0–0.13	0–0.03	0–0.31								
Melezitose												
Mean ± SD	0.18 ± 0.55	0.06 ± 0.18	0.01 ± 0.02	0.04 ± 0.06	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
Range	0–2.14	0–0.59	0–0.03	0–0.11								
Total	76.00 ± 2.36	74.93 ± 2.82	75.74 ± 1.43	76.38 ± 2.85	74.20	80.21	81.38	77.07	76.79	76.67	76.78	73.05

[a] F = fructose, G = glucose, M = mean and SD = standard deviation.

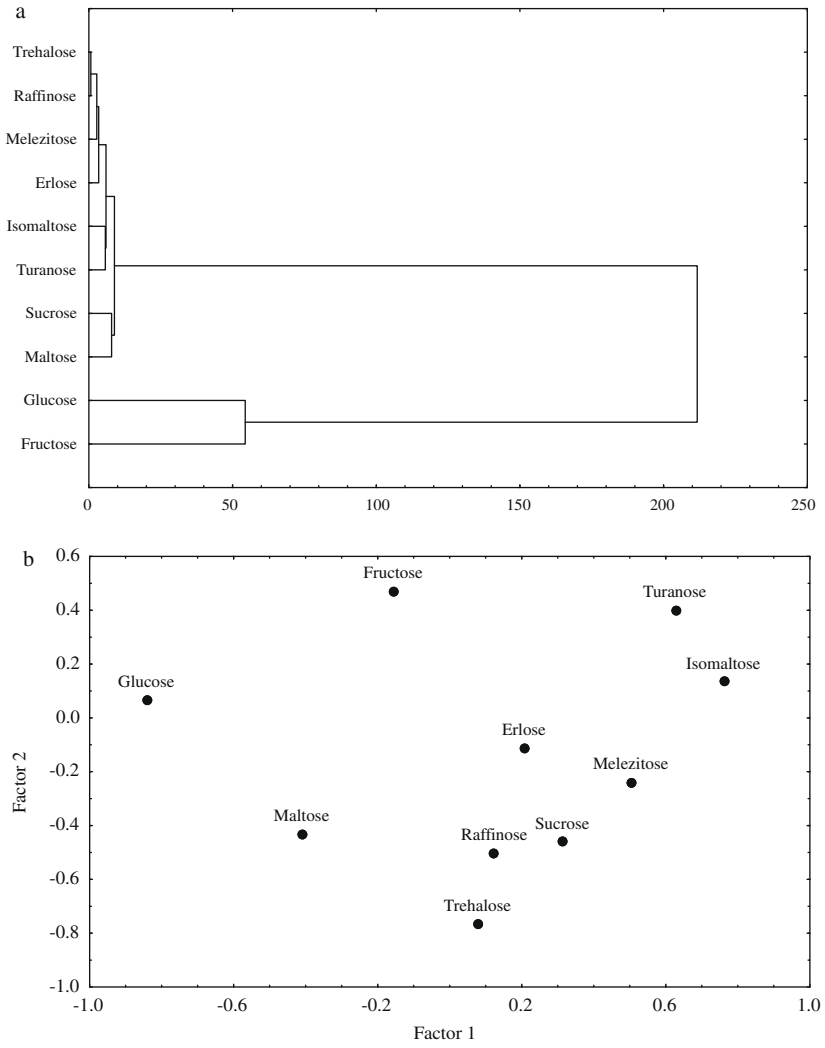


Fig. 2. (a) Hierarchical cluster analysis of sugars of honey samples obtained by Euclid's distances. (b) Plot of factorial weights in first factor versus factorial weights in second factor from the principal component analysis of 10 sugars.

sources from which the honey samples originated. The fructose/glucose for *Erica* and *Hedysarum* honeys are higher than the values reported for the Italian ones (Persano Oddo, Piazza, Sabatini, & Accorti, 1995). Honeys with fructose/glucose ratios remain liquid for longer periods. For instance, the *Apiaceae* Honey (H44) has low amount of glucose (24.63%) and the proportion of fructose is 37.99%. This honey type is always in liquid physical state because fructose/glucose ratio exceeds 1.5%.

Four honey types (multifloral, *Eucalyptus*, *Erica*, *Apiaceae*) show the same average amount of fructose. The concentrations of fructose (38.27%) and glucose (33.89%) of *Erica* Algerian honeys are the same of those of Italian ones (Persano Oddo et al., 1995). However, monosaccharides levels of *Eucalyptus* and *Citrus* honeys are high with those obtained by Serrano, Villarejo, Espejo, and Jodral (2004). Anklam (1998) explained that honey of the same floral source can vary due to seasonal climatic variations or to a different geographical origin.

In relation to the disaccharides, maltose and sucrose are the major disaccharides. The first disaccharide is present in all samples, except *Eucalyptus* honey (H28), and ranging from 0.47% to 3.42%. Maltose is also the main disaccharide in Brazilian honeys (mean 3.05%) (Da Costa Leite et al., 2000) and Spanish honeys (mean 3.96%) (Mateo & Bosch-Reig, 1997). *Citrus* honey shows the highest content of maltose and this result is in agreement with data obtained by Mateo and Bosch-Reig (1998). Terrab, Vega-Pérez, Diez, and Heredia (2001) found also that *Citrus* honey had the highest percentage of this disaccharide (5.7%). The value obtained for *Eucalyptus* is lower than the value found by Mateo and Bosch-Reig (1997) for Spanish *Eucalyptus* honey. The amount of maltose can exceed 6%. In fact, Diez, Andrés, and Terrab (2004) obtained 6.6% of maltose for Moroccan honeys. Sanz et al. (2004) found that trehalulose was the main disaccharide along with maltulose, turanose, kojibiose, maltose and isomaltose.

Sucrose is present in 48 samples, ranging between 0.48 and 5.26%, the multifloral honeys H12 and H19 do not contain this disaccharide. *Hedysarum* honey shows the highest level of this disaccharide. Sucrose amounts of *Myrtaceae* and *Citrus* honeys are closer, 2.78 and 2.87, respectively. This disaccharide is only detected in *Heather* honey (mean value 4.12%) by Terrab, Diez, and Heredia (2003a) with respect to six other Moroccan unifloral honeys. Sucrose contents of *Citrus* and *Eucalyptus* samples are lower than the data obtained for Andalusian ones (Serrano et al., 2004). Anklam (1998) reported that sucrose content can decrease during the storage of honey due to the presence of the invertase.

According to Da Costa Leite et al. (2000), the reason for the variable levels of sucrose could be that a transglucosylation reaction is

initiated by transference of the α-D-glucopyranosyl unit from sucrose to an acceptor molecule. None of the samples exceeded the highest limit set for sucrose by the European community directive (Council of the European Union, 2002). These results confirm that these honeys are at an advanced stage of ripening and are authentic because the sucrose is the most important sugar from a legislative point of view.

Isomaltose and turanose are present in 39 and 45 samples, respectively and varies from 0.30% to 4.71%, and 0.29% to 2.02%, respectively. Isomaltose content of *Myrtaceae* and *Hedysarum* honeys are closer, 0.39% and 0.40%, respectively. The results for isomaltose are different with those of Persano Oddo et al. (1995) and Mateo and Bosch-Reig (1998) who showed that the isomaltose content did not exceed 1.5%. However, the maximum value of this disaccharide in Spanish honeys was 3.07% (Sanz et al., 2004).

The trehalose content is very low and the mean value does not exceed 0.01%. The samples H1 and H33 have the highest amount (0.14%). However, this sugar has been found at high level (mean value of 0.09%) in Moroccan *Eucalyptus* honey (Terrab, Diez, and Heredia (2003b). Melibiose is not detected in all samples analysed in the present study, whereas values of melibiose for Brazilian honeys were between 0.05% and 0.15% which are lower than those of other disaccharides analysed (Da Costa Leite et al., 2000).

Sucrose, trehalose and trisaccharides (erlose, raffinose, melezitose) are non-reducing saccharides.

Among the trisaccharides, erlose is present in 47 honey samples but raffinose and melezitose are present in 17 and 15 honey samples, respectively.

The values for erlose are in the range of 0.01–2.35% and higher than those reported by Pérez-Arquillué et al. (1995).

This sugar is present in 47 honey samples and it is not detected in two multifloral honeys (H21 and H24) and one *Erica* honey (H39). The multifloral honey H23 has the highest value.

Raffinose, present only in 17 of the honeys, does not exceed 0.35%. This sugar is not detected in six honey types. This trisaccharide is the major sugar in the nectar of the sunflower *(Helianthus annuus)*. Da Costa Leite et al. (2000) reported that raffinose could be a nectar constituent or could arise from honeydew contamination.

Melezitose, present in 15 samples, ranges between 0.03% and 2.14%. The multifloral honey (H51) has the maximum value of this trisaccharide. *Apiaceae* and *Anarrhinum* honeys have the same amount of this trisaccharide, 0.04%. This sugar is usually indicative of honeydew honey. Sanz et al. (2004) obtained a high concentration of melezitose (6.57%) in Spanish honeydew samples. The amount of erlose found by these authors is the same of that of multifloral honey (H23), 2.35%. The percentage of the melezitose is low, indicating that these are nectar honeys.

Principal component analysis and factorial discriminant analysis are applied to the sugar results in order to determine the differences among the honey types.

Table 3a shows the factor loading matrix obtained for the two factors and the variance explained by each of them. The first principal component accounts for 22.83% of the variance and the second for 17.03%.

The cumulative variance is approximately 40%, which shows that the honey types are not well distinguished by their sugar contents. A scatter plot was obtained correlating the factorial weights of features in the first factor against the factorial weights in the second factor. It can be seen from Fig. 2b that glucose, isomaltose and turanose present high correlation with the first factor, while trehalose has correlation with the second factor.

Fig. 3a represents the graphic distribution of the samples according to their factor scores and shows that only Apiaceae honeys are differentiated from the rest, tending to higher values of the first component.

Table 3
(a) Rotated factor loadings and explained and cumulative variance. (b) Results of factorial discriminant analysis of sugar content in some Algerian honeys. (c) Coefficients for classification functions of some Algerian honeys. (d) Classification matrix of some Algerian honeys on the basis of sugar content.

Variable	Factor 1	Factor 2
(a)		
Fructose	−0.157771	0.474126
Glucose	−0.843830	0.069313
Maltose	−0.411632	−0.429478
Sucrose	0.311027	−0.458441
Isomaltose	0.758940	0.136811
Turanose	0.626057	0.404301
Trehalose	0.077361	−0.761454
Erlose	0.205089	−0.107919
Melezitose	0.500732	−0.235063
Raffinose	0.118230	−0.499963
Variance explained (%)	22.83	17.03
Cumulative variance (%)	22.83	39.86

Variable	Wilks' λ	*F* statistic	*P* significance level
(b)			
Fructose	0.204609	0.209119	0.889254
Glucose	0.231400	1.502238	0.234783
Maltose	0.229519	1.411436	0.259449
Sucrose	0.235789	1.714043	0.185997
Isomaltose	0.318455	5.704058	0.003392
Turanose	0.216305	0.773619	0.518221
Trehalose	0.247005	2.255411	0.103035
Erlose	0.226252	1.253758	0.308513
Melezitose	0.224664	1.177081	0.335535
Raffinose	0.356814	7.555536	0.000700

Variable	Honey type			
	Multifloral	*Eucalyptus*	*Erica*	*Apiaceae*
(c)				
Fructose	23.904	23.827	23.675	24.526
Glucose	18.124	17.588	18.514	18.421
Maltose	5.199	4.438	3.604	8.646
Sucrose	26.658	27.328	26.034	29.724
Isomaltose	46.330	44.810	45.838	52.600
Turanose	−22.585	−22.878	−25.146	−18.612
Trehalose	−282.508	−263.195	−258.047	−388.789
Erlose	8.052	6.975	8.327	2.199
Melezitose	8.052	6.975	8.327	2.199
Raffinose	78.176	74.274	61.486	140.268
Constant	−789.882	−769.128	−789.820	−856.448

Honey type	% Correct	Multifloral	*Eucalyptus*	*Erica*	*Apiaceae*
(d)					
Multifloral	96	24	1	0	0
Eucalyptus	60	4	6	0	0
Erica	50	2	0	2	0
Apiaceae	100	0	0	0	3
Total	83.33	30	7	2	3

Discriminant analysis is used to choose the parameters with higher discriminant power. The variables studied are shown in Table 3b, as well as Wilks' λ which indicates the contribution of each variable to the discrimination. As can be seen, the latter does not exceed 0.36. From this, it can be concluded that the selected parameters have low discriminant power.

Table 3c shows the standardised coefficients for canonical variables. It can be seen that raffinose is the most powerful parameter in the discrimination. Fig. 3b shows the perfect separation of the Apiaceae honeys from the rest. The functions that allow the classification of these types are shown in Table 3d. By applying these functions to the samples, their validity can be verified according to the agreement percentages of the cases in their corresponding group (Table 3d). It can be observed that the *Erica* honeys show the lowest agreement percentage (50%).

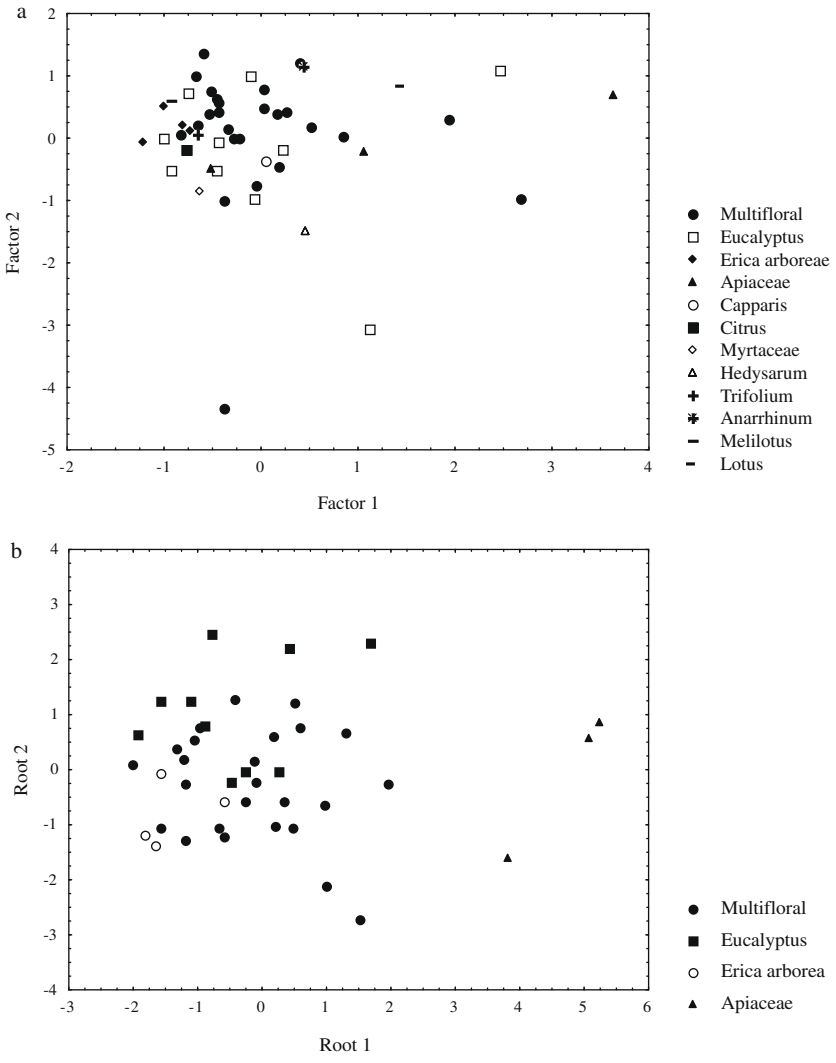


Fig. 3. (a) Plot of first factor versus second factor for the classification of honeys according to their floral type. (b) Factorial discriminant analysis of some Algerian honeys.

4. Conclusion

In this work, the sugar profiles are determined in multifloral and unifloral honeys from different regions of Algeria. The amount of total sugars of honey samples confirms that sugars are the main constituents honey, and vary with honey type. Therefore, honey is a source of energy that is instantly usable. Sugars composition is affected by contributions of the plant and environmental conditions. Reducing sugars, mainly fructose and glucose, represent the largest portion of honey composition, but small quantities of other sugars are also present such as sucrose, maltose and others. The sugar profiles show that these honey samples are nectar honeys and they are not adulterated. Seven sugars are detected in *Capparis* honey. Using multivariate statistical treatments, Apiaceae honeys can be separated from all other honeys using the sugar profiles. In order to widen the acquaintance of the composition of Algerian honeys, others analysis (physicochemical characteristics, antioxidant activity) are in progress.

Acknowledgements

Thanks are due to all the beekeepers who have provided the honey samples of this study.

References

Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry, 63*(4), 549–562.

Bogdanov, S., Martin, P., & Lüllmann, C. (1997). Harmonised methods of the European Honey Commission. *Apidologie*(extra issue), 1–59.

Bogdanov, S., Ruoff, K., & Persano Oddo, L. (2004). Physico-chemical methods for the characterisation of unifloral honeys: A review. *Apidologie, 35*, 4–17.

Cavia, M. M., Fernandez-Muino, M. A., Gomez-Alonso, E., Montes-Pérez, M. J., Huidobro, J. F., & Sancho, M. T. (2002). Evolution of fructose and glucose in honey over one year: Influence of induced granulation. *Food Chemistry, 78*, 157–161.

Cotte, J. F., Casabianca, H., Chardon, S., Lheritier, J., & Grenier-Loustalot, M. F. (2003). Application of carbohydrate analysis to verify honey authenticity. *Journal of Chromatography A, 1021*, 145–155.

Council of the European Union (2002). Council Directive 2001/110/ec of 20 December relating to honey. *Official Journal of the European Communities, 10*, 47–52.

Da Costa Leite, J. M., Trugo, L. C., Costa, L. S. M., Quinteiro, L. M. C., Barth, O. M., Dutra, V. M. L., et al. (2000). Determination of oligosaccharides in Brazilian honeys of different botanical origin. *Food Chemistry, 70*, 93–98.

De la Fuente, E., Sanz, M. L., Martinez-Castro, I., & Sanz, J. (2006). Development of a robust method for the quantitative determination of disaccharides in honey by gas chromatography. *Journal of Chromatography A, 1135*, 212–218.

Devillers, J., Morlot, M., Pham-Delègue, M. H., & Doré, J. C. (2004). Classification of monofloral honeys based on their quality control data. *Food Chemistry, 86*, 305–312.

Diez, M. J., Andrés, C., & Terrab, A. (2004). Physicochemical parameters and pollen analysis of Moroccan honeydew honeys. *International Journal of Food Science and Technology, 39*, 167–176.

Esti, M., Panfili, G., Marconi, E., & Carmela Trivisonno, M. (1997). Valorization of the honeys from the Molise region through physico-chemical, organoleptic and nutritional assessment. *Food Chemistry, 58*(2), 125–128.

Goodall, I., Dennis, M. J., Parker, I., & Sharman, M. (1995). Contribution of high-performance liquid chromatographic analysis of carbohydrates to authenticity testing honey. *Journal of Chromatography A, 706*, 353–359.

Lazaridou, A., Biliaderis, C. G., Bacandritsos, N., & Sabatini, A. G. (2004). Composition, thermal and rheological behaviour of selected Greek honeys. *Journal of Food Engineering, 64*, 9–21.

Louveaux, J., Maurizio, A., & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World, 59*, 139–157.

Low, N. H., & Sporns, P. (1988). Analysis and quantification of minor di and trisaccharides in honey, using capillary gas chromatography. *Journal of Food Science, 53*(2), 558–561.

Makhloufi, C., Schweitzer, P., Azouzi, B., Persano Oddo, L., Choukri, A., Laaredj, H., et al. (2007). Some properties of Algerian honeys. *Apiacta, 42*, 73–80.

Mateo, R., & Bosch-Reig, F. (1997). Sugar profiles of Spanish unifloral honeys. *Food Chemistry, 60*(1), 33–41.

Mateo, R., & Bosch-Reig, F. (1998). Classification of Spanish unifloral honeys by discriminant analysis of electrical conductivity, color, water content, sugars and pH. *Journal of Agricultural and Food Chemistry, 46*(2), 393–400.

Molan, P. C. (1996). Authenticity of honey. In P. R. Ashurst & M. J. Dennis (Eds.), *Food authentication* (pp. 259–303). London: University Press.

Morales, V., Corzo, N., & Sanz, M. L. (2008). HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups. *Food Chemistry, 107*, 922–928.

Ouchemoukh, S., Louaileche, H., & Schweitzer, P. (2007). Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food Control, 18*, 52–58.

Pérez-Arquillué, C., Conchello, P., Arifio, A., Juan, T., & Herrera, A. (1995). Physicochemical attributes and pollen spectrum of some unifloral Spanish honeys. *Food Chemistry, 54*, 167–172.

Persano Oddo, L., Piazza, M. G., Sabatini, A. G., & Accorti, M. (1995). Characterization of unifloral honeys. *Apidologie, 26*, 453–465.

Popek, S. (2002). A procedure to identify a honey type. *Food Chemistry, 79*, 401–406.

Sanz, M. L., Gonzalez, M., Lorenzo, C., Sanz, J., & Martınez-Castro, I. (2005). A contribution to the differentiation between nectar honey and honeydew honey. *Food Chemistry, 91*, 313–317.

Sanz, M. L., Polemis, N., Morales, V., Corzo, N., Drakoularakou, A., Gibson, G. R., et al. (2005). In vitro investigation into the potential prebiotic activity of honey oligosaccharides. *Journal of Agricultural and Food Chemistry, 53*, 2914–2921.

Sanz, M. L., Sanz, J., & Martınez-Castro, I. (2002). Characterization of o-trimethylsilyl oximes of disaccharides by gas chromatography–mass spectrometry. *Chromatographia, 56*, 617–622.

Sanz, M. L., Sanz, J., & Martınez-Castro, I. (2004). Gas chromatographic–mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey. *Journal of Chromatography A, 1059*, 143–1483.

Serrano, S., Villarejo, M., Espejo, R., & Jodral, M. (2004). Chemical and physical parameters of Andalusian honey: classification of *Citrus* and *Eucalyptus* honeys by discriminant analysis. *Food Chemistry, 87*, 619–625.

Terrab, A., Diez, M. J., & Heredia, F. J. (2003a). Palynological, physico-chemical and colour characterization of Moroccan honeys: III. Other unifloral honey types. *International Journal of Food Science and Technology, 38*, 395–402.

Terrab, A., Diez, M. J., & Heredia, F. J. (2003b). Palynological, physico-chemical and colour characterization of Moroccan honeys: I. River red gum (*Eucalyptus camaldulensis* Dehnh) honey. *International Journal of Food Science and Technology, 38*, 379–386.

Terrab, A., Vega-Pérez, J. M., Diez, M. J., & Heredia, F. J. (2001). Characterisation of northwest Moroccan honeys by gas chromatographic–mass spectrometric analysis of their sugar components. *Journal of the Science of Food and Agriculture, 82*, 179–185.

Weston, R. J., & Brocklebank, L. K. (1999). The oligosaccharide composition of some New Zealand honeys. *Food Chemistry, 64*, 33–37.

African Journal of Biotechnology Vol. 10(17), pp. 3451-3454, 25 April, 2011
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Antioxidant activity and separation of phenolic compounds of *Origanum glandulosum* from north Algeria by high performance liquid chromatography (HPLC)

Oukil Naima, Bedjou Fatiha*, Maiza-Benabdesselam Fadila and Chibane Mohamed

Faculty of Life and Nature Sciences -University Abderrahmane MIRA of Béjaia – Terga Ouzemmour – 06000- Béjaia-Algeria

Accepted 10 March, 2011

Origanum glandulosum **is an endemic plant of North African area (Algeria and Tunisia), used against infections like whooping cough and bronchitis. In this study, its antioxidant activity is evaluated. The results showed that the scavenging effect against DPPH is equal to 6730 mg/ml (IC_{50}). However, the scavenger effect of H_2O_2 estimated at 60.78% is very strong compared to the effects of BHA, BHT and α-tocopherol estimated respectively at 19, 25 and 23%. The scavenger effect of H_2O_2 is very strong compared to the scavenging of DPPH. A high scavenging effect is generally due to high content of phenolic compounds, phenolic acids and flavonoids. The results may suggest that *O. glandulosum* extract possesses compounds with antioxidant activity; these substances can play an important role in the prevention of free radical induced diseases.**

Key words: Phenolic compounds, *Origanum glandulosum,* HPLC, antioxidant, flavonoids.

INTRODUCTION

Origanum glandulosum is an endemic widely distributed taxon of the North African region (Algeria and Tunisia). It is considered as brushwood (Quezel and Santa, 1963; Leclerc, 1990; Bendahou et al., 2008). Several studies have shown that *O. glandulosum* taxon is rich in essential oils, phenolic compounds such as flavonoids and phenolic acids (Benzanger-beauquesne et al., 1980, 1990; Nakiboglu et al., 2007). In Algeria, this plant is used against infections like whooping cough, cough, fever and bronchitis (Bendahou et al., 2008).

The purpose of this study was the estimation of the total phenolic content of *O. glandulosum* using the Classical Folin-Ciocalteu reagent, the determination of their antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH assay), the H_2O_2 scavenging effects and the analysis of the chemical composition of methanolic extract by HPLC chromatography, to estimate the number of major components of this plant. The choice of this investigation is based on two criteria; firstly, there are few studies in this domain in Algeria and the second criterion is the traditional utilization of *O. glandulosum.*

*Corresponding author. E-mail: fadilamaiza@yahoo.fr. Tel: 00213771449217.
Fax: 00213214762.

Abbreviations: BHA, Butyl hydroxyanisol; **BHT,** butyl hydroxyltoluene; **DPPH**, 1,1 diphenyl-2-picryl hydroxyl.

MATERIALS AND METHODS

Plant materials

Samples of aerial parts (leaves, twigs and flowers) of individual plants were collected in the region of Boukhlifa (Béjaia) (36°41' 19°, 39' N; 5°06' 26°, 56' S; alt.1.00 Km) during June 2007. Identification of plants was made by the botanical laboratory of Béjaia University.

Crude extracts

The medicinal plants collected were harvested, dried and ground to

fine powder by a Kenwood multi-mill**.** Phenolic compounds were extracted according to the method described by Sousek et al. (1999). Dry aerial plant material was extracted in Soxhlet apparatus with methanol for 30 min. The solution was used for the analysis of the total phenolic compounds and the estimation of its flavonoid content.

Chemical reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagents were purchased from sigma Aldrich..

Determination of total phenolic content

The total phenolic content was estimated using the Folin-Ciocalteu colorimetric method according to Javanmardi (2003) with minor modifications. Appropriately diluted test sample (0.5 ml) was reacted with 2.5 ml of Folin-Ciocalteu (1/10 N) reagent. The reaction was then neutralized with 2 ml of saturated sodium carbonate (75 g/l) and allowed to stand for 15 min in the dark at 45°C. Later the absorbance of the resulting blue color was measured at 765 nm. Quantification was done on the basis of a standard curve with gallic acid. Results were expressed as gram of gallic acid equivalent (GAE) per 100 g dry weight.

Determination of flavonoid contents

The flavonoid content was estimated using the method of Djerridane et al. (2006). 1.5 ml of ammonium chloride (2%) was added to the same volume of the test sample. The absorbance was measured at 430 nm after a period of incubation at room temperature for 15 min. The control is prepared with the solution of ammonium chloride and the same volume of methanol.

Determination of antioxidant activity

DPPH assay

Antioxidant activity of the samples was determined using the method of Gachtar et al. (2007). 50 µl of the sample was added to 5 ml of DPPH solution (0.004%). The mixture was allowed to stand for 30 min in the dark at room temperature and the absorbance was measured at 517 nm.

H_2O_2 assay

The scavenging effect of H_2O_2 was determined according to the method of Atmani et al. (2009). 1.2 ml of H_2O_2 (40 mM) was added to 2 ml of the sample. After a period of incubation for 10 min at room temperature, the absorbance was measured at 230 nm.

HPLC analysis

HPLC analysis was carried out on a Perkin Elmer series 200 system with C18 (discovery Supelco ODS), 5 µm nucleosil 250 x 16

RESULTS AND DISCUSSION

Phenolic compounds and flavonoid contents

The evaluation of phenolic compounds and flavonoids show that *O. glandulosum* contains 55.15 and 6.88 mg/g of dry weight respectively. The content of phenolic compounds of *O. glandulosum* is three fold lower than that obtained for the aqueous extract of *Origanum vulgare* of Spain (175 mg/g) (Rodriguez-meizoso et al., 2006) and lower than that obtained for *O. vulgare* of Slovenia (Skerget et al., 2005). However, it is higher than that obtained for methanolic extract of *O. vulgare* of Poland (22.21 mg/g) (Capecka et al., 2005).

However, the content of flavonoids for *O. glandulosum* obtained in this study is largely higher than that obtained for *O. vulgare* of Slovenia (Skerget et al., 2005). These differences can be explained by the method of extraction of active substances used in every study, the difference between plant species and the climatic conditions (Fiamegas et al., 2004).

Antioxidant activity

DPPH assay

The IC50 evaluated for the methanolic extract of *O. gladulosum* is equal to 6730 µg/ml. However, the scavenger effect compared to that obtained for gallic acid (IC50 = 43 µg /ml), is not negligible (Table 1). A high scavenger effect is generally due to high content of phenolic compounds, phenolic acids and flavonoids (Samarth et al., 2008; Kouri et al., 2007, Capecka et al., 2005). The content of flavonoids in this study is very low. This partly explains the low scavenging effect of *O. glandulodum* extract.

Scavenger effect of H_2O_2

The scavenger effect of H_2O_2 by methanolic extract of *O. glandulosum* was estimated at 60.78% for a concentration of 66 µg/ml. The synthetic antioxidants like BHA, BHT and α-tocopherol have scavenging effects of 19, 25 and 23%, respectively (Table 2). These effects are largely lower than that obtained for the extract. This can be explained by the presence of phenolic compounds in the methanolic extract of *O. glandulosum*, which can liberate electrons (Balasundram et al., 2005; Elmastas et al., 2006).

HPLC analysis

Phenolic compounds can be defined as a large series of chemical constituents possessing at least one aromatic ring, bearing hydroxyl and other sub-constituents (Ribereau-Gayon, 1968). RP-HPLC analysis is the most used method for the identification of plant phenolic compounds. Because of the diversity and complexity of natural phenolics in medicinal plants, it is difficult to characterize every compound and elucidate its structure.

However, it is not difficult to identify the major categories

Table 1. Effects of methanolic extract of *O. glandulosum* and positive control gallic acid on the free radical (DPPH) scavenging.

Sample	DPPH, IC50 (mg/ml)
Methanolic extract	6730 ± 5.0
Gallic acid	43 ± 0.5

Table 2. Scavenging effect of H_2O_2 by methanolic extract and positive controls (BHA, BHT and α-tocopherol).

Sample	H_2O_2, IC50 (%)
BHA	19 ± 0.3
BHT	25 ±0.25
α-tocopherol	23 ±0.23

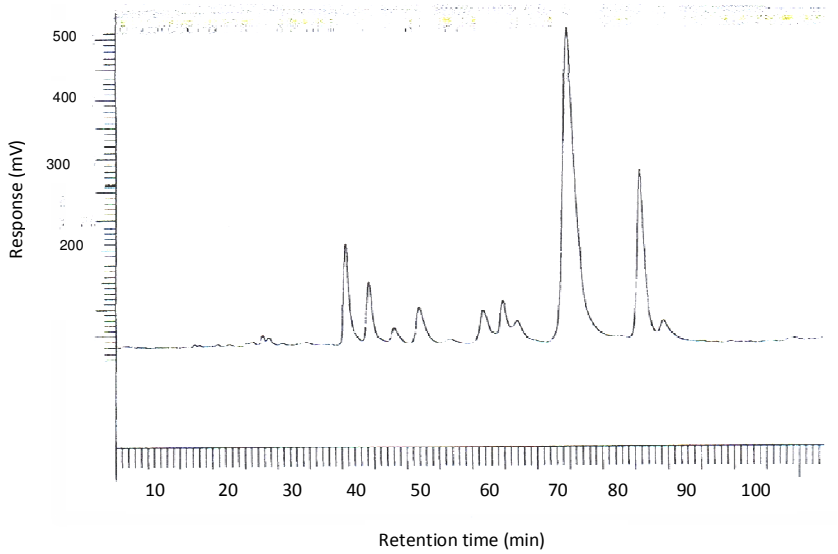


Figure 1. HPLC chromatogram of *O. grandulosum.*

of phenolic compounds.

In the present study, the identification of phenolics of *O. glandulosum* by HPLC was done by the comparison of their retention time with those obtained by Proestos et al. (2006). A typical HPLC chromatogram of *O. glandulosum* is presented in Figure 1. According to the literature, the phenolic compounds of *O. glandulosum* can be successively gallic acid (35 min), vanillic acid (40 min), coumaric acid (50 min), rutin (60 min), ferrulic acid (70 min) and naringenin (85 min).

Conclusion

Free radicals may play an important role in the origin of life and biological evolution. This implies their beneficial effects on the organisms. For example, oxygen radicals exert critical actions such as signal transduction and gene transcription. However, free radicals and other related species could also cause the oxidation of biomolecules which leads to cell injury and death.

In recent decades, the phenolic compounds have been of great interest as they have been in natural products. *O. glandulosum* exhibits a high antioxidant effect compared to that obtained with BHA, BHT and α-tocopherol. Therefore, the results may suggest that *O. glandulosum* possesses compounds with antioxidant properties like ferrulic and gallic acid which could be isolated and then used as antioxidants for the prevention and treatment of free radical related disorders.

REFERENCES

Balasundram N, Ai TY, Sambanthamurthi R, Sundram K, Samman S

(2005). Antioxidant Properties of palm fruit extract. Asia Pac. J. Clin. Nutr. 4, 446-324.
Bendahou M, Muselli M, Grignon-Dubois M, Benyoucef M, Desjobert JM, Bernardini AF (2008) Antimicrobial activity and chemical composition of Origanum glandulosum Desf. essential oil and extract obtained by microwave extraction: Comparison with hydrodistillation Food Chemistry. 106(1): 132-139.
Benzanger-beauquesne L, Pinkas M, Torck M, Trotin F (1980). Plantes médicinales des regions tempérées. Paris : ED. Malouine.
Capecka E, Mareczek A, Leja M (2005). Antioxidant activity of fresh end dry herbs of some Lamiaceae species. Food chemistry, 93: 223-226.
Djerridane A, Yousfi M, NAdjemi B, Boutassouna D, Stocker P, Vidal N (2006). Antioxidant activity of some Algerian medicinal plants extract containing phenolic compounds. Food chemistry, 97: 654-660.
Elmastas M, Gulçin I, Isildak O, Kufrevioglu OI, Ibaoglu K, Aboul-Enein HY (2006). Radical Scavengin Activity and ontioxidant Capacity of Bay Leaf Extract. J. Iranian chem.. Soc., 3: 258-266.
Fiamegos YC, Nanos CG, Vervoort JJM, Stalikas CD (2004). Analytical procedure for the in-vial derivatization-extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass selective detection. J. Chromatogr. A, Including electrophoresis and other separation methods 1041 (2004)1-2. (ISSN 0021-9673): Pp. 11-18.
Javanmardi J, Stushnoff C, Locke E, Vivanco JM (2003). Antioxidant activity and total phenolic content of Iranian Ocimum accessions.Food Chemistry, 83: 547-550.
Kouri R, Tsimogiannis D, Bardouki H, Oreopoulou V (2007). Extraction and analysis of antioxidant components from Origanum dictamnus. Innovative Food Sci. Emerging Technol., 8: 155-162.
Leclerc H (1999). Précis de phytothérapie: Essai de thérapeutique par les plantes françaises Paris: 5ème Ed. Masson.
Marculescu A, Laur V, Daniela H, Dragulescu C, Antonie J, Olah N (2007). Polyphenols Analyses From *Tymus* Species. Proc. Rom. Acad. 3 : 117–121.
Nakiboglu M, Urek RO, Kayali HA, Tarhan L (2007). Antioxidant capacities of endemic *Sideritis sipylea* and *Origanum sipyleum* from Turkey. Food Chemistry, 104: 630-635.
Proestos C, Sereli D, Komaitis M (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. Food Chemistry, 95. 44-52.
Quezel P, Santa S (1963). Nouvelle flore de l'Algérie et des régions désertiques meridionales. Tome 2. Paris : Ed. CNRS.
Ribereau-Gayon P (1968). Notions générales sur les composés phénoliques, méthodes générales d'études des composés phénoliques. In composés phénoliques des végétaux. Ed. Dunod, Paris, p: 1-27.
Rodríguez-Meizoso I, Marin FR, Herrero M, Señorans FJ, Reglero G, Cifuentes A, Ibáñez E. (2006). Subercritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization. J. Pharm. Biomed. Anal., 41: 1560-1565.
Samarth RM, Panwar M, Kumar M, Soni A, Kumar M, Kumar A (2008). Evaluation of antioxidant and radical-scavenging activities of certain radioprotective plant extracts. Food Chemistry, 106: 868-873.
Skerget M, Kotnik P, Hadolin M, Hras AR, Simoni M, Knez Z (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry, 89: 191-198.
Sousek J, Guédon D, Adam T, Bochorakova H, Taborska E, Valka I, Simanek V (1999). Alkaloids and organic acids content of eight *Fumaria* species. Phytochem. Anal., 10: 6 -11.

Diversity and evolution of the *Hordeum murinum* polyploid complex in Algeria

Malika Ourari, Abdelkader Ainouche, Olivier Coriton, Virginie Huteau, Spencer Brown, Marie-Thérèse Misset, Malika Ainouche, and Rachid Amirouche

Abstract: Population diversity and evolutionary relationships in the *Hordeum murinum* L. polyploid complex were explored in contrasted bioclimatic conditions from Algeria. A multidisciplinary approach based on morphological, cytogenetic, and molecular data was conducted on a large population sampling. Distribution of diploids (subsp. *glaucum*) and tetraploids (subsp. *leporinum*) revealed a strong correlation with a North–South aridity gradient. Most cytotypes exhibit regular meiosis with variable irregularities in some tetraploid populations. Morphological analyses indicate no differentiation among taxa but high variability correlated with bioclimatic parameters. Two and three different nuclear sequences (gene coding for an unspliced genomic protein kinase domain) were isolated in tetraploid and hexaploid cytotypes, respectively, among which one was identical with that found in the diploid subsp. *glaucum*. The tetraploids (subsp. *leporinum* and subsp. *murinum*) do not exhibit additivity for 5S and 45S rDNA loci comparative with the number observed in the related diploid (subsp. *glaucum*). The subgenomes in the tetraploid taxa could not be differentiated using genomic in situ hybridization (GISH). Results support an allotetraploid origin for subsp. *leporinum* and subsp. *murinum* that derives from the diploid subsp. *glaucum* and another unidentified diploid parent. The hexaploid (subsp. *leporinum*) has an allohexaploid origin involving the two genomes present in the allotetraploids and another unidentified third diploid progenitor.

Key words: Hordeum murinum, polyploidy, FISH–GISH, molecular evolution, bioclimate.

Résumé : La diversité des populations et les relations évolutives dans le complexe polyploïde *Hordeum murinum* L., ont été explorées dans les conditions bioclimatiques contrastées d'Algérie. Une démarche pluridisciplinaire reposant sur des données morphologiques, cytogénétiques et moléculaires, a été appliquée à un large échantillonnage de populations. La distribution des cytotypes diploïdes (subsp. *glaucum*) et tétraploïdes (subsp. *leporinum*) est fortement corrélée au gradient d'aridité Nord-Sud. La majorité d'entre eux, présente une méiose régulière avec quelques irrégularités chez certains tétraploïdes. Les analyses morphologiques ne montrent pas de différenciation entre taxons mais une variabilité corrélée au bioclimat. Deux et trois séquences nucléaires différentes (gène codant pour une protéine kinase) ont été identifiées respectivement chez les cytotypes tétraploïdes et hexaploïdes; l'une d'elles étant identique à celle du diploïde *glaucum*. Les tétraploïdes (sous-espèces *leporinum* et *murinum*) ne présentent pas d'additivité du nombre de loci ADNr 5S et 45S par rapport au diploïde (subsp. *glaucum*). Les génomes tétraploïdes ne se différencient pas non plus par le GISH. Les résultats soutiennent une origine allotétraploïde des deux sous-espèces *leporinum* et *murinum* qui dériveraient de la subsp. *glaucum* et d'un parent diploïde inconnu. Le taxon hexaploïde (subsp. *leporinum*) impliquerait les deux génomes tétraploïdes et un troisième génome diploïde inconnu.

Mots-clés : Hordeum murinum, polyploïdie, FISH–GISH, évolution moléculaire, bioclimat.

[Traduit par la Rédaction]

Introduction

Complexes including related diploid and polyploid taxa are particularly common in the Poaceae family where hybridization and genome duplication are widespread phenomena. This is particularly well illustrated in the genus *Hordeum* where 32 diploid ($2n = 2x = 14$), tetraploid ($2n = 4x = 28$), and hexaploid ($2n = 6x = 42$) species and infraspecific taxa

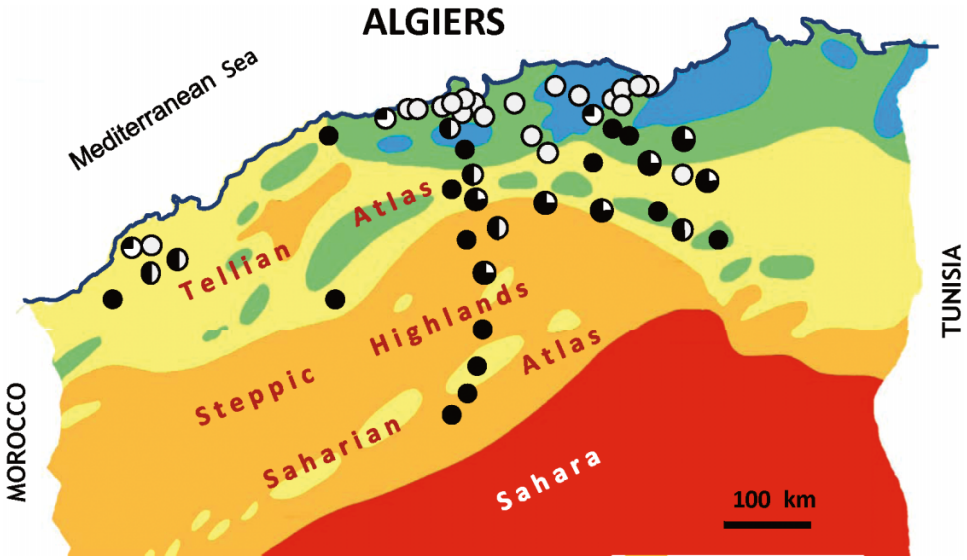
Received 6 November 2010. Accepted 14 April 2011. Published at www.nrcresearchpress.com/gen on XX August 2011.

Paper handled by Associate Editor F. Belzile.

M. Ourari. Université de Rennes 1, UMR CNRS 6553 Ecobio, Campus Scientifique de Beaulieu, 35042 Rennes CEDEX, France; Université Abderrahmane Mira, Faculté des Sciences de la Nature et de la Vie, Route de Targa Ouzemour, 06000, Béjaia, Algérie.
A. Ainouche, M.-T. Misset, and M. Ainouche. Université de Rennes 1, UMR CNRS 6553 Ecobio, Campus Scientifique de Beaulieu, 35042 Rennes CEDEX, France.
O. Coriton and V. Huteau. Institut national de la recherche agronomique, UMR 118 Amélioration des Plantes et Biotechnologies Végétales, BP 35327, Le Rheu, 35653 Le Rheu CEDEX, France.
S. Brown. Institut des Sciences du Végétal CNRS, 91190 Gif-sur-Yvette CEDEX, France.
R. Amirouche. Université des Sciences et de la Technologie Houari Boumediene, Laboratoire de Biologie et Physiologie des Organismes, BP 32 El-Alia, 16111, Bab-Ezzouar, Algiers, Algérie.

Corresponding author: R. Amirouche (e-mail: ramirouche@usthb.dz and ramirouche@hotmail.com).

Fig. 1. Distribution of the sampled populations of *Hordeum murinum* in North Algeria and relative frequencies of the 2*x* and 4*x* cytotypes. Bioclimatic areas are indicated by color: blue, humid (H), green, subhumid (SH), yellow, semiarid (SA), brown, arid (A), and red, saharian (see also Table 1). Populations entirely diploid (2*x*) or tetraploid (4*x*) are represented by filled circle or open circle, respectively.



are encountered (Nishikawa et al. 2002). In this study, we focused on the evolutionary relationships and population diversity in the *Hordeum murinum* L. complex from the Mediterranean region.

Hordeum murinum is composed of annual and predominantly autogamous plants, recognised either as three separate species (Baum and Bailey 1990) or as three subspecies clustered in a single specific complex comprising three subspecies with different ploidy levels: subsp. *glaucum* (Steudel) Tzvelev ($2n = 2x = 14$); subsp. *murinum* s. str. ($2n = 4x = 28$); and subsp. *leporinum* (Link.) Arcangeli ($2n = 4x = 28$, $2n = 6x = 42$). For a long time, the intraspecific relationships in this complex, the taxonomic rank, and the nature of polyploidy remained ambiguous and controversial. Morphological and isozyme analyses (Giles 1984; Giles and Lefkovitch 1986; Konishi 1989; Savova-Bianchi et al. 2002) emphasized the difficulty for separating these taxa and showed no significant differentiation between and within the diploid and tetraploid populations. However, karyological and cytogenetic data in *H. murinum* s.l. (Rajhathy and Morrison 1962; von Bothmer and Jacobsen 1986; von Bothmer et al. 1986, Linde-Laursen et al. 1995; Kankanpää et al. 1996) generated various lines of evidence, suggesting an allopolyploid origin. Several molecular phylogenetic analyses, performed in the global context of genus *Hordeum*, highlighted the differentiation between the diploid (subsp. *glaucum*) and polyploid (subsp. *leporinum* and subsp. *murinum*) genomes (Kochieva et al. 2001; Terzi et al. 2001; Svitashev et al. 1995; Baum and Johnson 1999; Komatsuda et al. 1999; El-Rabey et al. 2002; Taketa et al. 1999, 2000; Nishikawa et al. 2002). Some authors (e.g., de Bustos et al. 1999; El-Rabey et al. 2002) pointed out the need of reconsidering their taxonomic status. Further comparative analyses based on genome size (Jakob et al. 2004) and internal transcribed spacer sequences (Blattner 2004) raised doubts about the autopolyploid versus allopolyploid origin. Recently, new studies have focused on the origin of polyploidy in the *H. murinum* complex. Eilam et al. (2009), on the basis of the DNA amount, considered the 4*x* *leporinum* and *murinum* as diploidized autopolyploids, whereas Tanno et al. (2010) underlined their allopolyploid nature. Sequences of nuclear and chloroplast DNA allowed Jakob and Blattner (2010) to conclude that the polyploids derive from hybridization involving the genome of subsp. *glaucum* on the one hand and the genomes of two extinct diploid species on the other hand.

All the above mentioned studies take little account of the diversity within diploids and polyploids, if we except the work based on AFLP of El-Rabey et al. (2002) who revealed a continuum between the Egyptian samples of subsp. *murinum* and subsp. *leporinum*. Amirouche and Misset (2003) have studied hordein polymorphism, showing a strong differentiation among diploid populations along a gradient of aridity in North Africa.

The aim of this study is to evaluate the diversity among subspecies and cytotypes by sampling a large number of populations through the North–South bioclimatic gradient of Algeria using various lines of data from morphology, cytogenetics, and nuclear DNA sequences. More specifically, our goals are (*i*) to examine the distribution of cytotypes in Algeria, (*ii*) to evaluate the diversity within and among diploid and tetraploid subspecies based upon morphological analysis and meiotic behaviour of chromosomes in natural populations of *H. murinum* in Algeria, and (*iii*) to estimate the nature of the polyploidy in this complex by using molecular cytogenetics, nuclear DNA content, and molecular phylogeny.

Materials and methods

Origin of material and sampling

Populations of subsp. *glaucum* and subsp. *leporinum* (4*x*) were sampled in 53 sites in northern Algeria (Fig. 1). Each location is characterized according to the Mediterranean bioclimatic types defined using Emberger's coefficient Q2 (Stewart 1974). Collection sites and major bioclimatic parameters are presented in Table 1. At each collection site, 15–20

individual plants were randomly collected (separated at least by 5 m). Young spikes were fixed for meiosis analysis, and seeds were collected for experimental cultures. The subsp. *murinum* was represented by samples collected from France under Mediterranean and Atlantic climates. Hexaploid accessions of subsp. *leporinum* were obtained from USDA seed collections (as indicated below).

Morphological analysis

A first study was based on 25 diploid populations of subsp. *glaucum*, 24 tetraploid populations of subsp. *leporinum* (including two samples from southern France), and 4 tetraploid populations of subsp. *murinum* sampled under Atlantic climate in Brittany (France). Twenty-two quantitative and qualitative morphological characters (Table 2) were measured on 7–8 individuals per population. A total of 420 individuals were analyzed. Principal component analysis (PCA) was performed on the 53 populations using a correlation matrix obtained after standardization, following the STAND., SIMINT., EIGEN., PROJ., and MXPLOT procedures of the NTSYS-pc package program (Rohlf 1990).

A second analysis incorporated four bioclimatic parameters (Alt., altitude; *P*, annual rainfall in millimetres; *m*, the average of the minimum temperature of the coldest month in degrees Celsius; and *M*, the average of the maximum temperature of the hottest month in degrees Celsius) as additional variables. This second analysis was carried out on the Algerian populations only.

Cytological analyses

Mitosis and meiosis

Chromosome counting and ploidy level estimations were determined on root tip meristems (mitotic metaphases) of germinating seeds. Root tips were pretreated in α-bromonaphtalene–saturated water solution at room temperature for 2 h. After fixation in ethanol – acetic acid (3:1, *v*/*v*) at 4 °C, treatment in 1.0 mol/L HCl was performed for 8 min at 60 °C, followed by the Feulgen staining procedure. For meiotic analysis, young spikes prior to anthesis were fixed in ethanol – formol – glacial acetic acid (6:3:1, *v*/*v*/*v*) for 48 h. Spikes were then washed and conserved in 70% ethanol at 4 °C. The same Feulgen staining method as with the mitotic metaphases was adopted. The anthers were squashed in a drop of 45% acetocarmine. Meiotic behaviour of chromosomes was studied in pollen mother cells (PMC) mainly at diakinesis, metaphase I, and anaphase I.

Flow cytometry and genome size estimation

DNA amounts were estimated according to the method of Galbraith et al. (1983). Leaf fragments from both the analyzed sample and a reference standard were finely chopped together in 600 µL of Galbraith buffer (0.1% Triton, 1% PVP, 10 mmol/L metabisulfite) and filtered. Thirty microlitres of propidium iodide at 50 µg/mL and 1 µL of RNase at 10 µL/mL were added to the filtrate, and the fluorescence measure of stained nuclei was realized using an argon laser (20 mW, 488 nm). *Pisum sativum* 'Long Express' (2C = 2*x* = 8.37 pg; 40.5% GC) was used as an internal standard for genome size estimation in subsp. *glaucum*. For subsp. *leporinum* (6*x* and 4*x*) and subsp. *murinum*, nuclei isolated from *Triticum aestivum* 'Chinese Spring' (2C = 6*x* = 30.9 pg; 43.7% GC) were used as an internal standard. Twenty three plants of the three *H. murinum* cytotypes were analyzed. Individual plants of the diploid (subsp. *glaucum*) and tetraploid (subsp. *leporinum)* taxa were collected under different ecological conditions in Algeria (Kherrata, Sétif, Mesrane, Ksar El Boukhari, Guelt Stel, Adekar, Gouraya, and Tikjda). The tetraploid (subsp. *murinum*) individuals were collected in Brittany (France), and the hexaploids (subsp. *leporinum*) were derived from seeds provided by USDA seed collections (accessions originating from Afghanistan, ID: 12910; Turkey, ID: 82).

GISH and FISH protocols

In situ hybridization was performed on mitotic chromosomes from roots of germinated seeds. The root-tips were treated in α-bromonaphtalene solution at 4 °C for 24h and fixed with ethanol – acetic acid (3:1, *v*/*v*). Roots tips were stained in 2% acetocarmine solution and squashed in a drop of 45% acetic acid. For genomic in situ hybridization (GISH), total genomic DNA from subsp. *leporinum* (4*x*), subsp. *murinum*, and subsp. *glaucum* were used as a probe and labelled by nick translation with biotin-14-dATP (Invitrogen Life Technology, Carlsbad, California). Total genomic DNA from subsp. *leporinum* (4*x*), subsp. *murinum*, and subsp. *glaucum* were autoclaved to provide fragments of 100–300 bp to be used as a blocker. A series of test assessments were conducted to determine the amount of blocking DNA (*leporinum/glaucum*, *glaucum/leporinum* on subsp. *leporinum* chromosomes; *murinum/glaucum*, *glaucum/murinum* on subsp. *murinum* chromosomes) that have been tested to discriminate between the genomes. Fifty microlitres of denatured probe mixture, containing 100 ng of labeled probe and a 50-, 100-, 200-fold excess of blocking DNA, was applied to each slide and covered with a cover slip. Chromosome preparation and predenatured (92 °C for 6 min) probes were denatured at 85 °C for 10 min and allowed to hybridize overnight at 37 °C in a moist chamber. After hybridization, slides were washed in 50% formamide in 2× SSC at 42 °C for 5 min , followed by several washes in 4× SSC–Tween. Biotinylated probes were immunodetected by Texas-red conjugated with avidin antibodies (Vector Laboratories, Burlingame, California). The chromosomes were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5 µg/mL 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a CoolSnap HQ camera (Photometrics, Tucson, Arizona) on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and analyzed using MetaVue (Universal Imaging Corporation, Downington, Pennsylvania).

A double-labelling fluorescence in situ hybridization (FISH) experiment was carried out with two DNA probes. The 45S rDNA probe (pTa 71) is a cloned 9-kb *Eco*RI fragment of a rDNA repeat unit (18S–5.8S–26S genes and spacers) isolated from *Triticum aestivum* (Gerlach and Bedbrook 1979), and the pTa 794 probe is a 410-bp *Bam*HI fragment of the 5S rDNA from *T. aestivum* (Gerlach and Dyer 1980). The two probes were labelled by random priming with Alexa-488 dUTP (Invitrogen Life Technology) and Alexa 594 (Invitrogen Life Technology), respectively.

Table 1. Bioclimatic characteristics of the collection sites of *Hordeum murinum* populations in Algeria.

Code	Location	Alt. (m)	*P* (mm)	*m* (°C)	*M* (°C)	Q2	Bioclimate
H01	Chiffa	300	735	3.1	28.5	100.44	SH
H02	Benchicao	1250	584	1.3	35.0	59.44	SH
H03	Benhar	810	252	1.1	37.0	24.08	SA
H04	Mesrane	800	264	0.9	38.0	24.41	A
H05	Hassi Bahbah	880	240	0.8	37.0	22.74	A
H06	Guelt Stel	750	195	0.9	36.6	18.74	A
H07	Bouguezoul	700	430	0.7	34.5	42.38	SA
H08	Tiaret	1050	582	2.6	32.8	66.10	SH
H09	Frenda	1300	394	2.5	33.3	43.88	SA
H10	Tessala	700	520	1.8	32.5	58.10	SH
H11	Bouhadjar	150	414	1.9	33.2	45.66	SA
H12	Terga	10	330	4.8	31.0	43.20	SA
H13	El Amria	90	380	4.8	31.0	43.20	SA
H14	El Amria	90	380	8.9	31.2	43.20	SA
H15	Ait Méraou	950	1037	2.9	31.6	123.93	H
H16	Ain El Hammam	1080	1145	2.7	32.0	134.04	H
H17	Souk El Had	550	840	3.2	34.2	92.94	H
H18	Tassaft	1150	1080	2.8	32.6	124.31	H
H19	Tikjda I	1700	1190	0.9	29.0	145.26	H
H20	Tikjda II	1100	1005	1.1	30.5	117.25	H
H21	Tikjda III	900	645	1.6	30.5	76.55	H
H22	Tikjda IV	600	545	2.8	34.9	58.24	H
H23	Bouira	550	480	3.0	36.1	49.74	SH
H24	Rocher du Moine	420	380	1.6	34.5	39.62	SA
H25	Yachir	1050	441	3.3	36.2	45.98	SA
H26	Smala	1100	570	3.9	31.8	70.08	SH
H27	Msila	450	195	2.9	36.9	19.67	A
H28	Mont Dira	860	537	2.0	33.4	58.66	SH
H29	Mamora	1100	507	2.3	34.0	55.38	SH
H30	Douera	150	768	7.9	29.9	119.74	SH
H31	Berbessa	60	690	8.1	29.5	110.59	SH
H32	Sahel	190	630	8.3	30.0	99.58	SH
H33	Tipaza	10	690	5.9	31.7	91.73	SH
H34	Djelfa	1250	284	–1.8	35.5	26.12	A
H35	Bousaada	990	260	3.7	37.1	26.70	A
H36	Chott Beida	1200	415	1.2	34.5	42.75	SA
H37	Khellil	850	397	1.2	33.7	41.90	SH
H38	Sétif	1180	457	0.8	32.5	49.45	SH
H39	Bougaa	930	700	2.2	31.5	81.95	SH
H40	Cherchell	130	534	7.6	28.5	87.64	SH
H41	Cherchell	60	560	7.8	29.0	89.10	SH
H42	Blida	325	600	2.5	35.2	78.11	SH
H43	Berrouaghia	980	570	0.6	34.2	58.19	SH
H44	Ksar El Boukhari	860	370	2.6	36.7	37.22	SA
H45	Had Sahari	720	250	1.0	37.6	25.02	A
H46	Senalba nord	1350	392	–0.9	33.4	39.20	SA
H47	Ténès (le cap)	120	330	4.8	35.0	37.48	SA
H48	Mazafran	10	630	8.3	32.4	89.66	SH
H49	Ouled Fayet	190	650	7.9	30.2	99.98	SH
H50	Dely Brahim	160	650	7.9	30.2	99.98	SH
H51	Bab Ezzouar	10	630	7.6	31.7	89.66	SH
H52	Meurdja	450	675	3.5	35.2	88.15	H
H53	Bordj Menaiel	320	580	7.3	33.8	91.50	SH
H54	Naciria	240	580	7.3	31.0	83.94	SH
H55	Adekar	950	750	3.0	30.0	95.28	H
H56	Gouraya	600	650	7.6	30.2	98.65	H
H57	Kherrata	400	1003	6.0	30.8	138.72	H

Note: Alt., altitude; *P*, annual rainfall; *m*, the average of the minimum temperature of the coldest month; *M*, the average of the maximum temperature of the hottest month; Q2, Emberger's Mediterranean bioclimatic coefficient, calculated according to Stewart (1974) $Q2 = 3.43P/(M - m)$ from which the bioclimatic type is defined: H, humid; SH, subhumid; SA, semiarid, and A, arid.

Table 2. Morphological characters used in the numerical analyses of *Hordeum murinum* populations.

	Characters
A	Culm length (cm)
B	First upper internode length (cm)
C	Second upper internode length (cm)
E	Stubble diameter in the middle of the second upper internode (mm)
F	Upper leaf length (cm) (flag length of the upper leaf)
G	Upper leaf width (mm)
	Spike length and characters of the spikelets
H	Spike length (cm)
I	Lemma length of the lateral floret (mm), awn included
J	Lower glume length of the lateral spikelet (mm), awn included
K	Upper glume length of the lateral spikelet (mm), awn included
X	Ciliation of the lateral floret palea:
	Clearly unilateral
	Weakly unilateral
	Moderately unilateral
	Clearly bilateral
L	Lemma length of the central floret (mm), awn included
M	Lower glume length of the central spikelet (mm), awn included
N	Length of the rachilla (mm)
O	Pedicel length of the lateral spikelet (mm)
V	Pedicel length of the central spikelet (mm)
P	Upper glume width of the lateral spikelet (mm)
Q	Palea length of the lateral floret (cm)
R	Width of the lower glume of the central spikelet (mm)
S	Lemma width of the central floret (mm)
T	Palea length of the central floret (cm)
U	Length of caryopsis (mm)

DNA sequencing

Three samples of subsp. *glaucum* collected in contrasted bioclimatic and ecological conditions in Algeria were selected; these samples were collected from Sétif (semiarid), Kherrata (humid), and Guelt Stel (arid). Two tetraploid samples of subsp. *leporinum* were collected in Algeria in the humid bioclimate of the Tikjda mountain (1600 m) and at Gouraya (600 m). The tetraploid sample of subsp. *murinum* was from Brittany in France. The hexaploid cytotype originated from Afghanistan (USDA collection, ID: 12910). Representatives of related *Hordeum* species; *Hordeum marinum*, *Hordeum spontaneum*, *Hordeum bulbosum*, and *Hordeum vulgare*, were sampled in Algeria (Guelt Stel, Sétif, Adékar, and Hoggar, respectively).

Genomic DNA was extracted from 100 mg of fresh young leaves using the CTAB method adapted from Doyle and Doyle (1987). A potentially variable nuclear region was amplified using forward (F) and reverse (R) primers defined by Feltus et al. (2006): MA-5′(F) 5′-CGTGCTCATCAAAGAAGG-3′ and (R) 3′-CCCCAACTATACTGAGTTC-5′. This region, called PRSC1–005, was identified on chromosome 1 in rice as unspliced genomic protein kinase domain. PCR conditions were as follows: 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min; and 72 °C for 10 min. The purified PCR products were checked on agarose gel electrophoresis for the diploid and polyploid samples. When sequence heterogeneity was suspected (e.g., more than two amplified bands), the amplicons were cloned prior to sequencing. The PCR products from all the polyploid samples were cloned to isolate the duplicated nuclear copies.

DNA fragments were ligated to the pGEM-T Promega A3600 vector at 4 °C through T4 DNA ligase and transferred to 50 µL of competent cells (*Escherichia coli* DH5α). Transformation took place by electroporation at 1800 V. Twelve clones per individual were sequenced. The sequences produced in this study are deposited in the GenBank database at NCBI under the following accession numbers JF733552–JF733604.

All sequences generated from samples of the *H. murinum* complex were introduced and aligned in a data matrix using the BioEdit programme (Hall 1999). Also, sequences from closely related taxa; *H. marinum*, *H. bulbosum*, *H. vulgare*, and *H. spontaneum*, were included in the data matrix to serve as outgroup, as based on previous phylogenetic studies (de Bustos et al. 2002; Jakob and Blattner 2006, 2010; Sun et al. 2009). Sequence alignment was performed with the software MUSCLE (Edgar 2004). This alignment required inference of gaps of various lengths that were coded as binary (presence/absence) characters following the method of Simmons and Ochoterena (2000). Sequence lengths, the number of variable and parsimony informative characters, and the pairwise sequence divergence among the taxa were determined using PAUP* software v. 4.0b10 (Swofford 2002).

The aligned sequence data matrix was subjected to phylogenetic reconstruction using maximum parsimony (MP) and

maximum likelihood (ML) methods. MP analysis was performed using PAUP* with an heuristic search. The consistency index (CI) (Kluge and Farris 1969) and retention index (RI) (Farris 1989) were calculated. Robustness of the clades was estimated with the bootstrap method using 1000 replicates of heuristic searches (Felsenstein 1985). For the ML analysis, the appropriate nucleotide substitution model of sequence evolution was determined using the jModelTest program (Posada and Crandall 1998; Posada 2008, 2009) and the corrected Akaike information criterion (Posada and Buckley 2004). The best fit model selected by jModelTest, the General Time Reversible including Invariant sites model (GTR+I model), was then employed to analyze the data matrix with the PhyML program v. 3 (Guindon and Gascuel 2003; Guindon et al. 2010). In this analysis, all parameters were optimized and 1000 bootstrap replicates performed.

Results

Chromosome counts and cytotype distribution

Chromosome numbers were determined for each collected sample. Fifteen populations were found to be diploid ($2n = 2x = 14$) corresponding to subsp. *glaucum* and 26 were tetraploid ($2n = 4x = 28$) corresponding to subsp. *leporinum*. Mixed populations were identified in 13 sites containing both $2x$ and $4x$ ploidy levels in varying frequencies. The frequencies of $2x$ and $4x$ populations appear correlated to bioclimatic parameters and vary along the North–South biogeographical gradient as shown in Fig. 1. The subsp. *glaucum*, which exhibits high affinity for continentality, is encountered in stable and arid habitats such as sand dunes of steppe and chotts. The subsp. *leporinum* ($4x$) shows a wide distribution from the coastal regions to the Tellian Atlas mountains in the subhumid and humid bioclimatic zones.

Morphological variability of the cytotypes

A principal component analysis (PCA) based on 22 quantitative and qualitative morphological characters was performed on 420 individuals. The three first components cumulate ~79.75% of the total variation with 61.34% for PC 1 (Fig. 2). Except for characters X (ciliation of palea sides) and V (pedicel length of the central floret), all measured variables of culm and spikelet characters and O (pedicel length of the lateral floret) were significantly correlated to PC 1 (Fig. 2*a*). PC 2 separated the culm characters in the positive part from the spikelet characters in the negative part.

Analysis of individuals does not show clear morphological differentiation between the diploid (subsp. *glaucum*) and tetraploid (subsp. *leporinum* and subsp. *murinum*) populations (Fig. 2*b*).

The diploids are very polymorphic and form a continuum along PC 1. Populations from the Steppic highlands are clearly isolated in the negative part. These plants are characterized by particularly small values for all characters related to stubble, spike, and spikelets. In these samples, the lateral spikelets were pedunculate and the palea of the lateral floret was ciliated or weakly unilateral ciliated. The remaining diploid populations, distributed near the tetraploids on the first two axes are collected in the mountainous subhumid sites or in drier conditions from the plains.

The tetraploid populations are relatively homogeneous according to PC 1, and they are characterized by intermediate to high values of spikes, spikelets, and caryopsis characters. The French populations of subsp. *murinum* form one group discriminated in the positive part of PC 2 by short culm values. In the negative part of this second component (PC 2), samples of the subsp. *leporinum* ($4x$) originating from mountain populations form a second isolated group.

Some bioclimatic parameters in particular appear to be involved in the population differentiation. Figure 2*a* clearly shows the importance of summer temperatures (*M*) and winter temperatures (*m*), as well as altitude (Alt.), in discriminating groups along PC 1, while the bioclimatic coefficient (Q2) and rainfall (*P*) have a minor role. This suggests that the morphological differences revealed by this analysis are mainly shaped by temperature-related ecological parameters.

Meiotic behaviour of chromosomes

Meiotic behaviour was analysed on 150–300 PMC per population, at different stages of meiosis. The different types of chromosome association (univalent, bivalent, and multivalent) and the different abnormalities such as chromatin bridges and lagging chromosomes were noted. The various meiotic configurations observed are illustrated in Fig. 3 and their frequencies given in Table 3.

In the diploid populations (subsp. *glaucum*), meiosis is regular with essentially ring bivalents at diakinesis and metaphase I (6.85 ring II + 0.14 rod II). In this group, except for a very low frequency of univalents (Table 3), no multivalent or other irregularity was found. In the polyploid populations (subsp. *leporinum* ($4x$) and subsp. *murinum*), meiosis is less regular than in the diploids. Some populations of subsp. *leporinum* ($4x$) from high altitude exhibit a higher rate of multivalents (average > 0.34) and many irregularities such as chromatin bridges, lagging chromosomes, and supernumerary chromosomes. Chromatin bridges at late anaphase I suggest the presence of chromosomal inversions.

Flow cytometry analysis

Twenty-three plants of the *H. murinum* cytotypes ($2x$, $4x$, and $6x$) were analyzed by flow cytometry. The nuclear 2C DNA amount in the studied subspecies is presented in Table 4. This table shows no significant variation neither among plants of the same cytotype nor between the tetraploid cytotypes (subsp. *murinum* and subsp. *leporinum*). However, the amount of nuclear DNA in the tetraploid cytotypes is higher than the expected (double) values compared with the extant related diploid genomes (subsp. *glaucum*). Similarly, the genome size observed for the hexaploid cytotype is not additive regarding values encountered in the diploid and tetraploid cytotypes.

Molecular cytogenetic analyses

Although molecular cytogenetic approaches such as GISH were previously used in *Hordeum* to elucidate the genomic composition of some polyploid taxa (Taketa et al. 1999, 2009), to our knowledge, no data are available to date for the *H. murinum* complex.

GISH was performed to identify and characterize the genomes of the tetraploid taxa (subsp. *leporinum* and subsp. *murinum*) compared with the diploids. Whatever blocking/probe ratios and probe/blocking DNA combination tested,

Fig. 2. Principal component analysis of 53 populations of *Hordeum murinum*. (*a*) Loading of the 22 morphological characters including the five bioclimatic parameters (see Tables 1 and 2). (*b*) Distribution of the 55 populations on the basis of morphological characters averaged per population. Spot colors represent populations belonging to different subspecies: red, subsp. *glaucum* (2*x*); blue, subsp. *leporinum* (4*x*); green, subsp. *murinum* (4*x*).

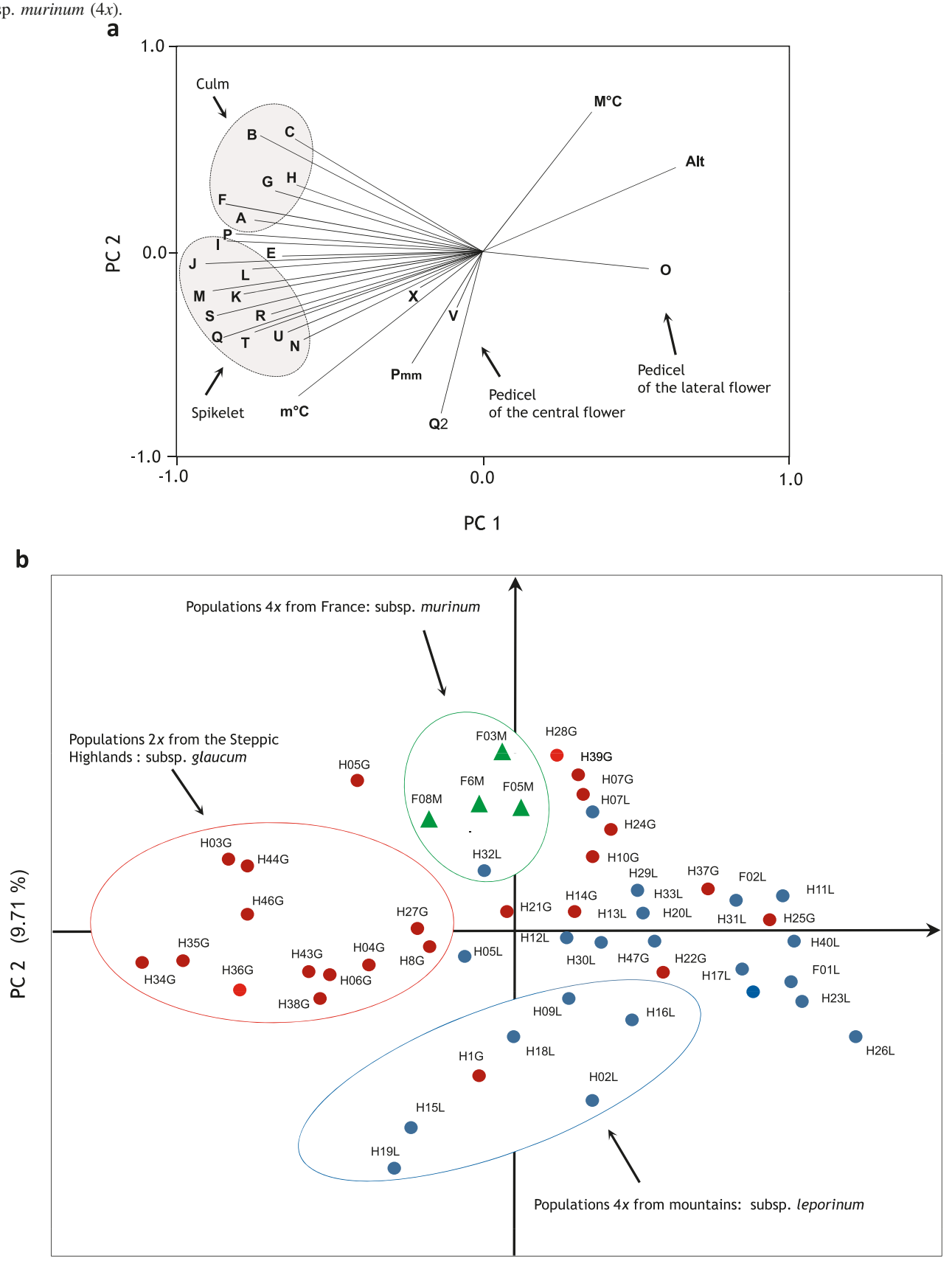
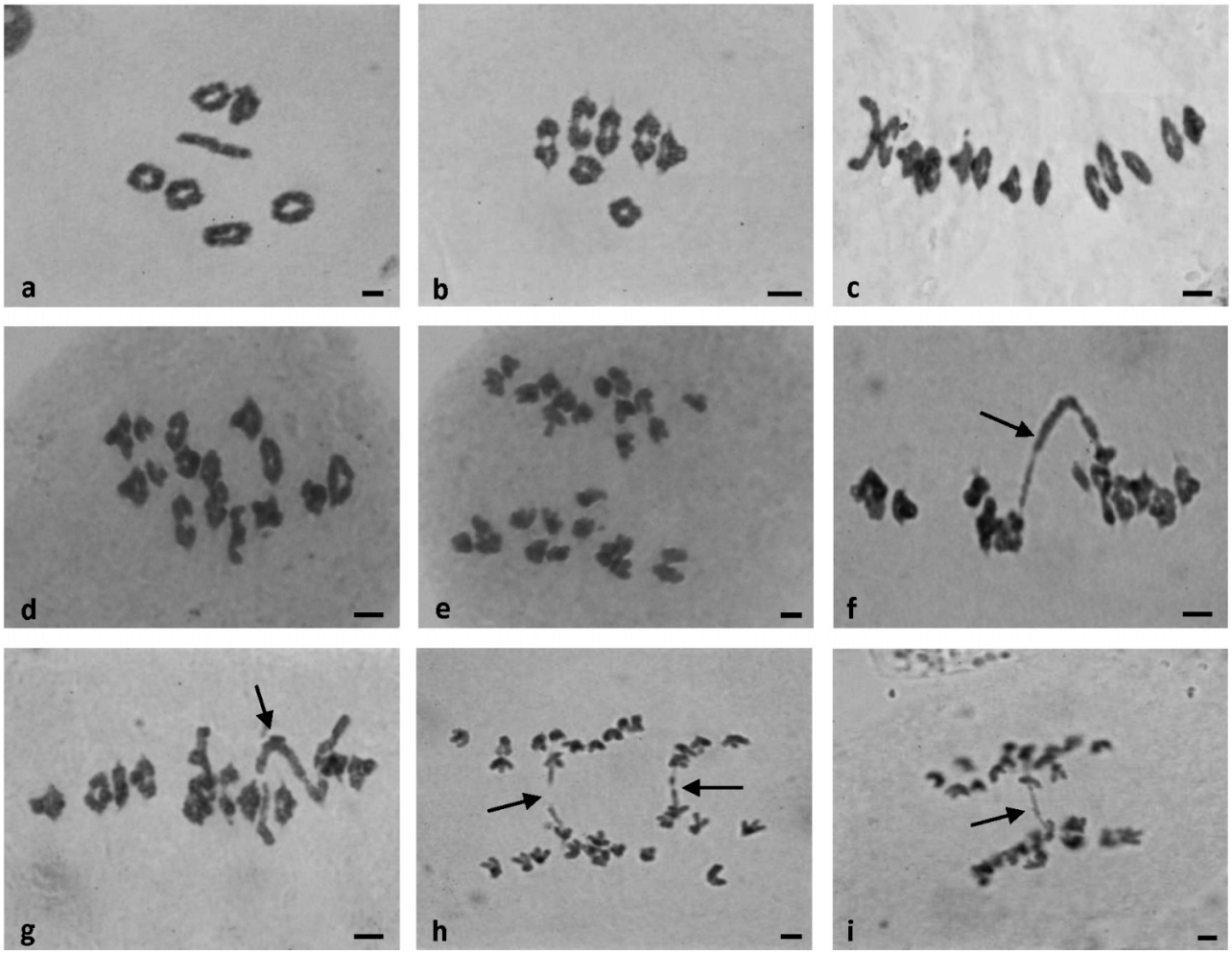


Fig. 3. Representative diploid and tetraploid meiotic configurations observed in natural populations of *Hordeum murinum* subspecies. (*a–b*) Diakinesis with 6 ring bivalents + 1 rod bivalent and with 7 ring bivalents in subsp. *glaucum*. (*c–d*) Metaphases I with 13 ring bivalents + 1 rod bivalent in subsp. *murinum*. (*e*) Regular anaphase I with $n = 14$ in subsp. *murinum*. (*f–g*) Metaphases I with multivalents (arrows) in subsp. *leporinum* (4*x*). (*h–i*) Anaphases I with chromosomes lagging and chromatin bridges (arrows) in subsp. *leporinum* (4*x*). Scale bar = 5 μm.



the two genomes present in the tetraploids showed no differentiation. In all cases, all chromosomes were labelled uniformly.

FISH analysis using both 5S and 45S rDNA probes revealed differences between diploids and tetraploids. Two 5S rDNA sites were detected in the diploid subsp. *glaucum* and 4 + 2 (four major sites and two minor sites) 5S sites in the tetraploid taxa subsp. *leporinum* and subsp. *murinum*. As well, four 45S rDNA sites in the diploid subsp. *glaucum* and 6 + 2 45S sites in the two tetraploid taxa subsp. *murinum* and subsp. *leporinum* were revealed. Figure 4*a–g* exhibits the in situ hybridization (GISH, FISH) results obtained on the tetraploid taxa subsp. *leporinum* and subsp. *murinum* and the diploid taxa subsp. *glaucum.*

Molecular phylogeny

Only one sequence type was found for the nuclear region analysed in each diploid (*H. marinum*, *H. spontaneum*, *H. vulgare*, *H. bulbosum*, and *H. glaucum*). Two different types of sequences (described below) were isolated after cloning in the tetraploids, whereas three sequence types were encountered in the hexaploid.

Alignment of the 53 amplified sequences from the eight studied taxa required the inference of 40 indels (insertions/deletions) with lengths ranging from 1 to 50 nucleotides. The length of this aligned matrix was 880 characters. When the indels were excluded and converted into coded characters, the new data matrix contained 835 characters, of which 627 were invariant and 128 were phylogenetically informative. Pairwise divergence among the analyzed sequences varied from 0.080 (*H. marinum – H. spontaneum*) to 0.00117 between some sequences from subsp. *glaucum*, subsp. *murinum*, and subsp. *leporinum* (4*x* and 6*x*). The MP analysis of this sequence generated 192 parsimonious trees. The strict consensus tree (tree length = 260, CI = 0.892, RI = 0.972) is shown in Fig. 5*a*. The analysis revealed that the *H. bulbosum – H. spontaneum – H. vulgare* group was sister to the rest of the studied taxa (100% bootstrap, 28 synapomorphies, and 2 informative indels).

Sequences of the *H. murinum* complex formed a sister

Table 3. Frequencies of the different meiotic chromosome associations per cell in the *Hordeum murinum* complex.

Subspecies	No. of populations	No. of PMCs	Chromosome associations/cell I	IId	IIa	IV
glaucum (2*x*)	12	222	0.001	0.14	6.85	—
leporinum (4*x*)						
Mountains	6	184	—	0.50	13.11	0.34
Plains	6	98	—	0.20	13.79	—
murinum (4*x*)	4	126	—	0.15	13.77	0.03

Note: PMCs, pollen mother cells; I, univalents; IId, rod bivalents; IIa, ring bivalents; and IV, quadrivalents.

Table 4. 2C DNA amounts in the *Hordeum murinum* complex.

Subspecies	No. of individuals	2C value (pg)	Expected 2C value (pg)
glaucum (2*x*)	11	8.27±0.14	—
leporinum (4*x*)	7	17.56±0.30	16.54
leporinum (6*x*)	3	26.44±0.40	24.81
murinum (4*x*)	2	17.25±0.25	16.54

clade to *H. marinum*, supported by 100% bootstrap, 12 synapomorphies, and 3 informative indels.

Sequences of the *H. murinum* complex are distributed in three lineages (A, B, C). Lineage A (100% bootstrap and 3 informative indels) includes all the sequences isolated in the diploid samples of subsp. *glaucum*, sequences from the tetraploids subsp. *murinum* and subsp. *leporinum*, and the hexaploid subsp. *leporinum*. The sequences of this clade were very similar, exhibiting 0 (most frequently) to 4 autapomorphies. Pairwise divergence in this clade varied from 0.0023 (*H. murinum* C2, *H. leporinum* C6, *H. leporinum* 6×C1, *H. glaucum* 6) to 0.016 (*H. glaucum* 13, *H. leporinum* 6×C4). Thus, in this lineage, no significant difference was detected between the taxa (subsp. *leporinum* (4*x*) and subsp. *glaucum*) sampled in Algeria (Tikjda, Gouraya, Guelt Stel, Kherrata, and Sétif) and the taxa sampled in France (subsp. *murinum*) and in Afghanistan (subsp. *leporinum* 6*x*).

Lineage B (97% bootstrap and 1 informative indel) includes only sequences isolated in the tetraploid and hexaploid cytotypes (subsp. *leporinum* (4*x*), subsp. *leporinum* (6*x*), and subsp. *murinum* 4*x*) that also appear very similar with 0–3 autapomorphies and pairwise distances ranging from 0.00 (*H. leporinum* C5, *H. leporinum* C3) to 0.005 (*H. leporinum* 6×C12, *H. leporinum* C7, *H. murinum* C11).

Lineage C includes only sequences isolated in the hexaploid cytotype (bootstrap 73%). Thus, both tetraploid subspecies carry the same two different sequence types distributed in clade A (with the diploid subsp. *glaucum*) and clade B, whereas the hexaploid exhibits the same two sequences encountered in the tetraploids plus one additional differentiated sequence type (in lineage C).

The tree resulting from the ML analysis, using the GTR+I model, is presented as a phylogram in Fig. 5*b*. Compared with the MP analysis, the *H. marinum* group's position was maintained as sister to the *H. murinum* complex with 100% of bootstrap support. Within the *H. murinum* complex, also the sequences were distributed in three distinct and well supported lineages, exactly corresponding to the A, B, and C assemblages circumscribed in the MP tree. In particular, the ML analysis significantly increased the bootstrap confidence of lineage C (from 73% in MP to 89.3% in ML), which only includes sequences from the hexaploid cytotype of *H. leporinum*. Moreover, whereas relationships among the three *H. murinum* lineages remained unresolved in the MP tree, the ML analysis revealed that lineages A and B likely derive from a common ancestor (77.7% bootstrap), and that they form together (A+B) the sister group of lineage C (99.1% bootstrap). Additionally, it can be noticed in the ML tree that among the three outgroups introduced in these analyses, *H. bulbosum* (rather than the *H. spontaneum* – *H. vulgare* complex) appeared as the closest relative (99.9% bootstrap) to the monophyletic *H. marinum* – *H. murinum* assemblage.

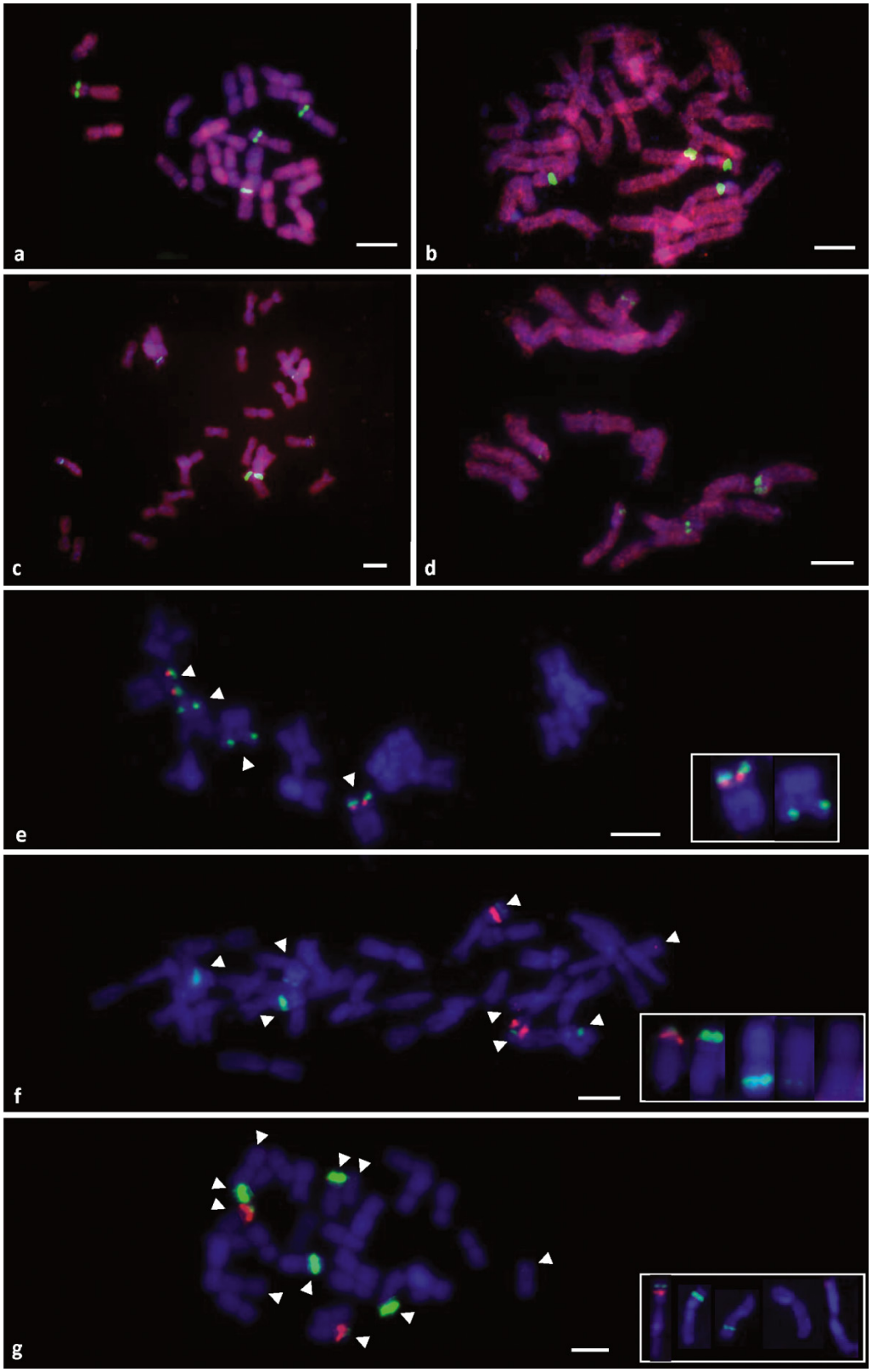
Discussion

In this paper, population diversity related to ecogeographical distribution and phylogenetic relationships were examined in the species of the *H. murinum* complex in Algeria that represents a large and poorly investigated region in North Africa. Phenotypic, cytogenetic, and molecular analyses were performed in natural populations across a large range of Mediterranean bioclimatic conditions.

Cytotype distribution and population diversity

Diploid ($2n = 2x = 14$) and tetraploid ($2n = 4x = 28$) cytotypes were encountered, belonging to subsp. *glaucum* and subsp. *leporinum*, respectively, as described by von Bothmer et al. (1995). Mixed populations occured in several sites containing both 2*x* and 4*x* ploidy levels, but no hexaploid cytotype was found. According to von Bothmer et al. (1995), the subsp. *murinum* occurs in northern and western Europe, from the Atlantic to the Caucasus area, usually not reaching the Mediterranean region. The subsp. *leporinum* (4*x*) is more thermophilous than subsp. *murinum* and rather occurs in the Mediterranean region, eastwards to Afghanistan. The diploid subsp. *glaucum* is restricted to xeric habitats in the southern Mediterranean region and eastwards to the Near East and Kashmir. One of the striking results of our study is the exis-

Fig. 4. GISH labelling of mitotic (metaphase) chromosome spreads in various subspecies of the *Hordeum murinum* complex. (*a–b*) Subsp. *murinum*, total genomic DNA from subsp. *murinum* was used as a probe (red) and subsp. *glaucum* as block (blue) (*a*) and subsp. *glaucum* was used as a probe (red) and subsp. *murinum* as block (blue) (*b*) with 1:50 ratio and 45S rDNA (green); (*c–d*) Subsp. *leporinum* (4*x*), total genomic DNA from subsp. *leporinum* was used as a probe (red) and subsp. *glaucum* as block (blue) (*c*) and subsp. *glaucum* was used as a probe (red) and subsp. *leporinum* as block (blue) (*d*) with 1:50 ratio and 45S rDNA (green). (*e–g*) Double-target FISH with 45S (green signals) and 5S (red signals) rDNA probes on subsp. *glaucum* (*e*), subsp. *leporinum* (4*x*) (*f*), and subsp. *murinum* (*g*). Scale bar = 5 μm.



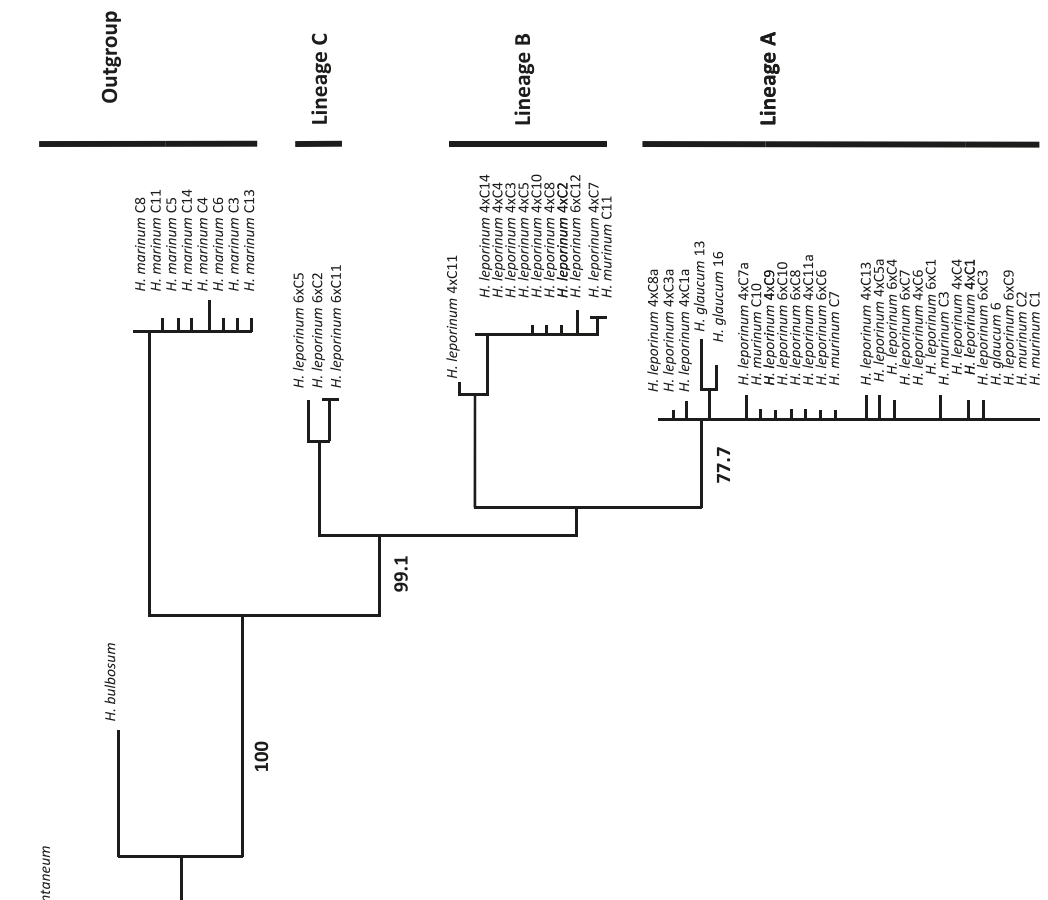


Fig. 5. Phylogenetic analyses of the *Hordeum murinum* complex based on the nuclear PRSC1-005 sequence. (*a*) Strict consensus tree of the 192 most parsimonious trees obtained with the maximum parsimony method. Tree length = 260, CI = 0.892, RI = 0.972, the bootstrap values are indicated above the branches, and the numbers of changes are represented in bold under the branches. (*b*) Maximum likelihood tree (the bootstrap values are indicated under the branches). Branch lengths are drawn according to the number of substitutions per position, with the bar indicating 0.1 substitutions per site.

tence in Algeria of a strong correlation between the distribution of 2*x* and 4*x* cytotypes and the North–South bioclimatic gradient. The frequencies of the diploids increased with aridity. Their chromosome behavior at meiosis was regular with mainly ring bivalents. The tetraploids are widespread in the northern regions where the bioclimatic conditions are most favourable. Such pattern of variation is in accordance with early observations of Reese (1958) who noticed a ploidy level range along a North–South gradient in North Africa and Europe with an obvious prominence of diploid taxa in the north African flora. However, samples from higher altitudes are characterized by an unexpected presence of quadrivalents (0.34) in contrast to the total lack of multivalent previously emphasized in several studies for subsp. *leporinum* (4*x*) and subsp. *murinum* (Gupta and Fedak 1985; von Bothmer et al. 1987, 1988; Jahan et al. 1992). Quadrivalents could lead to spontaneous structural rearrangements that may represent a significant source of structural and potentially adaptive diversity in these areas.

The bioclimatic parameters seem also to play an important role in structuring morphological variability. Thus, some populations of diploid subsp. *glaucum* from arid and semiarid sites of the Highlands differ from other diploid and tetraploid populations by their significant smaller culm and spikelet sizes.

Populations of subsp. *leporinum* (4*x*) are relatively polymorphic and clustered with diploid populations of subsp. *glaucum* from most favourable conditions, whereas some samples of *leporinum* (4*x*) from high altitudes tend to diverge from the other 4*x* populations. French populations of subsp. *murinum* are relatively more differentiated because of the geographical distance and the oceanic climate. However, the 23 morphological characters considered in our study did not allow significant discrimination among the three taxa, especially between subsp. *glaucum* and subsp. *leporinum* (4*x*). Booth and Richards (1978) reported no single diagnostic morphological character for subsp. *murinum* and subsp. *leporinum* (4*x*), but it seems that a combination of some characters can be useful, e.g., the pedicel length and the awn lemma of the central spikelet. However, on the basis of lodicule and epiblast characters, Baum and Bailey (1984*a*, 1984*b*) found that the diploid *H. glaucum* differs from the tetraploids *H. murinum* s. str. and *H. leporinum* that are close to each other over this diagnostic character.

In fact, according to Jacobsen and von Bothmer (1995) subsp. *murinum* is characterized by a sessile or subsessile pedicel of the central spikelet. The lemma awn of the central spikelet has the same length as the lemma awn of the lateral spikelet in subsp. *murinum*, whereas it is shorter in subsp. *leporinum* (4*x*) (Jacobsen and von Bothmer 1995). Thus, the three *H. murinum* subspecies are morphologically very similar to each other, which reinforce their treatment as the *H. murinum* complex (Covas 1949; Rajhathy and Morrison 1962; Jacobsen and von Bothmer 1995).

Origin of polyploids

Rajhathy and Morrison (1962) first postulated that the *H. murinum* complex was allopolyploid, based on studies of meiosis. They assumed that subsp. *glaucum* was one of the diploid progenitors of both tetraploids, subsp. *murinum* and subsp. *leporinum*, and that the hexaploid subsp. *leporinum* derives directly from the tetraploids. The allopolyploidy of the *H. murinum* complex is supported by a number of cytological studies based on meiosis (Morrison 1958; Rajhathy and Morrison 1962; Liu and Schooler 1965), karyotype (Richards and Booth 1976), and C-banding (Vosa 1976; Linde-Laursen et al. 1989) analyses. However, according to interspecific hybridization experiments among *Hordeum* species, von Bothmer et al. (1987, 1988) postulated that polyploid *H. murinum* species may have emerged via autopolyploidy. Diploidization of meiosis occurs in both allopolyploidy and autopolyploidy as a result of various mechanisms (Le Comber et al. 2010). In allopolyploids, the transition from polysomic to disomic inheritance takes place as an immediate consequence of polyploidization. Whereas in autopolypoids, diploidization is achieved through structural reorganization of chromosomes, loss of sequences involved in homology recognition, and initiation of meiotic pairing (Joppa et al. 1995; Belay and Merker 1997; Eilam et al. 2009) or through a polygenic control of the chromosome pairing (Jackson 1982; Sybenga 1999; Ramsey and Schemske 2002; Jenczewski and Alix 2004; Cifuentes et al. 2010). The recent results from cytological studies in successive generations of synthetic *Aegilops* and *Triticum* allopolyploid species showed that bivalent pairing was enhanced during each generation (Ozkan and Feldman 2009). A positive linear relationship was found between increased bivalent pairing and elimination of low-copy noncoding DNA sequences. These findings support the conclusion that rapid elimination of low-copy noncoding DNA sequences from one genome of a newly formed allopolyploid is an efficient way to quickly increase the divergence between homoeologous chromosomes and thus result in cytological diploidization.

In this study, we show that the tetraploids (subsp. *leporinum* and subsp. *murinum*) do not exhibit locus number additivity for both 5S and 45S rDNA loci, according to the number observed in the related diploid (subsp. *glaucum*). This could be explained by a hybrid origin involving parental species with a different number of loci and (or) by structural changes (i.e., gene loss) following polyploidization. Several cases of rDNA locus nonadditivity are reported in other species. The number of 45S rDNA loci in the allotetraploid *Brassica* is lower compared with the expected sum of loci in the progenitor diploid species (Maluszynska and Heslop-Harrison 1993). Changes in locus numbers have also been observed in synthetic *Nicotiana* (Skalická et al. 2003) and *Arabidopsis* (Pontes et al. 2004) allopolyploid lines.

The two basic genomes present in the tetraploids (subsp. *murinum* and subsp. *leporinum*) could not be identified by GISH. This suggests that these basic genomes are very similar. This similarity could either result from a recent divergence of the parental progenitors or from the presence of repetitive sequences (e.g., transposable elements) that could mask the differences that may exist between the parents; repetitive DNA was thus shown to comprise more than 70% of the barley genome (Vicient et al. 1999) that belongs to a sister lineage of *H. murinum*.

No difference in FISH patterns of both 5S and 45S rDNA sites was found between the two tetraploid cytotypes, suggesting a common origin. Both subsp. *murinum* and subsp. *leporinum* (4*x*) had (4 + 2) 5S and (6 + 2) 45S rDNA sites.

de Bustos et al. (1996) also found three major loci and one minor locus for the 18S–5.8S–26S rDNA locus in both subspecies, whereas Taketa et al. (1999) found 4 loci. For the 5S rDNA locus, de Bustos et al. (1996) found three pairs of major signals and one pair of minor signals, whereas Taketa et al. (1999) showed only three pairs. Despite this difference among the samples, the latter authors indicated no polymorphism for this site. However, it should be noted that intraspecific polymorphism of 5S rDNA site number was detected in *H. marinum* and *H. spontaneum* (Taketa et al. 1999). The difficulty in detecting minor signals of 5S rDNA sites in the Algerian samples may reflect their low copy number. When sequencing 5S rDNA loci, Baum and Johnson (1999) found a large number of sequences containing deletions (up to 50%) in *H. marinum*. Vershinin et al. (1996) reported that tandemly repeated DNA sequence organization is preserved in Triticeae, but that the copy number is drastically altered.

No difference in DNA amount was found between the tetraploid taxa (subsp. *leporinum* and subsp. *murinum*) in the studied samples. The similarity in nuclear DNA content between these taxa indicates that they share a common genome. Also, our results indicate that the polyploids contain a significantly higher amount of nuclear DNA than the expected additive amount. A similar finding was reported by Jakob et al. (2004) who suggested an allopolyploid origin for the tetraploids. However, this DNA increase could also be explained by the amplification of transposable elements subsequent to polyploidization as in the case of cotton (Hanson et al. 1999). In contrast, Eilam et al. (2009) noted in the *H. murinum* complex a lower genome size in the polyploids than expected from diploids. These authors explained the deviation from additivity by deletions occurring during the diploidization process.

Our phylogenetic analyses revealed the presence of two (in tetraploids) and three (in hexaploid) divergent sequences, revealing that three different lineages were involved in the formation of the polyploids in this complex. Both tetraploids exhibit identical sequences deriving from lineage A, which is closely related to the diploid subsp. *glaucum*, and from lineage B, deriving from another unidentified diploid parent. Hordein polymorphism of Algerian subsp. *leporinum* (4*x*) and French subsp. *murinum* populations also showed no significant difference between the two tetraploid subspecies (Amirouche and Misset 2003). AFLP data reported by El-Rabey et al. (2002) also indicated a close phylogenetic relationship between the two tetraploid subspecies in north African populations, pointing out a continuum between the two subspecies. The hexaploid taxa (subsp. *leporinum*) contains three sequence types (A, B, C), which also suggests an allotetraploid origin involving a tetraploid parent (AB) and another unidentified diploid (C) lineage, in agreement with recent findings obtained from other nuclear DNA sequences (Jakob and Blattner 2010; Tanno et al. 2010). As the non-*glaucum* sequences found in the polyploids do not relate to any known *Hordeum* taxon, these authors concluded that the ancestors carrying these sequences are nowadays extinct. However, a comprehensive study of diploid *H. murinum* populations through the natural range of the species is still lacking, so we cannot rule out the existence of other more or less differentiated *glaucum* lineages that could have provided the B and (or) C sequence types to the currently living polyploids. As an example of the diversity among subsp. *glaucum* populations, our six subsp. *glaucum* samples collected from an arid site (Guelt Stel) exhibited 11 nucleotide differences compared with the samples collected in more humid environments (16 from Sétif; 13 from Kherrata). Tanno et al. (2010) also found some sequence variants in different samples of the diploid subsp. *glaucum*.

Our molecular phylogenetic analysis also provides additional information regarding relationships of the *H. murinum* complex with other *Hordeum* lineages. The nuclear sequence analysed here indicates that the diploid *H. marinum* is sister to the *H. murinum* complex, and that the *H. vulgare* – *H. spontaneum* – *H. bulbosum* group has a basal position. In the literature published to date, conflicting results were reported regarding the relationships among these taxa (discussed in Blattner 2009). Various analyses based on nuclear sequences (Blattner 2004; Petersen and Seberg 2005; Kakeda et al. 2009; Sun et al. 2009; Jakob and Blattner 2006, 2010) considered the *H. murinum* complex a sister group to the *H. vulgare* – *H. bulbosum* complex (Blattner 2004; Jakob et al. 2004; Petersen and Seberg 2005; Kakeda et al. 2009; Sun et al. 2009; Jakob and Blattner 2010), whereas chloroplast and some nuclear (e.g., DMC1) sequences grouped *H. murinum* and *H. marinum* together (Provan et al. 1999; Terzi et al. 2001; de Bustos et al. 2002; Petersen and Seberg 2003, 2005; Jakob and Blattner 2006; Baum et al. 2010), as found in our analyses. Combined effects of different gene histories and possible reticulation events in the history of these taxa could account for the conflicting gene trees.

In conclusion, our study on diversity and evolution of the *H. murinum* polyploid complex, based on numerous populations sampled in various bioclimatic conditions in Algeria, provided a comprehensive picture of the cytotype distribution and abundance in this region. Results from morphological analysis confirmed the strong similarity between the three *H. murinum* subspecies *glaucum* (2*x*), *leporinum* (4*x*), and *murinum* (4*x*) but also demonstrated the consistent diversity among the diploid populations. These diploid populations are structured in small isolated populations whose abundance is strongly correlated with the North–South biogeographical gradient in Algeria. Meiosis was regular across the populations examined, although in some tetraploid populations of the subsp. *leporinum* an unexpected rate of multivalents and pairing irregularities was encountered. The striking correlation between the frequency of diploids and the bioclimatic parameters, the role of the transitional biogeographic area on their fragmentation and isolation in continental habitats (refuge), and their genetic diversity contradict the uniformity generally recognized for this taxon. Additionally, various lines of data from this study (GISH, FISH, and molecular phylogeny) support an allopolyploid origin for both tetraploid and hexaploid cytotypes.

Acknowledgements

This work is supported by the international Programme Hubert Curien CMEP-Tassili 08 MDU 724 “Polyploidy, Genome Evolution and Biodiversity” involving the Laboratoire de Biologie et Physiologie des Organismes, Université des Sciences et de la Technologie Houari Boumediene (Algeria)

and UMR CNRS Ecobio, Université de Rennes 1 (France). M.O. has benefited from the financial support of the University of Béjaia (Algeria).

References

Amirouche, R., and Misset, M.-T. 2003. Hordein polymorphism in diploid and tetraploid Mediterranean populations of the *Hordeum murinum* L. complex. Plant Syst. Evol. **242**(1–4): 83–99. doi:10.1007/s00606-003-0060-8.

Baum, B.R., and Bailey, G.L. 1984*a*. Taxonomic studies in wall barley (*Hordeum murinum*) and sea barley (*Hordeum marinum*). 1. Character investigation: assessment of new and traditional characters. Can. J. Bot. **62**(4): 753–762. doi:10.1139/b84-111.

Baum, B.R., and Bailey, G.L. 1984*b*. Taxonomic studies in wall barley (*Hordeum murinum* sensu lato) and sea barley (*Hordeum marinum* sensu lato). 2. Multivariate morphometrics. Can. J. Bot. **62**(12): 2754–2764. doi:10.1139/b84-368.

Baum, B.R., and Bailey, G.L. 1990. Key and synopsis of North American *Hordeum* species. Can. J. Bot. **68**(11): 2433–2442. doi:10.1139/b90-309.

Baum, B.R., and Johnson, D.A. 1999. The 5S rDNA gene in wall barley (*Hordeum murinum* L. sensu lato): sequence variation among repeat units and relationship to the Y haplome in the genus *Hordeum* (Poaceae, Triticeae). Genome, **42**(5): 854–866. doi:10.1139/g99-031. PMID:10584308.

Baum, B.R., Edwards, T., and Johnson, D.A. 2010. Codependence of repetitive sequence classes in genomes: phylogenetic analysis of 5S rDNA families in *Hordeum* (Triticeae: Poaceae). Genome, **53**(3): 180–202. doi:10.1139/G09-096. PMID:20237596.

Belay, G., and Merker, A. 1997. Cytogenetic studies in Ethiopian landraces of tetraploid wheat (*Triticum turgidurn* L.). II. Spontaneous chromosome translocations and fertility. Hereditas, **126**(1): 35–43. doi:10.1111/j.1601-5223.1997.00035.x.

Blattner, F.R. 2004. Phylogenetic analysis of *Hordeum* (Poaceae) as inferred by nuclear rDNA ITS sequences. Mol. Phylogenet. Evol. **33**(2): 289–299. doi:10.1016/j.ympev.2004.05.012. PMID:15336664.

Blattner, F.R. 2009. Progress in phylogenetic analysis and new infrageneric classification of the barley genus *Hordeum* (Poaceae: Triticeae). Breed. Sci. **59**(5): 471–480. doi:10.1270/jsbbs.59.471.

Booth, T.A., and Richards, A.J. 1978. Studies in the *Hordeum murinum* L., aggregate: disc electrophoresis of seed proteins. Bot. J. Linn. Soc. **76**(2): 115–125. doi:10.1111/j.1095-8339.1978.tb01501.x.

Cifuentes, M., Grandont, L., Moore, G., Chèvre, A.-M., and Jenczewski, E. 2010. Genetic regulation of meiosis in polyploid species: new insights into an old question. New Phytol. **186**(1): 29–36. doi:10.1111/j.1469-8137.2009.03084.x. PMID:19912546.

Covas, G. 1949. Taxonomic observations on the North American species of *Hordeum*. Madrono, **10**: 1–21.

de Bustos, A., Cuadrado, A., Soler, C., and Jouve, N. 1996. Physical mapping of repetitive DNA sequences and 5S and 18S–26S rDNA in wild species of the genus *Hordeum*. Chromosome Res. **4**(7): 491–499. doi:10.1007/BF02261776. PMID:8939360.

de Bustos, A., Soler, C., and Jouve, N. 1999. Analysis by PCR-based markers using designed primers to study relationships between species of *Hordeum* (Poaceae). Genome, **42**(1): 129–138. doi:10.1139/g98-109.

de Bustos, A., Loarce, Y., and Jouve, N. 2002. Species relationships between antifungal chitinase and nuclear rDNA (internal transcribed spacer) sequences in the genus *Hordeum*. Genome, **45**(2): 339–347. doi:10.1139/g01-158. PMID:11962631.

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**(1): 11–15.

Edgar, R.C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics, **5**: 113. doi:10.1186/1471-2105-5-113. PMID:15318951.

Eilam, T., Anikster, Y., Millet, E., Manisterski, J., and Feldman, M. 2009. Genome size in natural and synthetic autopolyploids and in natural segmental allopolyploids of several Triticeae species. Genome, **52**(3): 275–285. doi:10.1139/G09-004. PMID:19234556.

El-Rabey, H.A., Badr, A., Schäfer-Pregl, R., Martin, W., and Salamini, F. 2002. Speciation and species separation in *Hordeum* L. (Poaceae) resolved by discontinuous molecular markers. Plant Biol. **4**(5): 567–575. doi:10.1055/s-2002-35435.

Farris, J.S. 1989. The retention index and rescaled consistency index. Cladistics, **5**(4): 417–419. doi:10.1111/j.1096-0031.1989.tb00573.x.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, **39**(4): 783–791. doi:10.2307/2408678.

Feltus, F.A., Singh, H.P., Lohithaswa, H.C., Schulze, S.R., Silva, T.D., and Paterson, A.H. 2006. A comparative genomics strategy for targeted discovery of single-nucleotide polymorphisms and conserved-noncoding sequences in orphan crops. Plant Physiol. **140**(4): 1183–1191. doi:10.1104/pp.105.074203. PMID:16607031.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., and Firoozabady, E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science, **220**(4601): 1049–1051. doi:10.1126/science.220.4601.1049. PMID:17754551.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**(7): 1869–1885. doi:10.1093/nar/7.7.1869. PMID:537913.

Gerlach, W.L., and Dyer, T.A. 1980. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Res. **8**(21): 4851–4865. doi:10.1093/nar/8.21.4851. PMID:7443527.

Giles, B.E. 1984. A comparison between quantitative and biochemical variation in the wild barley *Hordeum murinum*. Evolution, **38**(1): 34–41. doi:10.2307/2408544.

Giles, B.E., and Lefkovitch, L.P. 1986. A taxonomic investigation of the *Hordeum murinum* complex (Poaceae). Plant Syst. Evol. **153**(3–4): 181–197. doi:10.1007/BF00983686.

Guindon, S., and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52**(5): 696–704. doi:10.1080/10635150390235520. PMID:14530136.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. **59**(3): 307–321. doi:10.1093/sysbio/syq010. PMID:20525638.

Gupta, P.K., and Fedak, G. 1985. Intergeneric hybrids between *Hordeum californicum* and *Triticum aestivum*. J. Hered. **76**(5): 365–368.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41**: 95–98.

Hanson, R.E., Islam-Faridi, M.N., Crane, C.F., Zwick, M.S., Czeschin, D.G., Wendel, J.F., et al. 1999. Ty1-*copia*-retrotransposon behavior in a polyploid cotton. Chromosome Res. **8**(1): 73–76. doi:10.1023/A:1009239522541. PMID:10730591.

Jackson, R.C. 1982. Polyploidy and diploidy: new perspectives on chromosome pairing and its evolutionary implications. Am. J. Bot. **69**(9): 1512–1523. doi:10.2307/2443113.

Jacobsen, N., and von Bothmer, R. 1995. Taxonomy in the *Hordeum murinum* complex (Poaceae). Nord. J. Bot. **15**(5): 449–458. doi:10.1111/j.1756-1051.1995.tb00177.x.

Jahan, B., Vahiny, A.A., and Ali, S.I. 1992. Chiasma frequency and chromosome associations in sixteen taxa of *Hordeum* species. Cytologia (Tokyo), **57**(1): 51–57.

Jakob, S.S., and Blattner, F.R. 2006. A chloroplast genealogy of *Hordeum* (Poaceae): long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Mol. Biol. Evol. **23**(8): 1602–1612. doi:10.1093/molbev/msl018. PMID:16754643.

Jakob, S.S., and Blattner, F.R. 2010. Two extinct diploid progenitors were involved in allopolyploid formation in the *Hordeum murinum* (Poaceae: Triticeae) taxon complex. Mol. Phylogenet. Evol. **55**(2): 650–659. doi:10.1016/j.ympev.2009.10.021. PMID:19850141.

Jakob, S.S., Meister, A., and Blattner, F.R. 2004. The considerable genome size variation of *Hordeum* species (Poaceae) is linked to phylogeny, life form, ecology, and speciation rates. Mol. Biol. Evol. **21**(5): 860–869. doi:10.1093/molbev/msh092. PMID:15014163.

Jenczewski, E., and Alix, K. 2004. From diploids to allopolyploids: the emergence of pairing control genes. Crit. Rev. Plant Sci. **23**(1): 21–45. doi:10.1080/07352680490273239.

Joppa, L.R., Nevo, E., and Beiles, A. 1995. Chromosomal translocations in wild populations of tetraploid emmer wheat in Israel and Turkey. Theor. Appl. Genet. **91**(5): 713–719. doi:10.1007/BF00220948.

Kakeda, K., Taketa, S., and Komatsuda, T. 2009. Molecular phylogeny of the genus *Hordeum* using thioredoxin-like gene sequences. Breed. Sci. **59**(5): 595–601. doi:10.1270/jsbbs.59.595.

Kankanpää, J., Mannonen, L., and Schulman, A.H. 1996. The genome sizes of *Hordeum* species show considerable variation. Genome, **39**(4): 730–735. doi:10.1139/g96-092. PMID:18469933.

Kluge, A.G., and Farris, J.S. 1969. Quantitative phylogenetics and the evolution of anurans. Syst. Zool. **18**(1): 1–32. doi:10.2307/2412407.

Kochieva, E.Z., Goryunova, S.V., and Pomortsev, A.A. 2001. RAPD analysis of the genome in species of the genus *Hordeum*. Russ. J. Genet. **37**(8): 905–910. doi:10.1023/A:1016777616536.

Komatsuda, T., Tanno, K.I., Salomon, B., Bryngelsson, T., and von Bothmer, R. 1999. Phylogeny in the genus *Hordeum* based on nucleotide sequences closely linked to the *vrs 1* locus (row number of spikelets). Genome, **42**(5): 973–981. doi:10.1139/g99-025. PMID:10584315.

Konishi, T. 1989. Biosystematic studies on the *Hordeum murinum* aggregate. Ber. Ohara Inst. Landwirtsch. Biol. Okayama Univ. **19**(4): 189–198.

Le Comber, S.C., Ainouche, M.L., Kovarik, A., and Leitch, A.R. 2010. Making a functional diploid: from polysomic to disomic inheritance. New Phytol. **186**(1): 113–122. doi:10.1111/j.1469-8137.2009.03117.x. PMID:20028473.

Linde-Laursen, I., von Bothmer, R., and Jacobsen, N. 1989. Giemsa C-banding karyotypes of *Hordeum marinum* and *H. murinum*. Genome, **32**(4): 629–639. doi:10.1139/g89-491.

Linde-Laursen, I., von Bothmer, R., and Jacobsen, N. 1995. Karyotype differentiation and evolution in the genus *Hordeum* (Poaceae). *In* Kew Chromosome Conference IV. *Edited by* P.E. Brandham and M.D. Bennett. Royal Botanic Gardens, Kew. pp. 233–247.

Liu, C.I., and Schooler, A.B. 1965. *Hordeum depressum* crosses with the *Hordeum murinum* complex. Can. J. Genet. Cytol. **7**(2): 291–295. doi:10.1139/g65-040.

Maluszynska, J., and Heslop-Harrison, J.S. 1993. Physical mapping of rDNA in *Brassica* species. Genome, **36**(4): 774–781. doi:10.1139/g93-102. PMID:18470023.

Morrison, J.W. 1958. *Hordeum murinum* in Holland. Acta Bot. Neerl. **7**: 654–664.

Nishikawa, T., Salomon, B., Komastuda, T., von Bothmer, R., and Kadowaki, K. 2002. Molecular phylogeny of the genus *Hordeum* using three chloroplast DNA sequences. Genome, **45**(6): 1157–1166. doi:10.1139/g02-088. PMID:12502262.

Ozkan, H., and Feldman, M. 2009. Rapid cytological diploidization in newly formed allopolyploids of the wheat (*Aegilops–Triticum*) group. Genome, **52**(11): 926–934. doi:10.1139/G09-067. PMID:19935917.

Petersen, G., and Seberg, O. 2003. Phylogenetic analysis of the diploid species of *Hordeum* (Poaceae) and revised classification of the genus. Syst. Bot. **28**(2): 293–306.

Petersen, G., and Seberg, O. 2005. Phylogenetic analysis of allopolyploid species. Czech J. Genet. Plant Breed. **41**(Special Issue): 28–37.

Pontes, O., Neves, N., Silva, M., Lewis, M.S., Madlung, A., Comai, L., et al. 2004. Chromosomal locus rearrangements are a rapid response to formation of the allotetraploid *Arabidopsis suecica* genome. Proc. Natl. Acad. Sci. U.S.A. **101**(52): 18 240–18 245. doi:10.1073/pnas.0407258102. PMID:15604143.

Posada, D. 2008. JModelTest: phylogenetic model averaging. Mol. Biol. Evol. **25**(7): 1253–1256. doi:10.1093/molbev/msn083. PMID:18397919.

Posada, D. 2009. Selection of models of DNA evolution with jModelTest. Methods Mol. Biol. **537**: 93–112. doi:10.1007/978-1-59745-251-9_5. PMID:19378141.

Posada, D., and Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike Information Criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. **53**(5): 793–808. doi:10.1080/10635150490522304. PMID:15545256.

Posada, D., and Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics, **14**(9): 817–818. doi:10.1093/bioinformatics/14.9.817. PMID:9918953.

Provan, J., Russell, J.R., Booth, A., and Powell, W. 1999. Polymorphic chloroplast simple sequence repeat primers for systematic and population studies in the genus *Hordeum*. Mol. Ecol. **8**(3): 505–511. doi:10.1046/j.1365-294X.1999.00545.x. PMID:10199011.

Rajhathy, T., and Morrison, J.W. 1962. Cytogenetic studies in the genus *Hordeum*. 6. The *murinum* complex. Can. J. Genet. Cytol. **4**(2): 240–247. doi:10.1139/g62-029.

Ramsey, J., and Schemske, D.W. 2002. Neopolyploidy in flowering plants. Annu. Rev. Ecol. Syst. **33**(1): 589–639. doi:10.1146/annurev.ecolsys.33.010802.150437.

Reese, G. 1958. Über die Polyploïdiespektren in der Nordsaharischen Wustenpflanzen. Flora, **144**: 598–634.

Richards, A.J., and Booth, T.A. 1976. Karyological indications of evolution in *Hordeum murinum* L. sensu lato. *In* Proceedings of Kew Chromosome Conference, Royal Botanical Garden, Kew, England, pp. 167–174.

Rohlf, F.J. 1990. NTSYS-pc: numerical taxonomy and multivariate analysis system. Version 1.60. Exeter Software, Setauket, New York.

Savova-Bianchi, D., Keller-Senften, J., and Felber, F. 2002. Isozyme variation of *Hordeum murinum* L. in Switzerland and test of hybridization with cultivated barley. Weed Res. **42**(4): 325–333. doi:10.1046/j.1365-3180.2002.00292.x.

Simmons, M.P., and Ochoterena, H. 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. **49**(2): 369–381. doi:10.1093/sysbio/49.2.369. PMID:12118412.

Skalická, K., Lim, K.Y., Matyásek, R., Koukalová, B., Leitch, A.R., and Kovarík, A. 2003. Rapid evolution of parental rDNA in a

synthetic tobacco allotetraploid line. Am. J. Bot. **90**(7): 988–996. doi:10.3732/ajb.90.7.988.

Stewart, P. 1974. Un nouveau climagramme pour l'Algérie et son application au barrage vert. Bull. Soc. Hist. Nat. Afrique du Nord. **65**: 239–248.

Sun, G., Pourkheirandish, M., and Komatsuda, T. 2009. Molecular evolution and phylogeny of the *RPB2* gene in the genus *Hordeum*. Ann. Bot. (Lond.), **103**(6): 975–983. doi:10.1093/aob/mcp020. PMID:19213797.

Svitashev, S.K., Vershinin, A.V., Trunova, S.A., Pershina, L.A., and Shumny, V.K. 1995. Molecular analysis of the genomes of wide hybrids in cereals. Hereditas, **122**(1): 25–31. doi:10.1111/j.1601-5223.1995.00025.x.

Swofford, D.L. 2002. PAUP: phylogenetic analysis using parsimony and other methods. Version 4.b10. Sinauer Associates, Sunderland, Massachusetts.

Sybenga, J. 1999. What makes homologous chromosomes find each other in meiosis? A review and an hypothesis. Chromosoma, **108** (4): 209–219. doi:10.1007/s004120050371. PMID:10460409.

Taketa, S., Harrison, G.E., and Heslop-Harrison, J.S. 1999. Comparative physical mapping of 5S and 18S–25S rDNA in nine wild *Hordeum* species and cytotypes. Theor. Appl. Genet. **98** (1): 1–9. doi:10.1007/s001220051033.

Taketa, S., Ando, H., Taketa, K., Harrison, G.E., and Heslop-Harrison, J.S. 2000. The distribution, organization and evolution of two abundant and widespread repetitive DNA sequences in the genus *Hordeum*. Theor. Appl. Genet. **100**(2): 169–176. doi:10.1007/s001220050023.

Taketa, S., Nakauchi, Y., and von Bothmer, R. 2009. Phylogeny of two tetraploid *Hordeum* species, *H. secalinum* and *H. capense* inferred from physical mapping of 5S and 18S–25S rDNA. Breed. Sci. **59**(5): 589–594. doi:10.1270/jsbbs.59.589.

Tanno, K., von Bothmer, R., Yamane, K., Takeda, K., and Komatsuda, T. 2010. Analysis of DNA sequence polymorphism at the *cMWG699* locus reveals phylogenetic relationships and allopolyploidy within *Hordeum murinum* subspecies. Hereditas, **147**(1): 34–42. doi:10.1111/j.1601-5223.2009.02142.x. PMID: 20416015.

Terzi, V., Pecchioni, N., Faccioli, P., Kucera, L., and Stanca, A.M. 2001. Phyletic relationships within the genus *Hordeum* using PCR-based markers. Genet. Resour. Crop Evol. **48**(5): 447–458. doi:10.1023/A:1012031413581.

Vershinin, A.V., Alkhimova, E.G., and Heslop-Harrison, J.S. 1996. Molecular diversification of tandemly organised sequences and heterochromatic chromosome regions in some Triticeae species. Chromosome Res. **4**(7): 517–525. doi:10.1007/BF02261779. PMID:8939363.

Vicient, C.M., Suoniemi, A., Anamthawat-Jónsson, K., Tanskanen, J., Beharav, A., Nevo, E., and Schulman, A.H. 1999. Retrotransposon *BARE-1* and its role in genome evolution in the genus *Hordeum*. Plant Cell, **11**(9): 1769–1784. doi:10.1105/tpc.11.9.1769. PMID:10488242.

von Bothmer, R., and Jacobsen, N. 1986. Interspecific crosses in *Hordeum* (Poaceae). Plant Syst. Evol. **153**(1–2): 49–64. doi:10.1007/BF00989417.

von Bothmer, R., Flink, J., and Landström, T. 1987. Meiosis in interspecific *Hordeum* hybrids. II. Triploid combinations. Evol. Trends Plants, **1**: 41–50.

von Bothmer, R., Flink, J., and Landström, T. 1988. Meiosis in interspecific *Hordeum* hybrids. IV. Tetraploid ($4x \times 4x$) hybrids. Genome, **30**(4): 479–485. doi:10.1139/g88-080.

von Bothmer, R., Flink, J., and Landström, T. 1986. Meiosis in interspecific *Hordeum* hybrids. I. Diploid combinations. Can. J. Genet. Cytol. **28**(4): 525–535. doi:10.1139/g86-077.

von Bothmer, R., Jacobsen, N., Baden, C., Jørgensen, R.B., and Linde-Laursen, I. 1995. An ecogeographical study of the genus *Hordeum*. Systematic and ecogeographic studies on crop genepool. N°7. International Plant Genetic Resources Institute, Rome.

Vosa, C.G. 1976. Chromosome banding patterns in cultivated and wild barleys (*Hordeum* spp.). Heredity, **37**(3): 395–403. doi:10.1038/hdy.1976.104.

OPEN ACCESS Freely available online

PLoS one

The Core Binding Factor CBF Negatively Regulates Skeletal Muscle Terminal Differentiation

Ophélie Philipot[¤], Véronique Joliot[¤], Ouardia Ait-Mohamed[¤], Céline Pellentz[¤], Philippe Robin[¤], Lauriane Fritsch[¤], Slimane Ait-Si-Ali*[¤]

Institut André Lwoff, FRE2944, CNRS and Université Paris-Sud, Villejuif, France

Abstract

Background: Core Binding Factor or CBF is a transcription factor composed of two subunits, Runx1/AML-1 and CBF beta or CBFβ. CBF was originally described as a regulator of hematopoiesis.

Methodology/Principal Findings: Here we show that CBF is involved in the control of skeletal muscle terminal differentiation. Indeed, downregulation of either Runx1 or CBFβ protein level accelerates cell cycle exit and muscle terminal differentiation. Conversely, overexpression of CBFβ in myoblasts slows terminal differentiation. CBF interacts directly with the master myogenic transcription factor MyoD, preferentially in proliferating myoblasts, *via* Runx1 subunit. In addition, we show a preferential recruitment of Runx1 protein to MyoD target genes in proliferating myoblasts. The MyoD/CBF complex contains several chromatin modifying enzymes that inhibits MyoD activity, such as HDACs, Suv39h1 and HP1β. When overexpressed, CBFβ induced an inhibition of activating histone modification marks concomitant with an increase in repressive modifications at MyoD target promoters.

Conclusions/Significance: Taken together, our data show a new role for Runx1/CBFβ in the control of the proliferation/differentiation in skeletal myoblasts.

Citation: Philipot O, Joliot V, Ait-Mohamed O, Pellentz C, Robin P, et al. (2010) The Core Binding Factor CBF Negatively Regulates Skeletal Muscle Terminal Differentiation. PLoS ONE 5(2): e9425. doi:10.1371/journal.pone.0009425

Editor: Axel Imhof, Ludwig-Maximilians-Universität München, Germany

Received August 21, 2009; **Accepted** February 3, 2010; **Published** February 25, 2010

Funding: This work was supported by the Association Française contre les Myopathies (AFM); the Fondation Bettencourt-Schueller; the Agence Nationale de la Recherche (ANR); the Ligue Nationale contre le Cancer; the Association pour la Recherche sur le Cancer (ARC), the CNRS; the Université Paris-Sud and the Université Paris Diderot. PO was recipient of fellowships from the Ministère de la Recherche and the ARC. AO was recipient of a fellowship from the ''Programme franco-algerien de formation supérieure en France''. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: slimane.aitsiali@univ-paris-diderot.fr

¤ Current address: UMR7216 Epigénétique et Destin Cellulaire, Centre National de la Recherche Scientifique (CNRS) and Université Paris-Diderot, Paris, France

Introduction

Runx1 (for Runt-related transcription factor 1, also known as AML1 for Acute Myeloid Leukemia 1, CBFA2 or PEPB2αB) belongs to a family of highly homologous heterodimeric transcription factors named Core Binding Factors or CBF (reviewed in: [1]). In addition to the Runx1 subunit which binds DNA directly, CBF is composed of a non-DNA-binding subunit named CBFbeta (CBFβ) [2]. Runx1 binds better DNA in the presence of CBFβ. *Runx1* was originally identified at a breakpoint on human chromosome 21 in the t(8;21) translocation, known as the most common target of chromosomal translocations in human leukemia [3,4]. Genetic studies showed that Runx1 is essential in the developing murine embryo for definitive hematopoiesis of all lineages [5,6].

There is now strong evidence that Runx proteins are also important for differentiation of multiple cell types, including osteoblasts [7], neurons [8,9], hematopoietic cells of all lineages [5,6,10] and skin epidermis and hair follicle stem cells [11,12]. Runx1 is also involved in promoting senescence in primary mouse fibroblasts [13], and in cell cycle regulation [14–16].

Runx proteins have the potential to either activate or repress transcription in a context dependent manner. Runx1 seems to promote proliferation in progenitor cells, whereas in differentiating cells it cooperates with tissue-specific transcription factors to regulate tissue-specific gene expression. For example, Runx1 cooperates with C/EBPα and C/EBPβ to regulate hematopoiesis and osteogenesis, respectively [17,18]. The dual role of Runx1 in regulating proliferation and differentiation could depend on differential interactions with protein partners, specific for each stage of cell development. The molecular mechanisms underlying such a switch in Runx1 function remain however to be deciphered.

Runx1 and CBFβ have also been linked to skeletal muscle differentiation [19–21], and prevention of muscle wasting [20]. In skeletal muscle, proliferation and differentiation are mutually exclusive. Indeed, skeletal muscle terminal differentiation begins with an irreversible withdrawal from the cell cycle, followed by muscle-specific marker expression [22]. Irreversible cell cycle exit involves a definitive silencing of proliferation-associated genes (reviewed in [23] and references therein). Terminal muscle differentiation is orchestrated by myogenic bHLH transcription factors, such as MyoD and Myf5, two master myogenic determination factors. MyoD is expressed in proliferating myoblasts, but is unable to activate its target genes even when

A

CBFβ peptide	Hits	% aa	% mass
SKFENEEFFR	3	5.5	6.2
AQQEDALAQQAFEEAR	3	8.8	8.4
VSQLLAVTGK	2	5.5	4.7
	Total	19.8	19.3

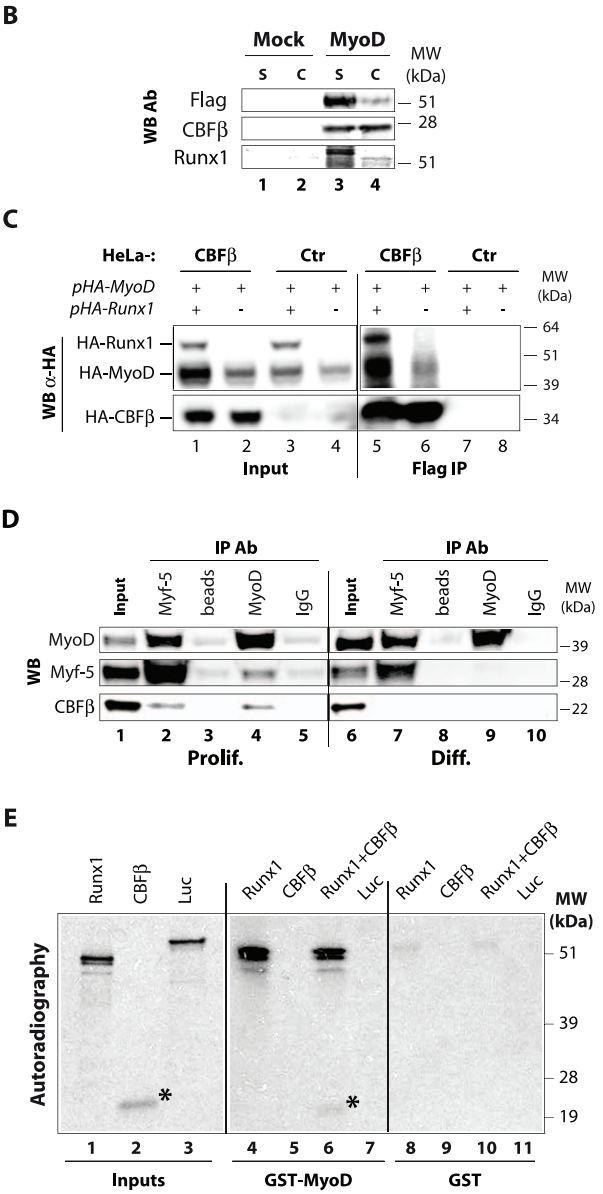


Figure 1. Runx1 and CBFβ interact with MyoD *in vitro* and in proliferating myoblasts. A. Peptide sequences identified by mass spectrometry in the MyoD complex corresponding to CBFβ protein. **B.** Western blot analysis of double-purified Flag-HA-MyoD (MyoD), or eluate from HeLa control cells (Mock) with the indicated antibodies (WB Ab). S: soluble; C: chromatin associated. **C.** HeLa cells stably expressing Flag-HA-CBFβ (eCBFβ) or HeLa control cells stably transfected with the empty vector (Ctr) were transiently transfected with expressing vectors for HA-tagged MyoD and/or HA-tagged Runx1. 24 hours post-transfection, cells were harvested and lysates were used for immunoprecipitation (IP) using Flag resin to precipitate Flag-HA-CBFβ. Precipitates were then subjected to western blot using HA Ab (WB α-HA) to simultaneously detect HA-Runx1, HA-MyoD, and Flag-HA-CBFβ (discriminated on the gel by their molecular weight). However, we have checked the identity of each HA-revealed band by using antibodies recognizing the native proteins (Figure S8). **D.** Nuclear extracts from proliferating (prolif.) or differentiating myoblasts (48 h, indicated as Diff.) were used for immunoprecipitation (IP) with antibodies (Ab) raised against MyoD (lanes 4 and 9) and Myf5 (lanes 2 and 7), with control beads (lanes 3 and 8) or with normal rabbit IgG (lanes 5 and 10) as negative controls. The resulting precipitates were then subjected to western blot analysis (WB) for the presence of MyoD, Myf5 and CBFβ. Input extracts were loaded to show endogenous protein levels (lanes 1 and 6). **E.** Runx1, CBFβ, or luciferase (Luc) were *in vitro* translated in the presence of ^{35}S-Methionine (inputs on lanes 1-3, respectively) and incubated with equivalent amounts of GST-MyoD beads (lanes 4–7) or GST beads (lanes 8–11). GST pull-down was then conducted as described in the Material and Methods section, and the radiolabeled proteins were detected by autoradiography. *: CBFβ.
doi:10.1371/journal.pone.0009425.g001

bound to their promoters [24,25]. MyoD therefore may have a repressive role at its target genes prior to initiating chromatin remodeling in differentiating cells [24,26,27]. In proliferating myoblasts, MyoD is associated with histone deacetylases (HDACs), the histone methyltransferase Suv39h1 and heterochromatin protein HP1, and might actively inhibit expression of its target genes by inducing a local repressive chromatin structure [24,28,29].

Here we show that CBF associates with MyoD preferentially in proliferating myoblasts, and knockdown of Runx1 or CBFβ accelerates cell cycle exit and terminal differentiation. Conversely, overexpression of CBF slows cell cycle exit and delays muscle differentiation. In proliferating myoblasts, the MyoD/CBF complex contains several chromatin modifying enzymes such as HDACs. In agreement with this, when overexpressed, CBFβ maintains histone H3 hypoacetylated, hypomethylated on lysine 4 and hypermethylated on lysine 9, on MyoD target promoters, along with HDAC1 recruitment, even in differentiation conditions. Finally, Runx1 is recruited to MyoD target genes preferentially in proliferating myoblasts, when these genes are repressed. Altogether, our data suggest that CBF transcription factor plays a pivotal role as a negative regulator of skeletal muscle terminal differentiation.

Results

CBF Subunits, Runx1 and CBFβ, Interact with MyoD in Proliferating Myoblasts

In an attempt to characterize MyoD protein partners, we carried out double-affinity purification of HA-Flag MyoD stably expressed in HeLa cells (see purification scheme on Figure S1). MyoD protein complex composition was then analyzed by mass spectrometry (MS) and western blot (WB). MS analysis of the purified protein complex revealed some already known partners of MyoD (Figure S2), such as Id, Pbx1, PC4 and E12/E47, and partners that had never been described to interact with MyoD. Indeed, MS analysis unveiled CBFβ protein within MyoD complex with a high number of peptides, covering almost 20% of its protein mass and amino acids content (Figure 1A). WB analyses confirmed that result and showed that Runx1 also co-purified with MyoD (Figure 1B).

We then performed a complementary experiment by transfecting HA-tagged MyoD and/or HA-tagged Runx1 into HeLa cells stably expressing Flag-HA-CBFβ (ectopic, eCBFβ). We showed that, indeed, MyoD co-precipitated with eCBFβ (Figure 1C, lane 6). Moreover, the simultaneous co-transfection of HA-tagged Runx1 resulted in its co-precipitation with CBFβ (Figure 1C, lane 5) and, more importantly, increased the MyoD co-precipitation (Figure 1C, compare lanes 5 and 6).

To further investigate the CBF/MyoD interaction, we turned to myogenic cells: the murine myoblastic cell line C2C12. Both

CBFβ and Runx1 are expressed in the C2C12 myoblasts and their protein level do not vary significantly during differentiation (Figure S3). We found that MyoD, and the other myogenic determination factor Myf5, co-precipitated with CBFβ preferentially in proliferating compared to differentiating C2C12 myoblasts (Figure 1D).

To assay whether MyoD has the ability to interact directly with CBF, we performed GST pull-down experiments, which showed that GST-MyoD strongly interacts with Runx1 (Figure 1E, lane 4), but not with CBFβ (Figure 1E, lane 5). The interaction of MyoD with Runx1 was specific; we did not detect any Runx1 signal in the presence of GST protein alone (Figure 1E, lane 8) nor any luciferase signal with GST-MyoD (Figure 1E, lane 7). Interestingly, GST-MyoD interacts with CBFβ only in the presence of Runx1 (Figure 1E, compare lanes 5 and 6), in agreement with our previous findings (Figure 1C). These results show that MyoD interacts directly with heterodimeric transcription factor CBF, *via* the Runx1 subunit.

The bHLH and the Transactivating Domains of MyoD, and the Transcription Regulation Domain of Runx1 Are Required for Their Interaction

In an attempt to delimit the domain of MyoD responsible for interaction with Runx1, we used HA-tagged mutants of MyoD transfected into HEK 293 cells. Anti-HA immunoprecipitation revealed an interaction of Runx1 and CBFβ with wild-type MyoD as expected, and only with a MyoD 82-318 mutant, which retains the bHLH and the C-terminal transactivating domains (Figure 2A). Runx1 and CBFβ failed to interact with truncated MyoD versions lacking either the bHLH, i.e., mutants Nter and Cter, or the C-terminal domain, i.e., mutants ΔCter, Nter and bHLH (Figure 2A). These experiments clearly show that the bHLH and the C-terminal transactivating domains are required for interaction with Runx1. Notably, MyoD deletion mutants that do not interact with Runx1 do not interact with CBFβ (Figure 2A). This result, combined with the results presented on Figure 1E, indicate that Runx1 is most likely the subunit that directly interact with MyoD.

To delimit the Runx1 domain involved in the interaction with MyoD, we performed a GST-pull down experiment (Figure 2B). Our results show that the transcription regulation domain located in the C-terminal part of Runx1 is required for the interaction with MyoD (Figure 2B). The interaction of MyoD with Runx1 was specific; we did not detect any Runx1 signal in the presence of GST protein alone nor any luciferase signal with GST-MyoD (Figure 2B).

CBF Negatively Regulates Cell Cycle Exit and Terminal Differentiation in Skeletal Myoblasts

We used siRNAs to decrease Runx1 level in order to investigate its role in differentiating myoblasts (Figure 3A, *see Runx1 quantification*). Downregulation of Runx1 resulted in a more efficient differentiation (Figure 3A); both the expression of muscle markers and the proportion of multinucleated cells were higher in Runx1-depleted cells (Figure 3A and Figure S4A). In particular, myogenin and MCK (Muscle Creatine Kinase) were expressed at higher levels in Runx1-depleted cells (Figure 3A). Interestingly, cyclin D1 level decreased more rapidly when Runx1 is downregulated (Figure 3A). Similarly, CBFβ downregulation induced an accelerated differentiation (Figure 3B, top panel) and a more rapid decrease in cyclin D1 (Figure 3B, lower panel). Indeed, we could detect MCK expression in CBFβ-depleted myoblasts as soon as 24 h after the induction of differentiation (Figure 3B, top panel). These cells moreover exhibited larger myotubes (Figure S4B). We confirmed these results in primary myoblasts (Figure 3C).

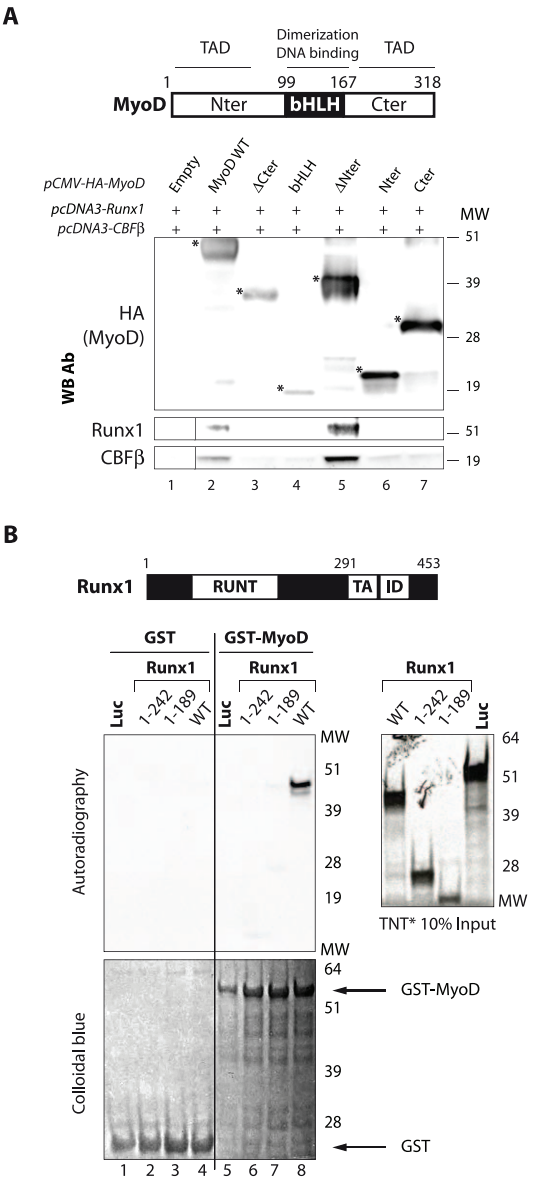


Figure 2. The domains of MyoD and Runx1 involved in their interaction. A. The bHLH domain and the C-terminal transactivating domain of MyoD are involved in the interaction with Runx1 and CBFβ. *Top panel*, Schematic diagram of MyoD functional domains, TAD: transactivation domain, bHLH: basic Helix Loop Helix. *Lower panel*, Runx1 and CBFβ interact with MyoD wild-type and with MyoD deletion mutant containing the bHLH and the C-terminal domain. *Lower panel*, expression vectors for wild-type MyoD and its deletion mutants were transfected in HEK 293 cells using Calcium phosphate precipitation as described in Material and Methods section. 48 h post-transfection, cells were lysed and lysates were subjected to anti-HA immunoprecipitation. Precipitates were then analyzed by western blotting using with the indicated antibodies (WB Ab). *: *specific bands*. **B.** The transcription regulation domain located in C-terminal part of Runx1 is required for the interaction with MyoD. *Top panel*, Schematic diagram of Runx1 protein. The Runt, transactivation (TA), and transcription inhibition (ID) domains are indicated. *Lower panel*, Runx1 and its deletion mutants, or luciferase (Luc) were *in vitro* translated in the presence of ^{35}S-Methionine (inputs shown on the right panel) and incubated with equivalent amounts of GST-MyoD or GST agarose beads (normalization is shown in the lower panel). GST pull-down was then conducted as described in the Material and Methods section, and the radiolabeled proteins were detected by autoradiography.
doi:10.1371/journal.pone.0009425.g002

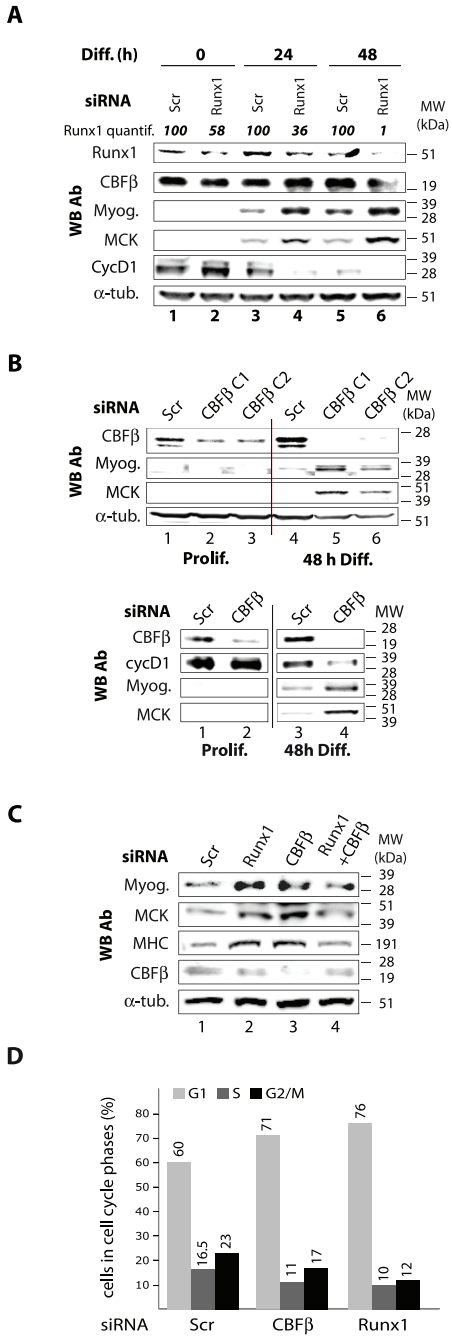


Figure 3. Downregulation of CBF subunits expression accelerates cell cycle exit and muscle terminal differentiation entry. A. C2C12 myoblasts were transfected with scrambled (Scr) or anti-Runx1 siRNAs. 48 h post-transfection (0 h, lanes 1–2), cells were placed in differentiation medium for 24 h (lanes 3–4) or 48 h (lanes 5–6). Cells were then analyzed by western blot with the indicated antibodies (WB Ab). The differentiation times are indicated in hours (h). Runx1 downregulation has been quantified (indicated as "Runx1 quantif.") using Bio1D application (Vilber Lourmat). α-tubulin is used as a loading control. **B.** As in A, except that we used two different CBFβ siRNAs (C1, C2). **C.** As in A and B, except that we used proliferating primary myoblasts instead of C2C12 myoblasts, and combined Runx1 and CBFβ siRNAs (lane 4). Note that all the kinetic studies of differentiation were carried out in the same 10 cm diameter cell culture dish for each sample. **D.** FACS analysis of the cell cycle distribution of C2C12 myoblasts transfected with the indicated siRNAs. Cells were analyzed 48 h post-transfection. Scr: scrambled siRNA.
doi:10.1371/journal.pone.0009425.g003

In agreement with our previous results, we have found that Runx1 or CBFβ downregulation led to a significant decrease of S-phase cells proportion concomitant with an increase in G1-phase cells (Figure 3D). This suggests that CBF positively regulates myoblasts proliferation.

As for the specificity of the siRNAs, we obtained the same phenotype with three different siRNAs that target CBF: the two targeting CBFβ subunit and the one targeting Runx1 subunit isoforms. Thus, the observed effects are unlikely due to any off-target effect.

To complete our analysis, we studied the effect of CBFβ overexpression on terminal differentiation. Overexpression of CBFβ in C2C12 myoblasts (C2C12-CBFβ) was well tolerated and did not lead to morphological abnormality (Figure S5). However, in differentiation conditions, C2C12-CBFβ cells showed a delay in cell cycle exit, as measured by early-G1 phase cyclin D1 level that decreased with a 24 to 48 h delay compared to control cells (Figure 4A), but not that of late-G1 cyclins A and E (Figure 4C). Note that proliferating C2C12-CBFβ cells contain more cyclin D1 protein then the control cells (Figure 4A), but not cyclins A2 and E (Figure 4C). The delayed cell cycle exit correlated with delayed expression of muscle markers such as myogenin (48 h delay), MCK (24 h delay), and MHC (Myosin Heavy Chain, not detected at 120 h) (Figure 4A). In contrast to control cells, C2C12-CBFβ cells exhibited smaller and mainly mononucleated myotubes (Figure 4B and Figure S5) with low expression of MCK and MHC (Figure 4B) in differentiation conditions. This suggests that differentiation kinetics was not completely impaired but greatly delayed when CBFβ was overexpressed. Further analysis of cell cycle regulators expression showed that, in addition to a delayed decrease in cyclin D1 (Figure 4A and C), cyclin D3 and p21 expression is delayed in C2C12-CBFβ cells compared to C2C12 control cells (Figure 4C).

Altogether, these results suggest that CBF plays a dual role during skeletal muscle differentiation by regulating cell cycle withdrawal and expression of muscle markers.

CBF Transcription Factor Is Located to MyoD Early Target Genes and Regulates Negatively Their Expression

The fact that CBF is a transcription factor which interacts with MyoD preferentially in proliferation conditions, led us to investigate whether it would be targeted to MyoD target genes to repress their transcription. Using an *in silico* approach, we first found that on early target gene promoters of MyoD, Runx1 and MyoD binding sites were adjacent (Figure S6A). In order to test the effective recruitment of Runx1 onto MyoD target promoters, we performed ChIP experiments. Our results showed a preferential enrichment in Runx1 on *myogenin*, *p21* and *cycD3* promoters in proliferating compared to differentiating myoblasts (Figure 5A). These MyoD target genes are expressed early in differentiating but not in proliferating myoblasts (Figure S6B).

Our ChIP assays showed that Runx1 was not located on late target genes of MyoD, such as *Desmin*, *MHC* and *MCK* (data not shown), while it was on early muscle differentiation genes *myogenin*, *p21* and *cycD3*. In addition, we did not find Runx1 binding sites adjacent to E-boxes on late MyoD target genes' promoters. These findings suggest that Runx1 would essentially regulate early events of skeletal muscle terminal differentiation. Taken together, our results strongly suggest that Runx1 could be recruited onto MyoD early target genes to regulate negatively their expression in proliferating myoblasts.

To gain insights into the mechanism of action of CBF on MyoD target genes, we purified CBFβ protein complex from proliferating C2C12-CBFβ cells *via* its Flag tag. As expected, CBFβ co-purified

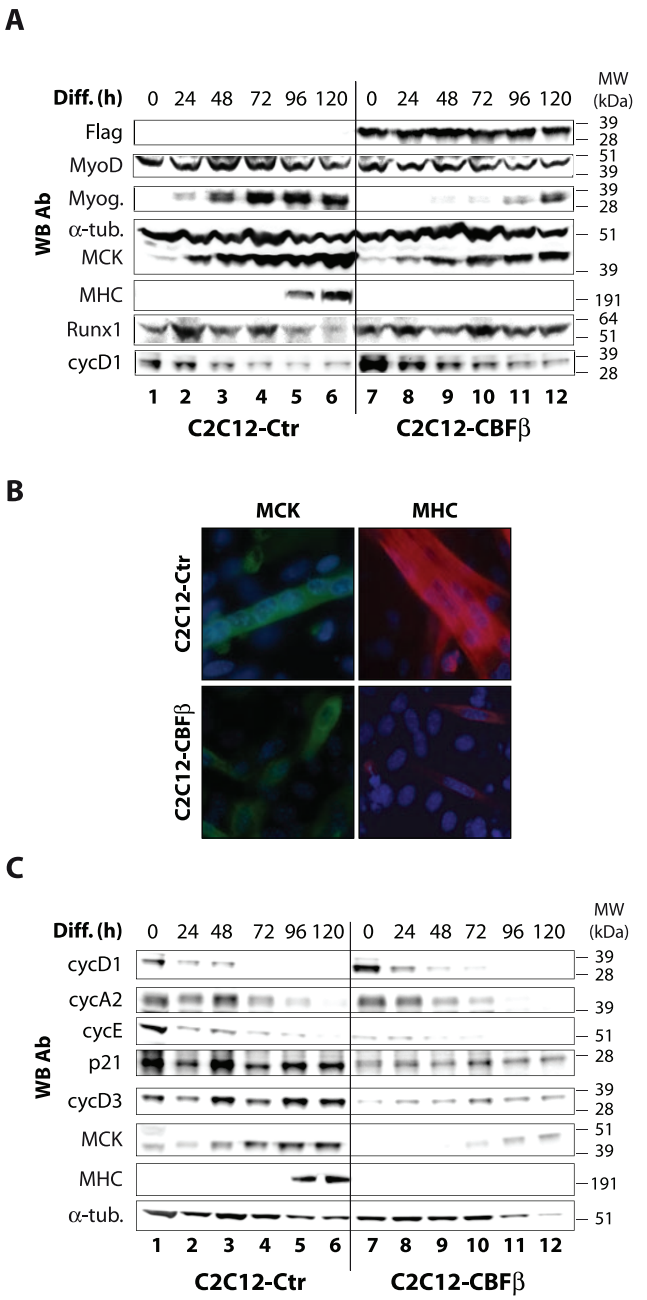


Figure 4. Overexpression of CBFβ delays cell cycle exit and muscle terminal differentiation. A–C. C2C12 cells stably overexpressing Flag-HA-CBFβ (C2C12-CBFβ) or control cells (C2C12-Ctr) were differentiated at the indicated times (in A and C, in hours), and analyzed by western blotting with the indicated antibodies (WB Ab) (A, C) or by immunofluorescence (IF) (63× magnification) (B). The kinetic studies were carried out in the same 10 cm diameter cell culture dish (A, C). IF experiments using anti-MCK or anti-MHC antibodies were performed 48 h and 72 h respectively after induction of differentiation. Cells were DAPI-stained prior to fluorescent microscopy analysis (63× magnification).
doi:10.1371/journal.pone.0009425.g004

with the myogenic factor MyoD and with its dimerization partner Runx1 (Figure 5B). The other partners that co-purified specifically with CBFβ are proteins known to be involved in transcriptional repression: the histone 3 lysine 9 (H3K9) methyltransferase Suv39h1, the heterochromatin protein HP1β, and the histone deacetylases HDACs 1, 2 and 3 (Figure 5B). These interactions could be mediated by Runx1. Indeed, these proteins are already known partners of Runx1 on the one hand [30], and repressors of MyoD activity on the other hand [31–33].

Given the association of CBFβ with chromatin-modifying enzymes, we studied the chromatin status of three target gene promoters of MyoD in differentiating C2C12-CBFβ using chromatin immunoprecipitation (ChIP). Our results showed that, in contrast to control cells in which histone H3 acetylation (a mark associated with transcription activation) on *myogenin*, *cyclin D3* and *p21* promoters increased in differentiation compared with proliferation conditions, histone H3 acetylation levels at these promoters did not vary in C2C12-CBFβ cells (Figure 5C). More generally, in differentiation conditions, we found that activating marks (histone H3 acetylation, histone H3 lysine 4 tri-methylation) are abnormally lower on *myogenin* and *cyclin D3* promoters in C2C12-CBFβ cells compared to control cells (Figure 5D). Concomitantly, repressive marks (histone H3 lysine 9 tri-methylation, presence of HDAC1) are higher (Figure 5D).

These results are in agreement with our findings showing that CBF associates with chromatin modifying enzymes, such as HDACs, Suv39h1 and HP1β (Figure 5B), which are known to repress MyoD activity in proliferating myoblasts. Thus, CBF has an effect on the chromatin structure of three early target genes of MyoD and contributes to maintain a repressive chromatin state.

Runx1 Represses MyoD Transcriptional Activity

To investigate the effects of Runx1 on MyoD transcriptional activity, we used a luciferase reporter gene under the control of the *myogenin* promoter, which is a direct target promoter of MyoD that harbors a Runx-binding site adjacent to MyoD-binding site (Figure S6A). Co-transfection experiments were performed in HeLa cells line that does not express MyoD endogenously. We observed that the co-transfection of Runx1-expressing plasmid together with MyoD expression vector resulted in the inhibition of *myogenin* promoter activity in a dose-dependent manner (Figure 6). The inhibitory effect of Runx1 is specific and is MyoD-dependent. Indeed, it was not seen with Renilla–luciferase expression under a *CMV* promoter (used as a normalization control for transfection efficiency). Thus, Runx1 inhibits MyoD activity.

Discussion

Here we show that CBF transcription factor, composed of Runx1 and CBFβ subunits, is expressed in proliferating myoblasts where it interacts with MyoD. Modulating the expression levels of either Runx1 or CBFβ impaired cell cycle exit and terminal myogenic differentiation.

Runx1/CBFb Interact with MyoD

Mass spectrometry analysis revealed that CBFβ is part of MyoD complex. Further western blot analyses of the same MyoD complex revealed the presence of Runx1 subunit. We have narrowed our study to Runx1 because of all the three Runx proteins, to our knowledge, Runx1 is the only one linked to skeletal muscle [20]. In addition, *Runx1* is one of the MyoD target genes in myoblasts [34]. Among the other Runx members, Runx2 is mainly involved in osteogenesis, and it has already been shown that Runx2 expression is not detectable in myoblasts [35]. Moreover, ectopic expression of Runx2 in myoblasts triggers osteogenic transdifferentiation [35–37]. Finally, concerning Runx3, it has been shown that it is not expressed in skeletal muscle and is mainly linked to neurogenesis [38–42]. Thus, we explored the role of Runx1/CBFβ during muscle skeletal terminal differentiation. However, Runx1 has been reported to play an

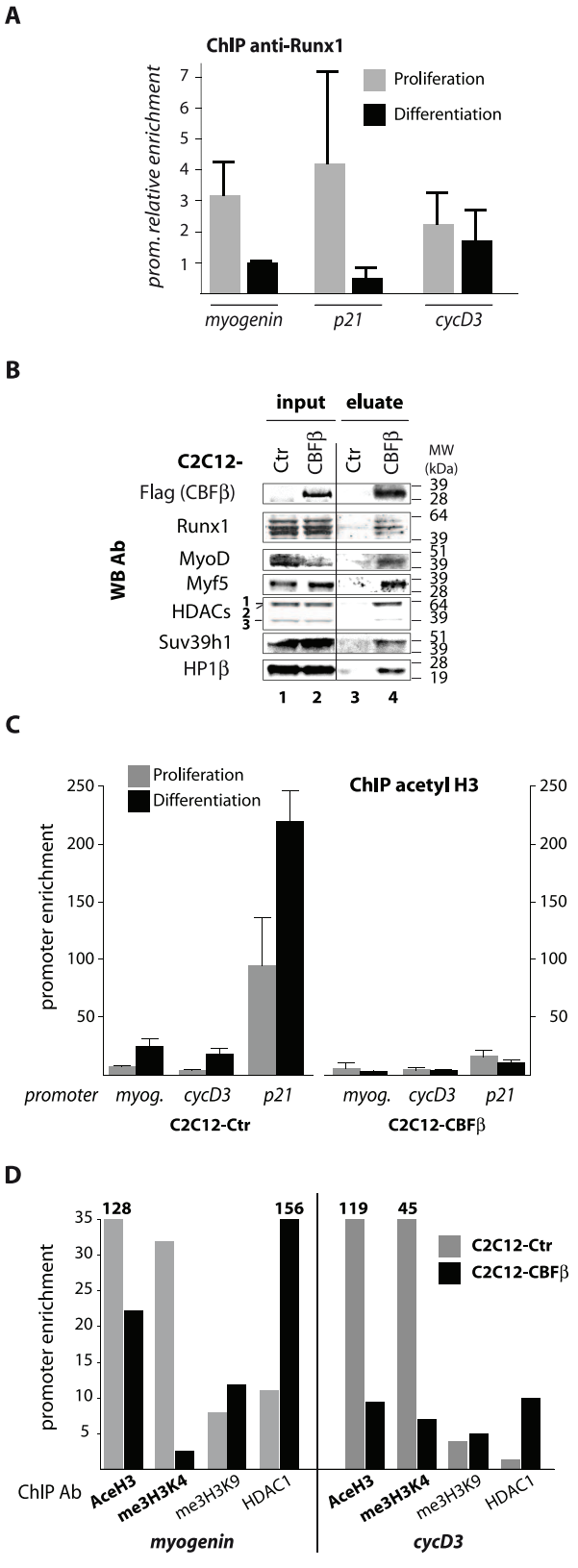


Figure 5. CBF acts at the chromatin level to regulate MyoD target genes. A. ChIP using anti-Runx1 Ab were performed from either proliferating (grey bars) or differentiating myoblasts (black bars). We quantified copy numbers of the *myogenin*, *p21* and *cyclin D3* promoter regions harboring the MyoD and Runx1 target sequences, compared to *36B4* gene, which was used as a reference gene. Results are the mean of 3 independent experiments. **B.** Western blot analysis with the indicated antibodies (WB Ab) of Flag-purified Flag-HA-CBFβ stably expressed in C2C12 cells (CBFβ), or from C2C12 control cells (Ctr). Both inputs are probed in lanes 1–2 and eluates are shown in lanes 3–4. **C.** Chromatin immunoprecipitation (ChIP) experiments using anti-acetyl H3 antibody were performed from either proliferating (grey bars) or differentiating (black bars) C2C12 control (left) and C2C12-CBFβ (right) cells. We quantified copy numbers of the *myogenin* (*myog.*), *cyclin D3* and *p21* promoter regions harboring the MyoD target sequences. Results are the mean of three measurements. **D.** ChIP experiments using antibodies against acetyl histone H3 (AceH3), trimethylated histone 3 lysine 4 (me3H3K4), trimethylated histone 3 lysine 9 (me3H3K9), Histone Deacetylase 1 (HDAC1) were performed from differentiating C2C12 control (grey bars) or C2C12-CBFβ (black bars) cells (48 h differentiation time). We quantified copy numbers of the *myogenin* and *cyclin D3* promoter regions harboring the MyoD target sequences. Results with the transcription activating marks (AceH3 and me3H3K4) were normalized using the expressed housekeeping gene *36B4*, while the repressive marks (me3H3K9 and HDAC1) were normalized using the repressed major satellite repeats. Results are the mean of three measurements.
doi:10.1371/journal.pone.0009425.g005

important role in protecting denervated, fully differentiated, myofibers from atrophy and autophagy [20]. In this paper [20], Runx1 has been assigned a role of a growth-promoting factor of muscle cells to limit muscle wasting. In this very elegant study, the authors generated *knock-in* homozygous mice carrying inactive *Runx1* in skeletal muscle cells. However, *Runx1* inactivation was conducted under the indirect control of *MCK* promoter, which is active only at late stages of terminal differentiation. Thus, with this system, we cannot see the effect of *Runx1* inactivation on early stages of terminal differentiation, where *MCK* promoter is not yet active.

Runx1 and CBFb Regulate Cell Cycle Exit and Terminal Differentiation

Runx1 or CBFβ downregulation in myoblasts induced an accelerated cell cycle exit. Indeed, cyclin D1 protein disappears

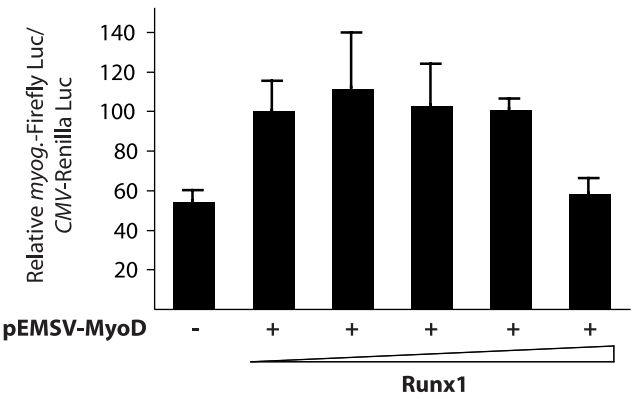


Figure 6. Runx1 represses MyoD-mediated transcription. Co-transfection into HeLa cells with a *myogenin* promoter-driven Firefly luciferase reporter plasmid (kind gift of V. Sartorelli, NIH) without or with a fixed amount of MyoD expression vector (800 ng), and increasing amounts of vector expressing Runx1. The quantities of Runx1 expression vector used were: 0, 30, 60, 150, and 300 ng. The total amount of plasmid was normalized when necessary to 300 ng with the empty vector. The inhibitory effect of Runx1 was specific, indeed, it was not seen with a Renilla luciferase expression under a *CMV* promoter (used as a normalization control for the transfection). Results are the mean of 2 independent experiments performed each in triplicates.
doi:10.1371/journal.pone.0009425.g006

24 h earlier in differentiation conditions. In addition, Runx1 or CBFβ downregulation led to a an increase in G1-phase cells, suggesting that CBF promotes myoblasts proliferation. Conversely, CBFβ overexpression delayed the early-G1 phase cyclin D1 disappearance in differentiation conditions (cyclin D1 is still detectable 72 hours after the induction of differentiation), but not that of late-G1 and early-S phase cyclins A2 and E1. Note that proliferating C2C12-CBFβ cells contain more cyclin D1 protein, but not cyclins A2 and E, than the control cells. Together, these results suggest that CBFβ overexpression impacts on early G1 markers. In addition, expression of the cell cycle exit regulators p21 and cyclin D3 [43,44] is delayed in differentiating CBFβ-overexpressing cells, suggesting a delayed or impaired cell cycle exit and terminal differentiation entry. In agreement with this, our ChIP results showed that in proliferating myoblasts, CBF (*via* Runx1) is recruited to repressed *p21* and *cyclin D3*, which encode cell cycle exit regulators [43,44]. Together, these results suggest that CBF regulates positively proliferation, and negatively terminal differentiation, of skeletal myoblasts. Thus, CBF impacts on the proliferation/differentiation switch in myoblasts (see model on Figure S7).

In differentiation conditions, C2C12-CBFβ cells showed delayed molecular differentiation (expression of muscle markers), and delayed appearance of myotubes that are abnormally small and mainly mono-nucleated. One alternative explanation is that these cells managed to differentiate correctly, although in a delayed manner, but exhibited a specific block in fusion and multi-nucleation. Indeed, CBFβ subunit is retained in the cytoplasm by cortical filamins [45]. In muscle cells notably, structural proteins and cell adhesion proteins are required for the reorganization of the cell cytoskeleton during cell fusion to form myotubes [46]. Some studies do suspect a cytoplasmic role for CBFβ [47]. Thus, CBFβ overexpression in myoblasts, especially in the cytoplasm, could have a role in impairing the correct cytoskeleton reorganization during fusion. This could explain the phenomenon we observe in differentiating CBFβ-overexpressing myoblasts. Moreover, we observed a very low expression of myogenin transcription factor in myoblasts overexpressing CBFβ and since that myogenin is involved in cell fusion, this is an alternative explanation of the observed the mono- or di-nucleated myotubes.

CBF Regulates Muscle Differentiation via a Direct Interaction between MyoD and the Runx1 Subunit

Our results revealed that the role of CBF in myoblasts is likely to be partly mediated through direct interaction with MyoD. GST pull-down experiments showed that MyoD interacts directly with Runx1 subunit, but not with CBFβ. This is confirmed by the use of MyoD deletion mutants in living cells. Indeed, MyoD deletion mutants that fail to interact with Runx1 do not interact neither with CBFβ. MyoD/Runx1 interaction implicates the bHLH and the C-terminal transactivating domains of MyoD, and the transcription regulation domain of Runx1.

The preferential association of MyoD and CBF in proliferating myoblasts could mean that CBF might be acting as a negative co-factor of MyoD. Indeed, we provide evidence that CBF is recruited to early MyoD target genes, *via* Runx1, in proliferating myoblasts, where MyoD is mainly associated with transcriptional repressors [24,26,31]. This suggests that CBF may serve for assembly of a transcription repression complex at early MyoD target genes such as *myogenin*, *p21* and *cyclin D3* (see our model on Figure S7). As for example, such a mechanism could be involved in the repression of the skeletal muscle *acetylcholine receptor* gene, which contains a repressive E-box that mediates its repression in proliferating myoblasts [48]. In agreement with this, we found that in proliferating myoblasts, CBF associates with many chromatin modifying enzymes, such as Histone Deacetylases (HDACs 1, 2 and 3), the histone H3 lysine 9 (H3K9) methylase Suv39h1, and Heterochromatin Protein beta (HP1β), which are known to repress MyoD activity in proliferating myoblasts [24,31,49,50]; and already known to interact with Runx1 [30,51,52]. In agreement with this, in myoblasts overexpressing CBFβ, histone H3 acetylation and its trimethylation on lysine 4 (marks of active transcription) are delayed on early MyoD target genes in differentiation conditions. Concomitantly, these genes remain abnormally marked by histone repressive marks (me3H3K9, HDAC1).

Interestingly, although MyoD is expressed both in proliferating and differentiating cells, we found that the interaction between MyoD and CBF was lost in differentiating cells. In addition, we showed that overexpression of CBFβ in myoblasts led to stabilization of Runx1 subunit that could more efficiently repress MyoD transactivating activity, which induces a delay in terminal differentiation. In agreement with this, we have shown that Runx1 represses MyoD activity in a gene reporter assay.

We did not succeed to show the concomitant presence of Runx1 and MyoD on MyoD target genes, given that it has previously been demonstrated that in proliferating conditions, only a small fraction of MyoD contributes to the repressive remodeling of its target genes, prior differentiation. Alternatively, Runx1 could prevent the proper binding of MyoD and the recruitment of the transcriptional machinery. Notably, the displacement of Runx1 in differentiating conditions is concomitant with a strong binding of MyoD to its target promoters (data not shown).

MyoD and Runx1 are both subject to post-translational modifications. Notably, MyoD is phosphorylated during the cell cycle while it becomes acetylated during differentiation. Runx1 can also be phosphorylated, acetylated or methylated, while these modifications still need to be characterized in muscle cells and during muscle differentiation. We propose that these modifications could favor or impair MyoD interaction with Runx1, respectively.

Conclusion

Our findings concerning the role of CBF in the regulation of the proliferation/differentiation balance are in agreement with several reports. CBF was indeed implicated in skin epidermis and hair follicle differentiation [11,12], as well as in neuronal differentiation [8,9]. These data support an emerging role for Runx proteins in cell fate regulation in many cell lineages. Furthermore, MyoD was also shown to regulate osteogenic differentiation [53]. In addition, it has been shown that muscle satellite cells can differentiate into osteocytes or adipocytes under some conditions [54,55]. Thus, our results point to a model in which CBF and myogenic bHLH protein families could act in concert to induce cell-lineage-specific gene expression, dependent on the extra-cellular stimuli.

In summary, we propose that CBF transcription factor might participate in recruiting chromatin modifying enzymes to repress MyoD early target genes by locally inducing a repressive chromatin structure. Our data reveal a new critical role of CBF in the regulation of the balance between proliferation and differentiation in skeletal muscle cells. They also demonstrate a new mechanism of repression of differentiation genes in proliferating myoblasts.

Methods

Cell Culture

C2C12, HEK 293 and HeLa-S3 cells were cultured under standard conditions. C2C12 cells and mouse primary myoblasts were cultured and differentiated as described in: [56].

Stable Cell Lines Establishment and Plasmid Construction

A HeLa cell line stably expressing MyoD was established with a transgene encoding for full-length MyoD; and HeLa and C2C12 cell lines expressing CBFβ were established with a transgene encoding for full-length CBFβ. The transgenes were tagged with double-HA (Haemagglutinin) and double-FLAG epitopes at the N-terminus as described in [57].

Control cell lines transduced with the empty vector were also established. Murine CBFβ cDNA (a kind gift from Dr Nancy A. Speck) was amplified by PCR with specific primers with protruding restriction sites (fw-Pspx1: CCGCTCGAGCCG-CGCGTCGTCCCGGG, rev-Not1: ATTCTATATGCGG-CCGCTAACGAAGTTTGAGATCATCG, and sub-cloned into the XhoI-NotI sites in the pREV retroviral vector after Pspx1 and Not1 digestion (Pspx1 is compatible with Xho cloning site in the pRev vector) [57,58].

Protein Complex Purification

Flag-HA-MyoD complex purification from HeLa-MyoD cells was performed as described in: [31]. Briefly, we used retroviral transduction strategy to establish HeLa-S3 cell lines expressing double tagged Flag-HA-MyoD [31,57,58], or a control cell line transduced with the empty pREV vector has been established. We carried out double-affinity purification of Flag-HA-MyoD from HeLa cells (Figure S1), using either nuclear soluble or chromatin fractions. For this, cells were resuspended in a hypotonic buffer (10 mM Tris-HCl pH 7.65; 1.5 mM $MgCl_2$; 10 mM KCl) and disrupted with 20 strokes of a tight-fitting Dounce homogenizer. The cytosolic fraction was separated from nuclei by 7 min centrifugation at 4°C at 9000 rpm. The nuclear soluble fraction was obtained by incubation of the nuclear pellet in a high salt buffer (900 mM NaCl, 20 mM Tris pH 7.65, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA), to get 300 mM NaCl, for 30 min at 4°C and centrifugation at 10,000 rpm. The resulting pellet, which corresponds to chromatin fraction, was resuspended and digested with micrococcal nuclease (Sigma, Saint-Quentin Fallavier, France), until it consisted primarily of mononucleosomes [57]. Nuclear soluble and chromatin fractions were then ultracentrifugated at 32000 rpm for 1 h at 4°C. Tagged-MyoD complex were then purified using anti-FLAG antibody immobilized on agarose beads (Sigma). After elution with the FLAG peptide (Ansynth, The Netherlands), the bound complexes containing nucleosomes were further affinity-purified on anti-HA antibody-conjugated agarose (Sigma) and eluted with the HA peptide (Ansynth, The Netherlands). Double-immunopurified complexes were resolved on 4–12% SDS-PAGE bis-Tris acrylamide gradient gel in MOPS buffer (Invitrogen), and stained using either the SilverQuest kit (Invitrogen, Cergy-Pontoise, France) [31], or with Colloidal blue (Invitrogen) for mass spectrometry (MS) analyses. In the latest, bands corresponding to proteins were cut from the gel, trypsin-digested using 0.4 mg of sequencing-grade trypsin (Promega, Charbonnières, France), and identified by MS analysis.

To purify CBFβ complex from C2C12 cells, 3 grams of C2C12-CBFβ cell pellet were used to purify tagged CBFβ using a simple-affinity purification method using Flag resin.

Preparation of Nuclear Extracts

Cells were scraped in a minimal volume of PBS and centrifuged 2 min at 400 g. The pellet was resuspended in 5 volumes of: 20 mM HEPES pH 7, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, then lysed by addition of NP-40 up to 4.5%. Nuclei were immediately neutralized with addition sucrose buffer (50 mM HEPES pH 7, 0.25 mM EDTA, 10 mM KCl, 70% (m/v) sucrose). After centrifugation (5 min, 2000 g), nuclei were suspended in glycerol buffer (10 mM HEPES pH 8, 0.1 mM EDTA, 100 mM NaCl, 25% glycerol) to remove any trace of cytosolic components and centrifuged again. The nuclei were then resuspended in sucrose buffer n°2 (20 mM Tris pH 7.65; 60 mM NaCl; 15 mM KCl; 0.34 M Sucrose) then lysed in a final concentration of 250 mM NaCl using High Salt Buffer (20 mM Tris pH 7.65; 0.2 mM EDTA; 25% glycerol; 900 mM NaCl; 1.5 mM $MgCl_2$). The lysates were sonicated 3 times for 15 s with the BioRuptor (Diagenode, Liège, Belgium) on "High", then centrifuged 10 min at 13000 rpm to harvest the total nuclear extracts (supernatants). Protein concentration for each sample was estimated with BCA kit (Perbio, Brebières, France).

Transient Transfections, Flag-Affinity Precipitation of Flag-HA-CBFb, HA-MyoD Precipitation

For plasmid transfection, 25 µg of pRcCMV-HA-Runx1 (kind gift of Dr I. Kitabayashi, Japan), pCMV-HA-MyoD or pRC-CMV backbone were transfected into HeLa-CBFβ cells, using calcium phosphate pH 7.12, and Flag IPs was performed 24 h post-transfection (results presented on Figure 1C). Each IP was performed with 1.5 mg of total nuclear extracts and with 25 µL stock of ssDNA and BSA-pre-blocked Agarose Flag M2 resin from Sigma. IP was performed on wheel overnight at 4°C. Resin was then washed 5 times with TEGN buffer (20 mM Tris pH 7.65; 0.1 mM EDTA; 10% glycerol; 150 mM NaCl; 0.5% NP-40) and eluted by competition with high-purity Flag peptide at a final concentration of 0.2 mg/ml. The resin-free eluate was retrieved using Clean-up Post reaction columns (Sigma).

For interaction experiments using MyoD deletion mutants, HEK 293 cells were transiently transfected using calcium phosphate at pH 7.12. We used for a 10-cm dish 5 µg of a pCMV-3HA-MyoD or its deletion mutants (Cter 241-318, Nter 1-66, bHLH 82-172, ΔCter 1-240, ΔNter 82-318), or the empty vector, along with 5 µg of pcDNA3-Runx1 and 5 µg of pcDNA3-CBFβ vectors. 48 h post-transfection, cells were lysed in lysis buffer (300 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0,4% NP-40, 10 mM $MgCl_2$) to extract proteins. Anti-HA immunoprecipitation was then performed as described above.

siRNA Transfection

siRNAs were purchased from Sigma (Saint-Quentin Fallavier, France) and were transfected using Hi-Perfect reagent (Qiagen, Courtaboeuf, France) according to the manufacturer recommendations. We usually transfect 0.2 µmol of siRNA per 100 mm cell culture dish. CBFβ targeting siRNA sequences used are: C1: CCGGGAAUAUGUCGACUUA, and C2: UAACUUAGGUGGCG-GUGAU; Runx1 siRNA: CUGUGAAUGCUUCUGAUUU; and the scrambled siRNA: ACUUAACCGGCAUACCGGCTT.

Immunoprecipitation of Endogenous Proteins

For IP, we usually use 2 µg of antibodies, 10 µL protein A/G Sepharose beads from Perbio and 1.2 mg of nuclear extracts from C2C12 cells, or 0.5 mg from primary myoblasts. Elution was performed with 40 µl of 0.1 M glycine pH 2.5, 15 min at 25°C, the eluate was recovered using Spin cleaning-up post-reaction column (Sigma). Acidity was neutralized with Tris pH 8.0 before adding loading buffer.

Western Blotting

For western blotting, protein samples were resolved on pre-cast NuPage 4–12% bis-Tris acrylamide gradient SDS-PAGE gel (Invitrogen, Cergy-Pontoise, France). Proteins were then trans-

ferred onto nitrocellulose membrane during 1 h at 400 mA in transfer buffer (25 mM Tris, 150 mM Glycine, 0.1% SDS and 20% methanol). Membranes are blocked 1 hour in PBS-0.2% Tween, 10% skimmed milk and incubated overnight at 4°C with primary antibodies. Membranes were incubated with the appropriate secondary antibodies coupled to HRP and revealed using West Dura from Pierce (Perbio, Brebières, France) and ChemiSmart 5000 system (Vilber Lourmat, Marne-La-Vallée, France).

Plasmids, GST Fusions and GST Pull-Down

GST and GST-MyoD plasmid constructs were expressed in *Escherichia coli* strain BL21 and purified using glutathione-sepharose beads according to the manufacturer (Sigma, Saint Quentin-Fallavier, France). Purified proteins were quantified by coomassie staining after SDS–PAGE separation. *In vitro* transcription and translation (TNT) of pcDNA3-Runx1 and its deletion mutants aa 1-189 and 1-242, pcDNA3-CBFβ and luciferase were performed with Riboprobe *in vitro* transcription systems (Promega, Charbonnières, France) in the presence of ^{35}S-labelled methionine.

Agarose beads coated with equal amounts of GST or GST-MyoD (1 µg) were incubated with 10 µL of radioactive TNT reaction in reaction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% Triton) during 2 h at 4°C. Beads were washed 5 times with wash buffer (50 mM Tris pH 7.6, 300 mM NaCl, 0.5% Triton 100), resuspended and proteins resolved by SDS-PAGE gel and revealed by autoradiography.

Immunofluorescence

Cells were cultured in Labtecks permanox (Falcon) and fixed briefly with 4% formaldehyde in PBS. Residual formaldehyde was neutralized with 0.1 M Glycine pH 8.0, and washed with PBS. Cells were permeabilized and blocked using 1% BSA, 1% goat serum, 0.3% Triton-X100 in PBS. Primary and secondary antibodies were diluted in the permeabilizing/blocking solution and were washed with 0.3% Triton-X100 in PBS. Nuclei are stained with DAPI and the glass lid is fixed using an anti-fading polymerizing media from DakoCytomation (Dako, Trappes, France).

Antibodies

The anti-MyoD (C-20), anti-myogenin (M-225), anti-Myf5 (C-20), anti-CBFβ (FL-182), anti-cyclin A2 (C19, sc-596), anti-cyclin D1 (72-13G, sc-450), anti-cyclin D3 (C-16, sc-182), anti-cyclin E (sc-25303), anti-p21 (C-19, sc-397) and normal rabbit IgG antibodies were all purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit polyclonal anti-Suv39h1 (07-550), anti-trimethyl histone 3 lysine 9 (07-442) and rabbit anti-acetyl histone H3 (06-599) antibodies were obtained from Upstate Biotech (Lake Placid, NY, USA). Anti-HP1β (1MOD1A9AS) was from Euromedex (Souffelweyersheim, France). Anti-HDAC1 (pAB-053-050) was from Diagenode (Liège, Belgium). Anti-trimethyl H3K4 was from Abcam (Paris, France). Rabbit polyclonal anti-MCK antibody was developed by Dr H. Ito [59]. Anti-Flag and anti-α-tubulin antibodies were purchased from Sigma (Saint-Quentin Fallavier, France). Rat anti-HA antibody was purchased from Roche (Meylan, France). Mouse anti-Runx1 antibody (MAB10062) was purchased from Millipore (Saint Quentin en Yvelines, France) and mouse anti-HDAC 1-3 antibody (611125) from BD Biosciences (Le Pont de Claix, France). Goat anti-rat IgG Alexa-488-conjugated, anti-rabbit IgG Alexa-488 were from Invitrogen (Cergy-Pontoise, France) and anti-mouse IgG TRITC (T7657) were from Sigma (Saint-Quentin Fallavier, France).

FACS Analysis

C2C12 were transfected with the siRNAs as indicated in the Material and Method section. 48 hours post-transfection, cells were washed with PBS, then scraped in 500 µL of PBS. Cells were kept on ice while 4,5 ml of ethanol 70% were added. Then cells are kept at least overnight at −20°C. Propidium iodide (PI) staining proceeds as follows: cells are centrifuged and the pellet is washed with PBS. Cells are then centrifuged and resuspended in 2 mL PI solution (PI 25 ng/ml, RNase 200 ng/ml, Triton 0,1%) 30 minutes and kept in the dark. Cells are homogeneized by vortexing before analysis. We worked on a Beckman and Coulter FACS apparatus and we counted at least 3000 events for each condition.

Chromatin Immunoprecipitation (ChIP)

ChIP protocol and primers have been described in: [56]. The yet unpublished primers used are: Myogenin fw: GAATCA-CATGTAATCCACGGA, rev: ACGCCAACTGCTGGGTGCCA. Cyclin D3 fw: CTGCTTGCCTCTGTCTTCA; rev: GACCCATG-TCAGATGACTC. 36B4 fw: ATGTGCAGCTGATAAAGACTGG; rev: CTGTGATGTCGAGCACTTCAG.

Gene Reporter Assays

HeLa cells at 60% confluence were co-transfected by Calcium Phosphate co-precipitation with a *myogenin* promoter-driven Firefly luciferase reporter plasmid (kind gift of V. Sartorelli, NIH) without or with a fixed amount of MyoD expression vector (800 ng), and increasing amounts of vector expressing Runx1. The quantities of Runx1 expression vector used were: 0, 30, 60, 150, and 300 ng. The total amount of plasmid was normalized when necessary to 300 ng with the empty vector. A Renilla luciferase expression under a *CMV* (cytomegalovirus) promoter was used as a normalization control for the transfection. 24 h post-transfection, cells were lysed in a reporter lysis buffer (Promega, Charbonnières, France). Luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was then normalized to the level of Renilla luciferase and to the total protein amount.

Supporting Information

Figure S1 Schematic representation of the purification protocol used to purify the MyoD complex from HeLa cells.
Found at: doi:10.1371/journal.pone.0009425.s001 (0.38 MB EPS)

Figure S2 MyoD known partners identified by mass spectrometry in the MyoD complex.
Found at: doi:10.1371/journal.pone.0009425.s002 (0.05 MB DOC)

Figure S3 Expression of Runx1 and CBFβ proteins during muscle terminal differentiation. Cellular extracts from proliferating or differentiating C2C12 myoblasts (left panel) or from mouse primary myoblasts (right panel) were subjected to western blot analyses for the expression of CBFβ, Runx1, MyoD, myogenin (Myog.) and Muscle Creatine Kinase (MCK). α-tubulin is detected as a loading control. Differentiation times are indicated in hours on the top of each panel. Lanes 1 and 7 correspond to proliferating cells.
Found at: doi:10.1371/journal.pone.0009425.s003 (0.57 MB EPS)

Figure S4 Downregulation of CBF subunits expression accelerates muscle terminal differentiation entry. A. C2C12 myoblasts were transfected with control siRNA (Scrambled, Scr) or with Runx1 siRNA as indicated. 48 hours post-transfection (Proliferation) cells were placed in differentiation medium (Differentiation)

for 86 hours. Cells were then analyzed by microscopy (10× magnification). B. As in A, except that we used CBFβ siRNA.
Found at: doi:10.1371/journal.pone.0009425.s004 (11.55 MB EPS)

Figure S5 C2C12-CBFβ cells characterization. A. Expression level of CBFβ in C2C12 control (ctr) and in C2C12-CBFβ, in proliferating myoblasts (0 h) or at the indicate differentiation times (in hours), as measured by western blot using anti-CBFβ antibody. ex.: exogenous; end: endogenous. B. C2C12 cells stably overexpressing Flag-HA-CBFβ (C2C12-CBFβ) or control cells (C2C12-Ctr) were differentiated for 72 hours and analyzed by light microscopy (10× magnification).
Found at: doi:10.1371/journal.pone.0009425.s005 (10.67 MB EPS)

Figure S6 A. Runx1 and MyoD binding sites found in silico on MyoD target genes myogenin and p21. B. Western blot analysis (with the indicated antibodies) of cell extracts used for the ChIP experiment presented on Fig. 5A. α-tubulin (α-tub.) is used a loading control.
Found at: doi:10.1371/journal.pone.0009425.s006 (0.52 MB EPS)

Figure S7 Proposed model of skeletal muscle terminal differentiation regulation by CBF and MyoD. Note that in muscle system, proliferation inhibition or cell cycle exit is a pre-requisite to terminal differentiation. Thus, cell cycle exit regulators, such as p21 and cycD3, are activated early during terminal differentiation. Muscle specific early markers, such as myogenin, are also activated before late muscle markers. In proliferating myoblasts, Runx1/CBFβ (CBF dimer) proteins repress MyoD target genes, possibly via a direct interaction with MyoD. Thus, in proliferating myoblasts, CBF binds early MyoD target genes via Runx1 subunit and recruits chromatin modifying enzymes such as HDAC1, Suv39h1 and HP1. Upon triggering of terminal differentiation, CBF dissociates from the promoters and MyoD recruits activating chromatin modifying enzymes, such as HATs. HDAC1: Histone Deacetylase 1; HAT: Histone acetyltransferase.
Found at: doi:10.1371/journal.pone.0009425.s007 (0.54 MB EPS)

Figure S8 Control of the anti-HA western blot results presented on Figure 1C using antibodies against native proteins: MyoD, CBFβ and Runx1.
Found at: doi:10.1371/journal.pone.0009425.s008 (0.53 MB EPS)

Acknowledgments

We thank A. Polesskaya for technical help and critical reading of the manuscript. We thank F. Ferri and C. Guillemin for technical help. We thank C. Francastel, J. Weitzman, F. Hubé, C. Rougeulle, V. Mezger and P.A. Defossez for critical reading of the manuscript. The authors warmly thank Drs N. Speck, Y. Ito, V. Sartorelli, G. Mouchiroud, SA. Leibovitch, I. Kitabayashi, H. Ito, and L. Delva for sharing reagents.

Author Contributions

Conceived and designed the experiments: SASA. Performed the experiments: OP VJ OAM CP PR LF SASA. Analyzed the data: OP VJ OAM CP PR LF SASA. Contributed reagents/materials/analysis tools: OP VJ OAM PR LF SASA. Wrote the paper: SASA.

References

1. Mikhail FM, Sinha KK, Saunthararajah Y, Nucifora G (2006) Normal and transforming functions of RUNX1: a perspective. J Cell Physiol 207: 582–593.
2. Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, et al. (1996) The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell 87: 697–708.
3. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, et al. (1991) t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A 88: 10431–10434.
4. Golub TR, Barker GF, Bohlander SK, Hiebert SW, Ward DC, et al. (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 92: 4917–4921.
5. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, et al. (1996) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A 93: 3444–3449.
6. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84: 321–330.
7. Komori T, Kishimoto T (1998) Cbfa1 in bone development. Curr Opin Genet Dev 8: 494–499.
8. Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, et al. (2006) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49: 365–377.
9. Theriault FM, Nuthall HN, Dong Z, Lo R, Barnabe-Heider F, et al. (2005) Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. J Neurosci 25: 2050–2061.
10. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, et al. (2004) AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med 10: 299–304.
11. Raveh E, Cohen S, Levanon D, Negreanu V, Groner Y, et al. (2006) Dynamic expression of Runx1 in skin affects hair structure. Mech Dev 123: 842–850.
12. Osorio KM, Lee SE, McDermitt DJ, Waghmare SK, Zhang YV, et al. (2008) Runx1 modulates developmental, but not injury-driven, hair follicle stem cell activation. Development 135: 1059–1068.
13. Wotton SF, Blyth K, Kilbey A, Jenkins A, Terry A, et al. (2004) RUNX1 transformation of primary embryonic fibroblasts is revealed in the absence of p53. Oncogene 23: 5476–5486.
14. Britos-Bray M, Ramirez M, Cao W, Wang X, Liu PP, et al. (1998) CBFbeta-SMMHC, expressed in M4eo acute myeloid leukemia, reduces p53 induction and slows apoptosis in hematopoietic cells exposed to DNA-damaging agents. Blood 92: 4344–4352.
15. Cao W, Adya N, Britos-Bray M, Liu PP, Friedman AD (1998) The core binding factor (CBF) alpha interaction domain and the smooth muscle myosin heavy chain (SMMHC) segment of CBFbeta-SMMHC are both required to slow cell proliferation. J Biol Chem 273: 31534–31540.
16. Zhang L, Fried FB, Guo H, Friedman AD (2008) Cyclin-dependent kinase phosphorylation of RUNX1/AML1 on 3 sites increases transactivation potency and stimulates cell proliferation. Blood 111: 1193–1200.
17. Zhang DE, Hohaus S, Voso MT, Chen HM, Smith LT, et al. (1996) Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. Curr Top Microbiol Immunol 211: 137–147.
18. Lian JB, Balint E, Javed A, Drissi H, Vitti R, et al. (2003) Runx1/AML1 hematopoietic transcription factor contributes to skeletal development in vivo. J Cell Physiol 196: 301–311.
19. Gonzalez de Aguilar JL, Niederhauser-Wiederkehr C, Halter B, De Tapia M, Di Scala F, et al. (2008) Gene profiling of skeletal muscle in an amyotrophic lateral sclerosis mouse model. Physiol Genomics 32: 207–218.
20. Wang X, Blagden C, Fan J, Nowak SJ, Taniuchi I, et al. (2005) Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle. Genes Dev 19: 1715–1722.
21. Zhu X, Yeadon JE, Burden SJ (1994) AML1 is expressed in skeletal muscle and is regulated by innervation. Mol Cell Biol 14: 8051–8057.
22. Buckingham M (1996) Skeletal muscle development and the role of the myogenic regulatory factors. Biochem Soc Trans 24: 506–509.
23. Smith RC, Wills KN, Antelman D, Perlman H, Truong LN, et al. (1997) Adenoviral constructs encoding phosphorylation-competent full-length and truncated forms of the human retinoblastoma protein inhibit myocyte proliferation and neointima formation. Circulation 96: 1899–1905.
24. Mal A, Harter ML (2003) MyoD is functionally linked to the silencing of a muscle-specific regulatory gene prior to skeletal myogenesis. Proc Natl Acad Sci U S A 100: 1735–1739.
25. Ohkawa Y, Marfella CG, Imbalzano AN (2006) Skeletal muscle specification by myogenin and Mef2D via the SWI/SNF ATPase Brg1. Embo J 25: 490–501.
26. Zhang CL, McKinsey TA, Olson EN (2002) Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. Mol Cell Biol 22: 7302–7312.
27. de la Serna IL, Carlson KA, Imbalzano AN (2001) Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. Nat Genet 27: 187–190.
28. Fulco M, Schiltz RL, Iezzi S, King MT, Zhao P, et al. (2003) Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. Mol Cell 12: 51–62.
29. Buckingham M (2001) Skeletal muscle formation in vertebrates. Curr Opin Genet Dev 11: 440–448.

30. Reed-Inderbitzin E, Moreno-Miralles I, Vanden-Eynden SK, Xie J, Lutterbach B, et al. (2006) RUNX1 associates with histone deacetylases and SUV39H1 to repress transcription. Oncogene 25: 5777–5786.
31. Yahi H, Fritsch L, Philipot O, Guasconi V, Souidi M, et al. (2008) Differential cooperation between heterochromatin protein HP1 isoforms and MyoD in myoblasts. J Biol Chem 283: 23692–23700.
32. Andreu N, Garcia-Rodriguez M, Volpini V, Frecha C, Molina IJ, et al. (2006) A novel Wiskott-Aldrich syndrome protein (WASP) complex mutation identified in a WAS patient results in an aberrant product at the C-terminus from two transcripts with unusual polyA signals. J Hum Genet 51: 92–97.
33. Ayyanathan K, Lechner MS, Bell P, Maul GG, Schultz DC, et al. (2003) Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev 17: 1855–1869.
34. Ishibashi J, Perry RL, Asakura A, Rudnicki MA (2005) MyoD induces myogenic differentiation through cooperation of its NH2- and COOH-terminal regions. J Cell Biol 171: 471–482.
35. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, et al. (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20: 8783–8792.
36. Gersbach CA, Byers BA, Pavlath GK, Garcia AJ (2004) Runx2/Cbfa1 stimulates transdifferentiation of primary skeletal myoblasts into a mineralizing osteoblastic phenotype. Exp Cell Res 300: 406–417.
37. Tanaka T, Sato H, Doi H, Yoshida CA, Shimizu T, et al. (2008) Runx2 represses myocardin-mediated differentiation and facilitates osteogenic conversion of vascular smooth muscle cells. Mol Cell Biol 28: 1147–1160.
38. Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, et al. (2002) Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat Neurosci 5: 946–954.
39. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, et al. (1994) AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics 23: 425–432.
40. Levanon D, Bernstein Y, Negreanu V, Ghozi MC, Bar-Am I, et al. (1996) A large variety of alternatively spliced and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. DNA Cell Biol 15: 175–185.
41. Meyers S, Lenny N, Sun W, Hiebert SW (1996) AML-2 is a potential target for transcriptional regulation by the t(8;21) and t(12;21) fusion proteins in acute leukemia. Oncogene 13: 303–312.
42. Le XF, Groner Y, Kornblau SM, Gu Y, Hittelman WN, et al. (1999) Regulation of AML2/CBFA3 in hematopoietic cells through the retinoic acid receptor alpha-dependent signaling pathway. J Biol Chem 274: 21651–21658.
43. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, et al. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 267: 1018–1021.
44. Cenciarelli C, De Santa F, Puri PL, Mattei E, Ricci L, et al. (1999) Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. Mol Cell Biol 19: 5203–5217.
45. Yoshida N, Ogata T, Tanabe K, Li S, Nakazato M, et al. (2005) Filamin A-bound PEBP2beta/CBFbeta is retained in the cytoplasm and prevented from functioning as a partner of the Runx1 transcription factor. Mol Cell Biol 25: 1003–1012.
46. Buckingham M (1992) Making muscle in mammals. Trends Genet 8: 144–148.
47. Chiba N, Watanabe T, Nomura S, Tanaka Y, Minowa M, et al. (1997) Differentiation dependent expression and distinct subcellular localization of the protooncogene product, PEBP2beta/CBFbeta, in muscle development. Oncogene 14: 2543–2552.
48. Simon AM, Burden SJ (1993) An E box mediates activation and repression of the acetylcholine receptor delta-subunit gene during myogenesis. MolCell Biol 13: 5133–5140.
49. Mal AK (2006) Histone methyltransferase Suv39h1 represses MyoD-stimulated myogenic differentiation. Embo J 25: 3323–3334.
50. Puri PL, Iezzi S, Stiegler P, Chen TT, Schiltz RL, et al. (2001) Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. Mol Cell 8: 885–897.
51. Chakraborty S, Sinha KK, Senyuk V, Nucifora G (2003) SUV39H1 interacts with AML1 and abrogates AML1 transactivity. AML1 is methylated in vivo. Oncogene 22: 5229–5237.
52. Taniuchi I, Littman DR (2004) Epigenetic gene silencing by Runx proteins. Oncogene 23: 4341–4345.
53. Hewitt J, Lu X, Gilbert L, Nanes MS (2008) The muscle transcription factor MyoD promotes osteoblast differentiation by stimulation of the Osterix promoter. Endocrinology 149: 3698–3707.
54. Komaki M, Asakura A, Rudnicki MA, Sodek J, Cheifetz S (2004) MyoD enhances BMP7-induced osteogenic differentiation of myogenic cell cultures. J Cell Sci 117: 1457–1468.
55. Asakura A, Komaki M, Rudnicki M (2001) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 68: 245–253.
56. Ait-Si-Ali S, Guasconi V, Fritsch L, Yahi H, Sekhri R, et al. (2004) A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. Embo J 23: 605–615.
57. Robin P, Fritsch L, Philipot O, Svinarchuk F, Ait-Si-Ali S (2007) Post-translational modifications of histones H3 and H4 associated with the histone methyltransferases Suv39h1 and G9a. Genome Biol 8: R270.
58. Ouararhni K, Hadj-Slimane R, Ait-Si-Ali S, Robin P, Mietton F, et al. (2006) The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. Genes Dev 20: 3324–3336.
59. Ito H, Kamei K, Iwamoto I, Inaguma Y, Kato K (2001) Regulation of the levels of small heat-shock proteins during differentiation of C2C12 cells. Exp Cell Res 266: 213–221.

Pteridines
Vol. 22, 2011, pp. 97 - 104

Hepatotoxicity and Langerhans Islets Regenerative Effects of Polar and Neutral Lipids of *Nigella sativa* L. in Nicotinamide/streptozotocin-Induced Diabetic Rats

Sobhi Widad[1,2], Khettal Bachra[2], Belmouhoub Messaoud[2], Atmani Djebbar[3], Duez Pierre[4], Benboubetra Mustapha[1]

[1]Laboratory of Applied Biochemistry, Faculty of Sciences, University of Setif, Algeria, [2]Laboratory of Vegetal Biotechnology and Ethnobotany, Faculty of Life and Nature Sciences, University of Bejaia, Algeria, [3]Laboratory of Applied Biochemistry, Faculty of Life and Nature Sciences, University of Bejaia, Algeria, [4]Laboratory of Pharmacognosy, Bromatology and Human Nutrition, Faculty of Pharmacy, Université Libre de Bruxelles(ULB), Belgium.

Date received: 2011/08/10

Abstract

The extracted oil from *Nigella sativ*a seeds is reported to be effective against various diseases and chemically-induced hepatotoxicity and nephrotoxicity. The effect of oral administration of *Nigella sativa* total, polar and neutral oils was investigated on hepatoprotective status in streptozotocin/nicotinamide (STZ-N)-induced diabetic rats. The toxicity was assessed biochemically by monitoring aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptitase (γ-GT) and alkaline phosphatase (AP) activities as well as biluribin titre and histologically under light microscope. The study was also undertaken to evaluate the effect of oil fractions on the regeneration of pancreatic Langerhans islets in treated diabetic rats.
Biochemical analysis showed that lipid fractions from total oil of *Nigella sativa* seeds are not hepatotoxic. However, histological study of the liver demonstrated major and minor tissue damages with the neutral fraction exhibiting the most protective effect. At the end of the experiment period (17 days) of treatment with thymoquinone (25mg/kg bw/day) or neutral lipid fraction (100mg/kg bw/day), a positive effect on the regenerative of Langerhans islets, initially distorted by STZ, was observed. Thus, the hypoglycaemic effect of neutral lipid fraction could be a result of the regeneration of the pancreatic Langerhans islets.

Key words: *Nigella sativa seeds*, polar and neutral lipids, diabetes, streptozotocin, hepatotoxicity, regenerative effect on Langerhans islets.

Introduction

The hypoglycaemic effect of the seeds and oil of *Nigella sativa Linn*, family *Ranunculaceae*, an annual medicinal plant widely used in traditional medicine all over the world, is well documented. Different studies indicate that various extracts from the seeds and its major phenolic compound, thymoquinone, exhibit an anti-diabetic activity by either decreasing the levels of both blood glucose and lipids or increasing the release of insulin (1-3) or by the inhibitory effect on intestinal glucose absorption (4,5).

Beside the well established strong antioxidant properties, the oil of *Nigella sativa* seeds was effective against various diseases and chemically-induced hepatotoxicity and nephrotoxicity (6). The protective effect of the oil of *Nigella sativa* seeds and thymoquinone on liver and Langerhans islets was described in many studies (7,8). In fact, it was reported that *Nigella sativa* seeds uptake decreases lipid peroxidation, increases the antioxidant defence system and also prevents the lipid-peroxidation-induced liver damage in experimentally induced diabetic rabbits (9).

Volatile oil of *Nigella sativa* seeds reduces the level of glucose in streptozotocin/nicotinamide (STZ-N)-induced diabetic hamster and increases the rate of insulin production (10). Few years later, it has been reported that thymoquinone reduces the level of blood glucose and its hepatic production through a decrease in hepatic gluconeogenesis (11). The administration of

Correspondence to: Widad SOBHI, Department of Physical and Chemical Biology, Faculty of Life and Nature Sciences, University of Bejaia, Targa Ouzermour, 06000 Bejaia, Algeria, Tel. + 213 (0) 553688956.
E-mail: sobhiwidad@yahoo.fr

3mg/kg bw/day of thymoquinone abrogates the hyperglycaemic and hypo-insulinemic response to STZ in rats (8). Administration of volatile oil of *Nigella sativa* seeds prior to STZ induction of diabetes gave a protective effect by decreasing lipid peroxidation and serum nitric oxide (NO), and increasing antioxidant enzyme activity in diabetic rats with an increase in staining intensity for insulin and the number of preserved β-cell (2,12). Increasing glucose-induced insulin release from the β-cells of the Langerhans islets was also observed by using defatted whole extract and basic subfractions of the *Nigella sativa* seeds (13). The effect of total oil of *Nigella sativa* seeds on rat blood homeostasis and its hepatotoxicity has been well investigated (14,15).

Tested on isolated rat hepatocytes, thymoquinone (1mM) was found to be a hepato-protective agent, by reducing the leakage of cytosolic enzymes, alanine transaminase (ALT) and aspartate transaminase (AST), against the toxicity induced by t-butyl hydroperoxide (TBHP) (7).

Recently, Kanter reported that *Nigella sativa* and thymoquinone therapy causes morphologic improvement in neurodegeneration in frontal cortex, brain stem and hippocampus after chronic toluene exposure in rats (16,17). *Nigella sativa* and thymoquinone, used in the treatment of STZ-induced diabetic rats, increased significantly the area of insulin immunoreactive -cells and the histological evaluation of the tissues in treated diabetic animals showed fewer morphologic alterations (17,18). Thymoquinone demonstrated cyto- and genotoxic effects in a concentration-dependent manner (19).

As far as we are aware of, up to date no hepatotoxicity effect of *Nigella sativa* oil was reported. In this study, by looking at the biochemical hepatic markers and histologically on liver slides, we investigated the hepatotoxicity effect of *Nigella sativa* total oil, polar and neutral lipid fractions of total oil and thymoquinone. In parallel, we looked at the regenerative effect of these compounds on Langerhans islets in STZ-N-induced diabetic rats with comparison to the anti-diabetic drug metformin.

Material and methods

Plant materials and extraction procedure

Nigella sativa seeds were purchased from a local herb store in Bejaia, Algeria, and identified by Doctor Benabdelmoumene in the laboratory of plant biology (University of Bejaia, Algeria) where specimens are deposited for future reference. The seeds were powdered in a mixer. 40 g of the powdered seeds were added to 200 ml of methanol and extraction was carried out by steam distillation and dried under vacuum. The obtained extract was subject to a second extraction with the organic solvent hexane (three times 50 ml) then dried yielding a defatted methanolic extract and the lipid fractions.

The total oil was then applied on a silica gel 60 G (70-230 mesh, Merck) column (30cmx3cm). The total neutral lipid fraction (TNLF) was eluted by chloroform (5x100ml), whereas the total polar lipid fraction (TPLF) was obtained by acetone-methanol (50:50, 5x100ml).

Induction of diabetes

Animals:

Female healthy albino rats of Wistar strain, aged 8 weeks and weighting between 180 and 220g, were obtained from the Centre for Research and Development (CRD) at the pharmaceutical group company SAIDAL (Algiers, Algeria). They were housed in plastic cages with a 12/12-h light/dark cycle at an ambient temperature of 22-24°C and humidity of approximately 50%. Animals were fed standard diet and water *ad libitum*. All experiments were in compliance with the guidelines for the care and use of laboratory animals published by the US National Institute of Health (NIH publication No 85-23, revised 1985) with approval of SAIDAL ethic committee.

Experimental protocol:

Prior to experimentation, rats were maintained for three weeks on a normal diet before inducing diabetes by administration of N/STZ. The rats were randomly allotted into one of seven experimental groups:

Group I: control
Group II: diabetic untreated
Group III: diabetic treated with 100mg/kg bw/day of total volatile oil
Group IV: diabetic treated with 100mg/kg bw/day of total polar lipids
Group V: diabetic treated with 100mg/kg bw/day of total neutral lipids
Group VI: diabetic treated with 25 mg/kg bw/day of thymoquinone
Group VII: diabetic treated with 25 mg/kg bw/day of metformin

Diabetes was induced in all groups except group A by a single intra-peritoneal injection of nicotinamide (230 mg/kg) followed, after 15min, by a single intraperitoneal injection of STZ (65 mg/kg) freshly dissolved in 5mmol/L citrate buffer, pH 4.5 (2,12) according to the protocol reported by Masiello and co-workers (20) with slight modification. Animals of group I (control normal rats) and of group II (untreated diabe-

tic rats) received daily a volume of tap water equivalent to administered treatment volumes by intra-gastric gavage. Two days after STZ-N treatment, development of diabetes was confirmed by measuring blood glucose levels in a tail vein blood samples. Rats with blood glucose levels of 110 mg/dl or higher were considered as diabetic and included for the study. Serum glucose levels in control animals remained normal for the duration of the study (17 days). We took advantage of the partial protection of nicotinamide against the beta-cytotoxic effect of STZ to create a new experimental diabetic syndrome in adult rats that appears closer to non insulin-dependent diabetes mellitus (NIDDM) or type 2. This model compared to other available animal models, with regard to insulin responsiveness to glucose and sulfonylureas, yielded a maximum of animals with moderate and stable non-fasting hyperglycemia with a partial preservation of pancreatic insulin stores.

Biochemical analysis

Five days after N/STZ administration, we confirmed the development of diabetes by measuring the blood glucose using the glucose oxidase method (kit from Elitech Diagnostic, France) on overnight fasted rats. Animals showing fasting blood sugars (FBS) more than 110 mg/dl were considered as diabetic and included in the study.

At the end of experimental period (17th day), rats were fasted overnight and blood was taken, centrifuged and serum separated. Effect of each fraction on FBS level was evaluated. Serum levels of transaminases ALT and AST (kits QCA.S.A, Spain) were determined according to Bergmeyer method (21), gamma-glutamyl transpeptidase (γ-GT) (kits, QCA.S.A, Spain) was determined according to the Szass methods (1969), total bilirubin (kits, QCA.S.A, Amposta /Spain) according to the Jandrassik-Grof method (22) modified by Doumas (23) and alkaline phosphatase (AP) (kits, QCA.S.A, Amposta/Spain) according to the method described by Hausamen and his collaborators (24). Results were compared with that of tymoquinone and standard antidiabetic drug metformin.

Histopathological procedures

After the blood sample collection, all animals were killed under pentobarbital anaesthesia (60 mg/kg bw; Sigma, Poole, UK). Liver and pancreatic tissues were harvested and tissue fragments were fixed in 10% neutral formalin solution, embedded in paraffin, and sectioned at 3µm thickness using a microtome (Leica Microsystems, Leica RM 2125 RT). Slices were treated with xylene and ethanol, and then stained with haematoxylin and eosin (H&E). The preparations were evaluated by means of an optical bright-field microscope and photographed (Optiphot 2, Nikon, Tokyo, Japan).

Statistical analysis

The results were expressed as Mean ± SEM. The Student's t-test was used for analyzing the data between treated and control groups. Values of $p < 0.05$ were considered statistically significant.

Results

The use of N-STZ has induced diabetes in all rats but glycaemia was not as high as reported previously when induced by STZ alone (2,8,9,11,12,25,26). This is due to the protective effect of nicotinamide (230mg/kg bw) on the pancreatic β cells, to an extent

Table 1. Measurement of the hepatic activity of four enzymes and bilirubin concentrations in serum of normal, diabetic, and diabetic treated with total oil, polar and neutral lipid fractions of *Nigella sativa* seeds, in rats.
Group I: normal rats, **Group II:** diabetic rats, **Group III:** diabetic rats treated with 100mg/kg bw/day of total oil of *Nigella sativa* seeds, **Group IV:** diabetic rats treated with 100mg/kg bw/day of polar fraction of *Nigella sativa* seeds total oil, **Group V:** diabetic rats treated with 100mg:kg bw/day of neutral fraction total oil of *Nigella sativa* seeds, **Group VI:** diabetic rats treated with 25mg/kg bw/day of thymoquinone, **Group VII:** diabetic rats treated with 25mg/kg bw /day of metformin. Each group contains 7 rats.
AST = aspartate transaminase, ALZ = alanine transaminase, γ-GT = gamma-glutamyl transpeptidase, AP = alkaline phosphatase

ANIMALS	AST (IU/L)	ALT (IU/L)	γ-GT (IU/L)	Bilirubin (mg/L)	AP (IU/L)
Group I	56.5 ± 10.1	20.3 ± 4.04	1.35 ± 0.22	0.067 ± 0.007	332 ± 4.75
Group II	82.1 ± 2.05	40.0 ± 1.18	0.87 ± 0.10	0.021 ± 0.004	430 ± 74.7
Group III	47.2 ± 3.31	26.5 ± 2.95	0.83 ± 0.08	0.071 ± 0.005	304 ± 58.9
Group IV	44.3 ± 1.77	18.0 ± 3.32	0.81 ± 0.21	0.047 ± 0.007	300 ± 75.0
Group V	42.3 ± 1.92	20.4 ± 1.26	1.44 ± 0.41	0.059 ± 0.005	186 ± 55.0
Group VI	39.9 ± 2.05	20.9 ± 3.91	1.28 ± 0.51	0.049 ± 0.005	306 ± 83.2
GroupVII	60.7 ± 0.61	24.6 ± 0.43	3.58 ± 0.50	0.048 ± 0.007	311 ± 38.3

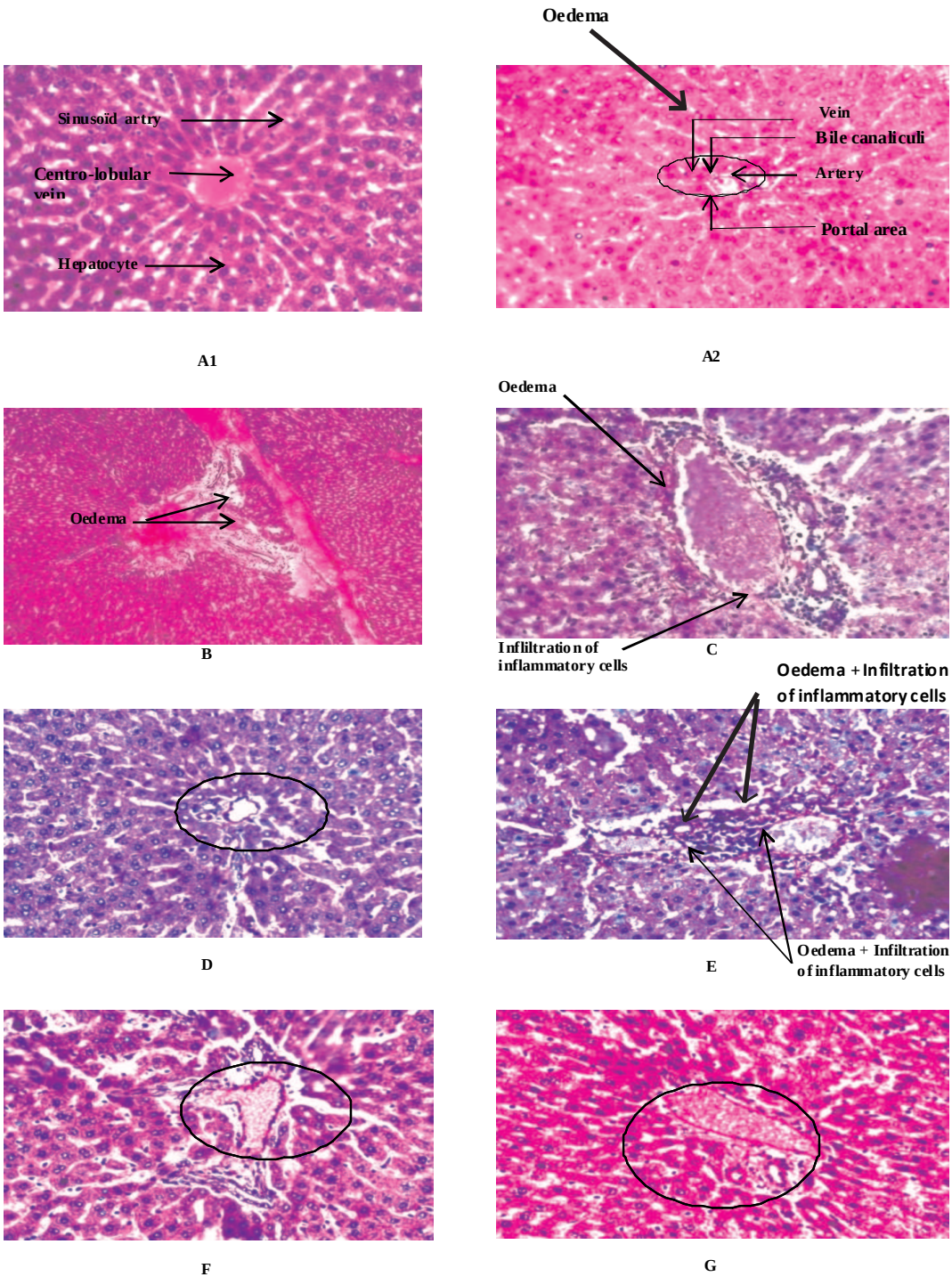


Figure 1. Effect of *Nigella sativa* extract on liver tissue, Histology of liver tissues from normal rats (A1&A2), untreated diabetic rats (B); diabetic rats treated (for 17 days) with total *Nigella sativa* seeds oil at100mg/kg/day (C), lipid polar (D) and neutral (E) fractions from total *Nigella sativa* seeds oil at100mg/kg bw/day; thymoquinone at 25 mg/kg bw/day (F) and metformin at 25 mg/kg bw/day (G). Diabetes was induced by N/STZ. Magnification (X425) Slides are stained with haematoxylin and eosin (H&E).

of 40 % or more in favor of the development of type II diabetes without the obesity effect (20). At the end of the experimental period, treatment with total oil, polar and neutral lipid fractions (at 100mg/kg bw/day for 17 days) caused a significant ($p < 0.01$) decrease in fasting blood glucose (FBG), by 17.7 %, 28.46% and 16.51%

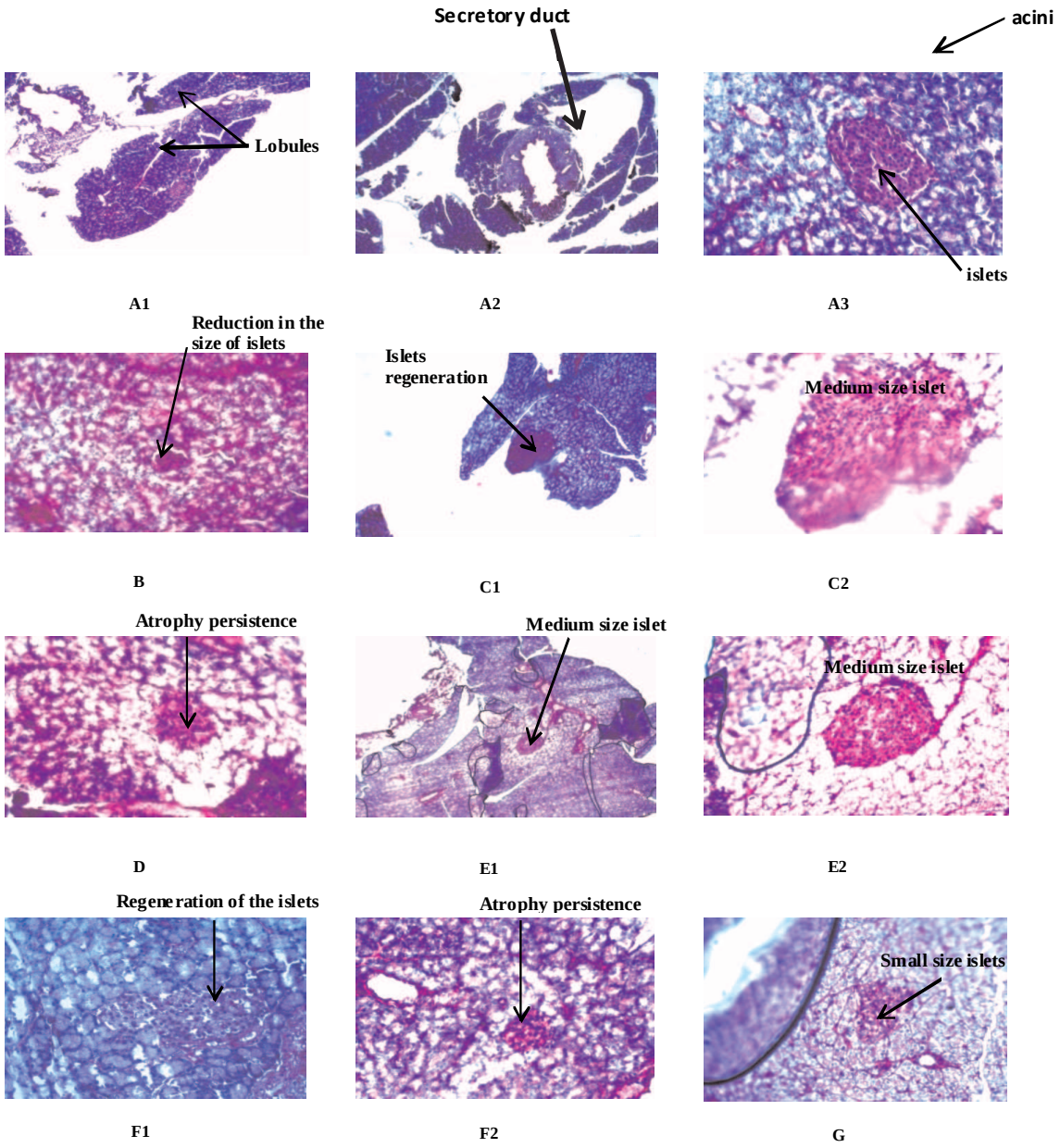


Figure 2. Effect on Nigella sativa extract on pancreatic tissue, Histology of pancreatic tissues from normal rats (A1, A2 & A3), untreated diabetic rats (B); diabetic rats treated (for 17 days) with total Nigella sativa seeds oil at100mg/kg bw/day (C1&C2), lipid polar (D) and neutral (E1&E2) fractions from total Nigella sativa seeds oil at100mg/kg bw/day; thymoquinone at 25 mg/kg bw/day (F1&F2) and metformin at 25 mg/kg bw/day (G). Diabetes was induced by N/STZ. Magnification: x125 for A1, C1, E1 and x425 for the others. Slides are stained with haematoxylin and eosin (H&E).

respectively, in diabetic rats (groups III, IV and V with 6 animals in each group). The antidiabetic drug metformin (at 25 mg/kg bw/day for 17 days) lead to 18.48% reduction in FBG (group VI, n=7) while thymoquinone (at 25 mg/kg bw/day for 17 days) did not show any significant effect on FBG which was reduced by less than 8% (group VII, n=6). Control normal rats (group I, n=9) and diabetic non treated rats (group I, n=6) were used as controls.

Table 1 illustrates the effect of total oil, polar and neutral lipid fractions on AST, ALT, γ-GT and AP enzyme activities and on total biluribin serum titer of diabetic rats at the beginning and at the end of experimental period, 17 days, of treatments (Table 1). It is clear that the three fractions as well thymoquinone and metformin had no significant effect on the levels of transaminases suggesting that they are not toxic for the liver. We found a significant increase in AST (82.06 ±

2.05 IU/L vs. 56.52 ± 10.12 IU/L for controls) and in ALT (39.98 ± 1.18 IU/L vs. 20.30 ± 4.04 IU/L for controls) enzymatic activities in untreated diabetic rats.

Apart from metformin that led to nearly 2.6 fold increase γ-GT activity (3.58 ± 0.50 IU/L vs. 1.35 ± 0.22 IU/L for controls), *Nigella sativa* oil extracts and thymoquinone did not alter the activity of this enzyme demonstrating their non cytolytic hepatic effect. Levels of bilirubin were not affected by treatment with either *Nigella sativa* oil extracts or the two standard molecules (metformin and thymoquinone). However, a significant drop in the level of bilirubin was noticed with untreated diabetic rats (0.021 ± 0.004 mg/dl vs. 0.067 ± 0.007 mg/dl in controls) (Table 1).

Treatment with neutral lipid fraction led to a significant reduction of AP activity, from 332 ± 4.75 IU/L to 186 ± 55.0 IU/L. Treatments with total oil, polar lipid fraction, thymoquinone and metformin did not show any significant changes. However, a significant increase in the activity of this enzyme to 430 ± 74.7 IU/L, was recorded in diabetic non treated rats (Table 1).

In contrast to the biochemical markers where no obvious indication of any hepatotoxicity by different fractions and molecules were observed, the histopathological analysis showed minor and major hepatic lesions (Figure 1). The histology of liver tissue was normal in control rats. Hepatic tissues of this group I present a normal morphological aspect with a lot of lobules, each of which is constituted of hepatocytes separated by small arteries converging towards the centrolobular vein. Lobules are small patches of connective tissue each containing a duct, a large vein, and a small artery which mark the corners where lobules come together, as shown in Figure 1A.

In non-treated diabetic rats, the most consistent findings were the enlargement of the portal area due to the formation of edema (Figure 1B). Treated diabetic rats with total oil of *Nigella sativa* seeds or with the neutral lipid fraction (100 mg/kg bw/day for 17 days) showed, in addition to the enlargement of the portal area observed in non treated rats, inflammatory manifestations due to the infiltration of mononuclear cells (Figure 1C and Figure 1D respectively) pointing out to the occurrence of hepatic lesions or hepatitis. The histology of livers from group IV, treated with polar lipids at 100 mg/kg bw/day for 17 days, showed no inflammatory cell infiltration (Figure 1E). Liver tissue of rats treated with thymoquinone (group VII), 25mg/kg bw/day for 17 days, present an enlargement of the portal area but without inflammatory infiltration (Figure 1F). Metformin treatment had minor effect on the liver histology, which appears to be normal with a much reduced enlargement of the portal area and without inflammatory cell infiltration (Figure 1G).

Histopathological investigation on pancreatic tissue revealed that the pancreatic islet cells were normal in control rats, a full parenchyma with lobular structure where lobules are separated by connective tissue. The islets are of normal size and surrounded by serous acini (Figure 2A1) and a normal extra-lobular excretory duct (Figure 2A2). In diabetic rats with no treatment, pancreatic tissue is abnormal with lobular atrophy and a marked diminution in number and size of islets (Figure 2B) which is a consequence of STZ toxic effect leading to a drop in insulin production and hence development of diabetes.

Pancreatic tissue of diabetic rats treated with total oil (100 mg/kg bw/day for 17 days) presented islets of medium size (Figure 2C). An obvious improvement is observed when compared to slides from untreated rats (Figure 2B) suggesting a regenerative effect. Treatment with polar lipid fractions from total oil of *Nigella sativa* seeds (100mg/kg bw/day, for 17 day) gave no improvement with the persistence of islets anomalies (Figure 2C). But, in the other hand, a significant improvement was observed with the neutral lipid fraction where islets distinctly increased in size (up to 3 fold) and number (Figure 2D). This supports the possibility of a regenerative potential effect of neutral lipids on pancreatic tissue. A more marked increase, particularly in size (up to 5.5 fold), was observed in rats treated with thymoquinone (25mg/kg bw/day, for 17 day) (Figure 2E). In contrast, treatment with metformin (25mg/kg/day, for 17 day) had no effect of the recovery in terms of the number and size of the islets (Figure 2F).

Discussion

The hypoglycaemia activity of extracts from the seeds of *Nigella sativa* in general and oils in particular, with emphasis on thymoquinone, the major constituent of volatile oils of the seeds, is well documented (2-5,9,11,25,27). Our data suggest that such hypoglycaemic effect is a result of (i) a hepatic regulation of carbohydrate metabolism (11), (ii) action on the intestinal absorption (5) or (iii) regeneration of insulin producing pancreatic islets (2,12,13,25,26,28). However, little is known about the toxicity of extracts from these seeds. Recently, It has been reported beside the neuroprotective effect of the oil of *Nigella sativa* seeds and thymoquinone on rats exposed to neurotoxique agents (16-18), and a protective effect of beta-cell damage in STZ-induced diabetic rats (28).

At the end of the experiment (day 17), a significant decrease in blood glucose level was observed in diabetic rats treated with total oil, neutral and polar lipid fractions. In contrast, thymoquinone did not show any

significant effect on glycaemia. In a quite similar study, Kanter and coworkers used 0.2ml/kg bw/day of essential oil from *Nigella sativa* seeds on a one month treatment showed a reduction in blood glucose and an increase in insulin production as a consequence of the regeneration of Langerhans islets (2,12).

High concentrations of total oil or thymoquinone from *Nigella sativa* seeds have been used to show their hypoglycaemiant effect on treated animals in STZ or alloxan induced diabetes (3,4,9,11).

Despite the fact that liver markers AST, ALT, γ-GT, AP and biluribin did not show any hepatotoxic effect which is in agreement with what has been reported previously, histological studies showed clear and apparent hepatic lesions on liver slides.

Histological comparison between liver tissues from different groups of rats showed that diabetic untreated rats present an enlargement of the portal areas which could be a result of sugar metabolic disorders responsible for hepatic edema. The treatment with either total oil or polar fraction worsens the case by an observed inflammatory cell infiltration in contrast to the recovery, with the reduction in edema size, obtained with the neural fraction. This suggests the presence of a compound (s) in the polar fraction behind the secondary inflammation process (cell infiltration) observed and that the neutral fraction possesses a hepatoprotective effect. Surprisingly, both thymoquinone and metformin did not show any protective effect on the liver of treated diabetic rats. We note that concentrations tested of each treatment: total oil, polar lipid fraction, neutral lipid fraction, thymoquinone and metformin induce a slight histopathological toxic effect, but those lesions could not modify the titer of the hepatic markers.

STZ induces degeneration of the pancreas with a lobular atrophy and a decrease in size and number of Langerhans islets (2,20). A recovery on the pancreatic tissues of rats treated with total oil or the neutral of lipid fraction was observed with a size increase of the islets due to either a regeneration and /or proliferation of the islets in contrast to the treatment with the polar fraction which did not show any positive effect, namely persistence of the abnormalities observed in non treated diabetic rats. Slight regenerative and proliferative effects were noticed on the pancreatic tissues of rats treated with thymoquinone but not with metformin.

Published papers support our findings: Meral and his coworkers noticed that *Nigella sativa* oil improves the antioxidant defense system (reduction in MDA and an increase in GSH and ceruloplasmin), of the liver in alloxan induced diabetes in rabbits with a normal hepatic histology (9). However, pancreatic slides from diabetic rabbits treated or not with total oil from *Nigella sativa* seeds showed degeneration, necrosis and infiltration with mononuclear cells beside edema formation (9). A protective effect of the volatile oil on β cells, by acting on the oxidative stress generated by NO following STZ action on the islets of Langerhans (2,12,25) with a light and electron microscopic studies (28) has been reported. Beside the hypoglycaemic effect, extracts from *Nigella sativa* seeds stimulate insulin production in isolated Langerhans islets (13).

Pancreatic protective effect of thymoquinone is well documented where the administration of 50mg/kg bw/day to STZ-induced diabetic hamsters reduces not only significantly blood sugar and glycosylated hemoglobin but acts equally on the reduction of gluconeogenesis (11). It protects the pancreas against NO generated oxidative stress (8) and showed a hepatoprotective effect on cultured hepatocytes (7). In our study, we observed that the regenerative effect on pancreatic islets of neutral lipid fraction was more important than that of thymoquinone.

In this study, we confirmed the hypoglycemic effect of *Nigella sativa* seeds oil and showed that it resides mainly on its polar lipid fraction. Although no changes on hepatic enzyme activities and biluribin titer were recorded, histological investigation showed liver damages on diabetic rats treated with *Nigella sativa* lipid extracts. Neutral lipid fraction of the oil showed a regenerative and/or a proliferative effect on STZ damaged pancreatic islets. This leads us to suggest that the hypoglycemic effect *Nigella sativa* is partially due to the positive action of the neutral oil fraction on the pancreatic tissue. The action of the polar oil fraction in lowering blood sugar could be explained by other mechanisms such as the action on intestinal sugar metabolism or by the regulation of such metabolism in the liver. The same thing can be stated for the hypoglycemic action of thymoquinone where its involvement on hepatic glycogenesis regulation has been reported (11).

References

1 Fararh KM, Atoji Y, Shimizu Y, Shiina T, Nikami H, Takewaki T. Mechanisms of the hypoglycaemic and immunopotentiating effects of *Nigella sativa L*. oil in streptozotocin-induced diabetic hamsters. Res Vet Sci 2004;77:123-129.

2 Kaleem M, Kirmani D, Asif M, Ahmed Q, Bano B. Biochemical effect of *Nigella sativa L*. seeds in diabetic rats. Indian J Exp Biol 2006;44:745-748.

3 Kanter M, Meral I, Dede S, Gunduz H, Cemek M, Ozbek H, Uygan I. Effects of *Nigella sativa L*. and *Urtica dioica L*. on lipid peroxidation, antioxidant enzyme systems and some liver enzymes in CCl4-

treated rats. J Vet Med A Physiol Pathol Clin Med 2003;50:264-268.
4 Houcher Z, Boudiaf K, Benboubetra M, Houcher B. Effects of methanolic extract and commercial oil of *Nigella sativa L*. on blood glucose and antioxidant capacity in alloxan-induced diabetic rats. Pteridines 2007;18:8-18.
5 Meddah B, Ducroc R, Faouzi MEA, Eto B, Mahraoui L, Benhaddou- Andaloussi A, Martineau LC, Cherrah Y, Haddad PS. *Nigella sativa* inhibits intestinal glucose absorption and improves glucose tolerance in rats. J Ethnopharmacol 2009;121:419-424.
6 Ali BH. The effect of *Nigella sativa* oil on gentamicin nephrotoxicity in rats. Am J Chin Med 2004;32:49-55.
7 Daba MH, Abdel-Rahman MS. Hepato-protective activity of thymoquinone in isolated rat hepatocytes. Toxicol Lett 1998;95:23-29.
8 El-Mahmoudy A.B, Shimizu Y, Shiina T, Matsuyama H, El-Sayed M, Takewaki T. Successful abrogation by thymoquinone against introduction of diabetes mellitus with streptozoticin via nitric oxide inhibitory mechanism. Int Immunopharmacol 2005;5:195-207.
9 Meral I, Yener Z, Kahraman T, Mert N. Effect of *Nigella sativa* on glucose concentration, lipid peroxidation, anti-oxidant defence system and liver damage in experimentally-induced diabetic rabbits. J Vet Med 2001;48:593-599.
10 Farah KM, Atoji Y, Shimizu Y, Takewaki T. Insulinotropic proprieties of *Nigella sativa* oil in streptozotocin-induced plus nicotinamide diabetic hamster. Res Vet Sci 2002;73:279-282.
11 Farah KM, Shimizu Y, Shiina T, Nikami H, Ghanem MM, Takewaki T. Thymoquinone reduces hepatic glucose production in diabetic hamsters. Res Vet Sci 2005;79:219-223.
12 Kanter M, Coskun O, Korkmaz A, Oter S. Effects of *Nigella sativa* on oxidative stress and β-cell damage in streptozotocin-induced diabetic rats. Anat Rec A Discov Mol Cell Evol Biol 2004;279: 685-691.
13 Rchid H, Chevassus H, Nmila R, Guiral C, Petit P, Chokairi M, Sauvair Y. *Nigella sativa* seed extracts enhance glucose-induced insulin release from rat-isolated Langerhans islets. Fund Clin Pharmacol 2004;18:525-529.
14 Zaoui A, Cherrah Y, Alaoui K, Mahassine N, Amarouch H, Hassar M. Effects of *Nigella sativa* fixed oil on blood homeostasis in rat. J Ethnopharmacol 2000;79:23-26.
15 Zaoui A, Cherrah Y, Mahassini N, Alaoui K, Amarouch H, Hassar M. Acute and chronic toxicity of *Nigella sativa* fixed oil. Phytomedicine 2000;91:69-74.
16 Kanter M. *Nigella sativa* and derived thymoquinone prevents hippocampal neurodegeneration after chronic toluene exposure in rats. Neurochem Res 2007;33:579-588.
17 Kanter M. Effect of *Nigella sativa* and its major constituent, thymoquinone on sciatic nerves in experimental diabetic neuropathy. Neurochem Res 2008;1:87-96.
18 Kanter M. Protective effects of *Nigella sativa* on the neural injury in frontal cortex and brain stem after chronic toluene exposure. Neurochem Res 2008;33:2241-2249.
19. Khader M, Bresgen N, Eckl PM. In vitro toxicological properties of thymoquinone. Food Chem Toxicol 2009;47:129-133.
20 Masiello P, Broca C, Gross, Roye M, Manteghetti M, Hillaire-Buys D, Novelli M, Ribes G. Experimental NIDDM, development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes 1998;47:224-229.
21 Bergmeyer, H.U, Scheibe, P, Wahlefeld, A.W.1978. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin. Chem, 24, 58-73
22 Jendrassik L, Gróf P. Simplified photometric methods for the determination of the blood bilirubin. Biochem Z 1938;297:81-89.
24 Hausamen TU, Helger R, Rick W, Gross W. Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. Clin Chim Acta 1967;15:241-245.
25 Kanter M, Meral I, Yener Z, Ozbek H, Demir H. Partial regeneration/proliferation of the β-cell in the islets of Langerhans by *Nigella sativa L*. in streptozotocin-induced diabetics rats. Tohoku J Exp Med 2003;201:213-219.
26 Kanter M, Sen S, Donmez S, Aktas C, Ustundag S, Erboga M. Protective effects of irbesartan and alpha lipoic acid in STZ-induced diabetic nephropathy in rats. Ren Fail 2010;32:498-505.
27 Le PM, Benhaddou-Andaloussi A, Elimadi A, Settat A, Cherrah Y, Haddad PS. Tthe petrolium ether extract of *Nigella sativa* exerts lipid-lowering and insulin-sensitizing actions in the rat. J Ethnopharmacol 2004;94:251-259.
28 Kanter M, Akpolat M, Aktas C. Protective effects of the volatile oil of *Nigella sativa* seeds on beta-cell damage in streptozotocin-induced diabetic rats: a light and electron microscopic study. J Mol Histol 2009;40:379-385.
29 Doumas BT, Perry BN, Sasse EA, Staumfjord JV Jr, Standardization in bilirubin assays: Evaluation of selected methods and stability of bilirubin solutions. Clin Chem 1973;19:984-993.

INLUENCE OF SAMPLING DATE ON REPRODUCTION IN THE LAND SNAIL *HELIX APERTA* KEPT UNDER CONTROLLED CONDITIONS OF TEMPERATURE AND PHOTOPERIOD

Saïda BENBELLIL-TAFOUGHALT[1*], Mohamed SAHNOUNE[1], Annette de VAUFLEURY[2] & Aissa MOALI[1]

RÉSUMÉ. — *Influence de la date d'échantillonnage sur la reproduction de l'escargot terrestre* Helix aperta *maintenu en conditions contrôlées de température et de photopériode*. — Les caractères reproductifs des escargots *Helix aperta* ont été étudiés sous quatre combinaisons de température et photopériode (20°C/16hL 8hD ; 20°C/8hL:16hD ; 15°C/16hL:8hD and 15°C/ 8hL:16hD). Trois échantillons ont été utilisés : Ech. 1 et Ech. 2 collectés à Annaba (Nord-Est Algérien) respectivement durant et après l'hibernation ; Ech. 3, précédemment analysé (données non publiées), collecté à Béjaïa (proche Nord-Est Algérien) durant l'estivation. Tous les escargots ont commencé à s'accoupler dès la 1ère semaine et à pondre durant la 3ème ou la 4ème semaine de leur mise en conditions de reproduction. Ech. 1 et Ech. 2 se sont distingués par leur plus courte période de reproduction (4-6 semaines) en comparaison avec Ech. 3 (5-7 semaines). D'une manière frappante, bien que les escargots collectés durant ou après hibernation (Ech. 1 et Ech. 2) se soient accouplés, dans la plupart des cas, à des taux plus élevés (56-87 %) que ceux échantillonnés durant l'estivation (32-92 %), leurs taux de pontes ont été dramatiquement plus faibles (6-25 % contre 12-80 %). Autrement dit, parmi les escargots qui se sont accouplés dans Ech. 1 et Ech. 2, seulement 11-36 % ont pondu, contre 38-87 % dans Ech. 3. Les nombres moyens d'œufs par ponte étaient plus élevés en Ech. 1 (293-323) et Ech. 3 (337-348) qu'en Ech. 2 (237-248) ($P < 0.05$). Inversement, les poids moyens des œufs par ponte étaient plus élevés en Ech. 2 (17.5-17.8 mg) qu'en Ech. 1 (16.1-16.3 mg) et Ech. 3 (16.3-16.6 mg) ($P < 0.05$). Après la période de reproduction, en conséquence des rendements reproductifs différentiels, les poids moyens des escargots ont très significativement augmenté en Ech. 1 et Ech. 2 ($P < 0.001$) et significativement baissé en Ech. 3 ($P < 0.05$). Les performances reproductives étaient plus affectées et les taux de mortalité plus élevés sous basse température et courte photopériode. La meilleure combinaison de ces deux facteurs était souvent 20°C/16hL:8hD, plus proche des conditions sur le terrain en automne, surtout durant la nuit, phase d'activité des escargots. Après la période de reproduction, la mortalité devenait de plus en plus élevée et les survivants moins actifs ou essayant de s'enfouir dans le sol des pots de ponte. Tous ces arguments plaident que les escargots *H. aperta* sont mieux adaptés à se reproduire en automne (jours décroissants et températures plus élevées) après une longue estivation stimulant la gamétogenèse qu'au printemps (jours croissants et températures basses) après l'hibernation inhibant la gamétogenèse.

SUMMARY. — Reproductive traits of *Helix aperta* snails were investigated under four combinations of temperature and photoperiod (20°C/16hL:8hD; 20°C/8hL:16hD; 15°C/16hL:8hD and 15°C/ 8hL:16hD). Three samples were used: Sample 1 and Sample 2 collected from Annaba (Northeastern Algeria) during and after hibernation respectively; Sample 3, previously studied (unpublished data), collected from Bejaïa (near Northeastern Algeria) during aestivation. All the snails began to mate on the first week and to lay on the 3rd-4th week of setting under conditions of reproduction. Sample 1 and Sample 2 were characterized by shorter reproduction periods (4-6 weeks) than Sample 3 (5-7 weeks). Strikingly, although most of the snails collected during and after hibernation mated at a high rate (56-87 %) in comparison with those collected during aestivation (32-92 %), their rates of egg-laying were drastically lower (6-25 % against 12-80 %).

[1] Laboratory of Ecology and Environment, Faculty of Nature and Life Sciences, University A. Mira of Bejaïa, 06000 Bejaïa, Algeria. E-mails: tafoughalt19@yahoo.fr; sahnounemohamed@yahoo.fr; rubiette_am@hotmail.com

[2] Department of Environmental Biology, EA 3184 USC INRA, University of Franche-Comté, Place Leclerc. F-25030 Besançon Cedex. E-mail: annette.devaufleury@univ-fcomte.fr

* Corresponding author. Phone number: (213) 777.00.80.66, Fax: (213) 34.21.47.62

Rev. Écol. (Terre Vie), vol. 66, 2011.

Among the snails that had mated, only 11-36 % laid eggs in Samples 1 and 2 against 38-87 % in Sample 3. The mean numbers of eggs per clutch were higher in Sample 1 (293-323) and Sample 3 (337-348) than in Sample 2 (237-248) ($P < 0.05$). Inversely, the mean egg weights per clutch were heavier in Sample 2 (17.5-17.8 mg) than in Sample 1 (16.1-16.3 mg) and Sample 3 (16.3-16.6 mg) ($P < 0.05$). After the reproduction period, as a consequence of differential reproductive yields, the mean weights of snails very significantly increased in Sample 1 and Sample 2 ($P < 0.001$) and significantly decreased in Sample 3 ($P < 0.05$). Reproductive performances were more affected and lethality rates higher under low temperature and short day photoperiod. The best combination of temperature and photoperiod was mostly 20°C/16hL:8hD, conditions closer to those in the field during autumn, especially overnight, the phase of snail activity. After the reproduction period, the lethality was becoming higher and higher and the surviving snails were less active or trying to burrow themselves into the soil of the egg-laying pots. All these arguments plead that *H. aperta* snails are better adapted to reproduce in autumn (decreasing days and higher temperatures) after a long aestivation stimulating gametogenesis than in spring (increasing days and lower temperatures) after hibernation inhibiting gametogenesis.

The terrestrial gastropod pulmonates are dependent on the abiotic factors that govern their life cycle. Most of these organisms observe periods of rest when their environment becomes hostile. Their growth and reproduction are strongly affected by their biotope conditions (Gomot *et al.*, 1982; Laurent *et al.*, 1984; Gomot & Deray, 1987).

Since the mid-60s, many researchers have taken an interest in studying the influence of environmental factors, in particular temperature and photoperiod, on the reproductive activity of land snails. The studies dealing with the effect of photoperiod on reproduction in helicid snails have yielded very divergent results from species to species. Thus, in *Cornu aspersum* (Stephens & Stephens, 1966; Charrier, 1980; Bailey, 1981; Enée *et al.*, 1982; Bonnefoy-Claudet *et al.*, 1987; Aupinel & Daguzan, 1989; Gomot *et al.*, 1990) and *Helix pomatia* (Gomot, 1990), it has been demonstrated that reproduction is stimulated by long-day photoperiods. However, in *Cepaea nemoralis*, reduction in reproduction activity is induced by both short-day and long-day photoperiods, with a more pronounced negative effect of the latter (Hunter & Stone, 1986).

Furthermore, the experiments of Gomot *et al*. (1986) and Griffond *et al.* (1992) on *Cornu aspersum* have clearly revealed that temperature has a very net effect on the multiplication and the development of the male line cells. On the other hand, in *Cornu aspersum aspersum* (Gomot *et al.*, 1989), *Helix pomatia* (Gomot, 1990) and *Cornu aspersum maxima* (Jess & Marks, 1998), temperature and photoperiod have been proved to exert a combined action on reproductive activity, with the stimulating long-day photoperiods compensating the negative effects of low temperatures.

Very few studies have been dedicated to *Helix aperta* Born (1778) (= *Cantareus apertus* Born, 1778). So, most of the eco-physiological aspects of its biology are not well known. This helicid species, native to the Mediterranean basin, is mentioned in the south of France, Italy, Turkey, Cyprus and North Africa (Kerney & Cameron, 1979; Schütt, 2001). According to Sacchi (1955, 1958), when temperatures are incompatible with its activity (below 7°C or beyond 27°C) or when relative humidity is outside its optimum (75-95 %), the *H. aperta* snail becomes inactive (i.e. enters endogenous aestivation or hibernation) and lives in a state of metabolism slowdown; this land mollusc is known as the "burrowing snail", coming up above ground only by rainy weather; by dry conditions, it burrows 7-15 cm deep into the ground and aestivates in a thick whitish operculum until rain softens the soil. *H. aperta* snails are poikilotherms (heterotherms) because their internal temperature varies with the temperature of their external surroundings; they are also eurytherms because they withstand strong variations in temperature of their external and internal environments.

The reproduction of *H. aperta* snail is rather poorly documented. Giusti & Andreini (1988) reported that individuals of this species, collected in autumn and in spring close to Orbetello and Castelnuovo Berardenga (Siena, Tuscany, Italy) and maintained under laboratory conditions similar to those in the field for continuous observations over three years (1982-1984), reproduce preferentially in autumn from early October to mid-December. In a recent work, de Vaufleury & Gimbert (2009) noted that reproduction of *H. aperta* snails, collected in late

summer at Bazina (Tunisia), is stimulated by short-day photoperiods (6 hours of Light – 18 hours of Dark) but inhibited under long-days (18 hours of Light – 6 hours of Dark). In addition, Tafoughalt-Benbellil *et al.* (2009) collected in autumn a sample of adult *H. aperta* snails at Bakaro (Bejaïa, Eastern Algeria) and bred them for several generations under controlled conditions; they have observed that, in snails of the fourth generation, the reproduction is strongly affected by photoperiod length and, moreover, temperature and photoperiod have interactive effects on both the number and duration of matings and layings, with a predominant influence of photoperiod.

In the present study, we aim to assess how time of sampling (during or after hibernation) affects the reproductive performances of *Helix aperta* snails in comparison with data on conspecific snails collected during aestivation.

MATERIALS AND METHODS

Geographic origin of snails and time of sampling

Two samples of adult snails identified as belonging to *Helix aperta* were collected at the same spot in the vicinity of the University of Annaba (Annaba, Northeastern Algeria). The first sample (Sample 1) was collected on the 3rd week of February 2007; at this time of year, the subjects were still inactive, i.e. in hibernation and hidden in the ground with shell blocked up by a thick whitish operculum; the snails were dug out of the soil using a small pick. The second sample (Sample 2) was drawn at the end of hibernation, on the fourth week of March 2007; the snails were collected early in the morning, by a rainy day; at the moment of sampling, the snails were active, crawling on the ground. Unpublished data on snails collected in September 2005 at Bakaro (Bejaïa, Northeastern Algeria) (Sample 3) were included in the present analysis. These unpublished data were obtained under exactly the same experimental conditions as for Sample 1 and Sample 2. At the moment of collect, the snails were still under aestivation, burrowed deep in the soil.

For the three samples, the snails presented roughly the same shell sizes, indicating a same stage of maturity. The meteorological characteristics of the sites of study are given in Table I.

Table I

Meteorological characteristics of the study sites. Precipitation values (P), maximal (M) and minimal (m) temperatures were recorded from January through December near the sites of study (Data from the Meteorological Stations of Bejaïa and Annaba, Algeria). The mid-month day lengths were estimated using sunrise and sunset times in the area (Calendar Talantikit, undated, Ed. Talantikit, Bejaia, Algeria)

Months	Bejaia 2005			Annaba 2007*			Day length (h:mn)
	P (mm)	M (°C)	m (°C)	P (mm)	M (°C)	m (°C)	
January	165.5	13.9	4.6	35.0	13.7	4.7	9:58
February	167.5	13.6	5.9	51.0	12.9	5.6	10:52
March	60.4	17.3	9.2	204.0	16.8	8.2	11:58
April	41.9	20.3	11.9	27.6	21.1	10.9	13:10
May	7.7	24.3	14.5	42.1	24.8	14.8	14:11
June	0.0	27.6	18.0	52.2	27.4	17.6	14:43
July	0.4	29.9	21.3	3.0	29.6	21.2	14:30
August	6.1	29.8	20.5	3.0	29.5	19.9	13:38
September	28.2	28.0	18.5	33.0	28.2	18.4	12:29
October	31.7	26.1	16.5	102.0	25.8	16.3	11:20
November	107.0	20.5	11.4	84.0	20.7	11.3	10:17
December	172.4	16.5	8.1	125.4	16.3	7.9	9:42

* *The temperature values in 2006 were very similar to those in 2007*

Rearing and monitoring techniques

Two 2.5x4.0x5.5 m rooms were used for the experiments, one at 20°C and the other at 15°C. The temperatures were controlled by means of 12000 BTU air-conditioners (Samha, Setif, Algeria). In each room, an opaque screen served to separate between the two photoperiods used (16hL:8hD and 8hL:16hD, in hours (h) of light (L) and dark (D)). The light was provided using daylight neons with an intensity of 50–100 lux. The neons were connected to an electronic chronometer clock set to automatically control the photoperiods.

We used the 'soilless' breeding technique developed by Daguzan (1981) and adopted after him by many other authors (e.g. Enée *et al*., 1982; Laurent *et al.*, 1984; Gomot & Deray, 1987) and also in heliciculture to raise the *Cornu aspersum* snail, another ubiquitous helicid living beside *Helix aperta* in Algeria.

All the experimental snails were housed in polythene containers with 3600 cm^3 volume at a density of 50 snails/m^2. A wet absorbent paper was placed on the floor of the containers to maintain humidity. During the experiment, the relative humidity oscillated between 90-95 %. Pots of 10 cm diameter and 8 cm height filled with wet light soil were placed in each container for egg-laying. Each sample was split into four groups. The groups were of 16 snails in Sample 1 and Sample 2, and 25 snails in Sample 3. The groups are coded A_1, B_1, C_1 and D_1 for Sample 1 (collected in February, during hibernation), A_2, B_2, C_2 and D_2 for Sample 2 (collected in March, after hibernation) and A_3, B_3, C_3 and D_3 for Sample 3 (collected in September, during aestivation). Sample 1 and Sample 2 snails were set to reproduction immediately after collect; those of Sample 3, before to be set to reproduction, were kept under optimal rearing condition (as described below, at 20°C/16hL:8hD) for three weeks. For each sample, the four groups of snails were simultaneously submitted to different combinations of temperature and photoperiod as follows: Groups A_i (20°C, 16hL:8hD), Groups B_i (20°C, 8hL:16hD), Groups C_i (15°C, 16hL:8hD), and Groups D_i (15°C, 8hL:16hD).

In order to determine the possible influence of weight on reproduction, all the snails involved in the study were individually weighed at the beginning and the end of the experiment, using an electronic balance with 0,01 g precision; this would also be useful to assess weight variation during the experiment. Before any weighing operation, each snail had been washed to remove any excrements or food possibly imprisoned in the pedal plate folds. In order to discern the individuals, they were marked with adhesive labels.

Throughout all the experiment, the animals were fed with the commercial product "*Helixal*" (Etablissements Chays, France) developed by Gomot-de Vaufleury (2000). To nourish the snails, 50 g of food were provided in each container on Petri dishes. Three times a week, at the same time, the containers were cleaned, the food renewed and the absorbent paper changed. The boxes location in the rearing room was changed every day.

To follow the matings and egg-layings, observations were made twice a day: the first early in the morning (8 a.m.) and the second in the afternoon (8 p.m.) (The laying operation lasts more than 12 hours). To avoid confusion, as soon as a snail was observed in an egg-laying position, the pot was removed to another container and replaced. Each clutch was identified by its parentage, date of laying, number of eggs, date of hatching and number of young hatched. Twice a week, the eggs were collected using a teaspoon and their numbers recorded; 30 % of the eggs of each clutch were individually weighed with an accuracy of 0.001 g. All the eggs were then incubated at 20°C in Petri dishes (9 cm diameter and 1.5 cm height). To maintain humidity during all the incubation period, a layer of wet absorbent paper was placed in the bottom of the Petri dishes. To aerate the eggs, the lids of the Petri dishes were perforated with 10 small holes (1 mm diameter). After hatching, the numbers of the newly emerged snails were counted. Each week, the number of dead animals in each group was monitored. The length of reproduction period was estimated by recording the beginning of matings and the end of egg-layings. After the last clutch, the snails were kept under observation for 5 weeks during which no mating occurred but rather inactivity and mortality drastically increased.

Statistical analysis

The data were subjected to statistical evaluation using Statistica 5.5 (StatSoft Inc., 1999). The mean weights of snails of the different groups and the mean number of eggs per clutch were expressed as means ± standard deviation (M±SD). The differences between the mean weights of snails in the different groups, mean weights of snails within each group at the start and the end of reproduction period, mean numbers of eggs per clutch and mean egg weights per clutch were tested using Anova, Student (t) or Lsd tests. For comparison of numbers of matings and numbers of layings between the corresponding groups of samples the chi-square (χ^2) test was applied. The Pearson's correlation coefficient served to estimate the relation between numbers of matings and numbers of egg-layings, as well as the relation of wet weight of snails with each of numbers of eggs per clutch and egg wet weights. Comparison between proportions test was used to compare the various percentages reported in the results.

RESULTS

The main results and their statistical evaluation are condensed in Figures 1 & 2, and Table II.

Mating and egg-laying activities

For all the three samples, the mating activity began on the first week after setting to reproduction; it lasted 5 weeks for all groups of Sample 1 (collected in February) and a week shorter at 20°C for Sample 2 (collected in March) (Fig. 1). The longest (6 weeks) and the shortest (2 weeks) periods of mating activity were observed in Sample 3 (collected in September) at 20°C/16L:8D and 15°C/8L:16D respectively (Tab. II, line 3).

The first clutch was laid on the 3rd week (at 20°C) or the 4th week (at 15°C) after setting to reproduction for all groups, including Sample 3 (Fig. 1 and data not shown). The laying activity lasted 3-4 weeks for most groups. The longest period of laying activity (5 weeks) was

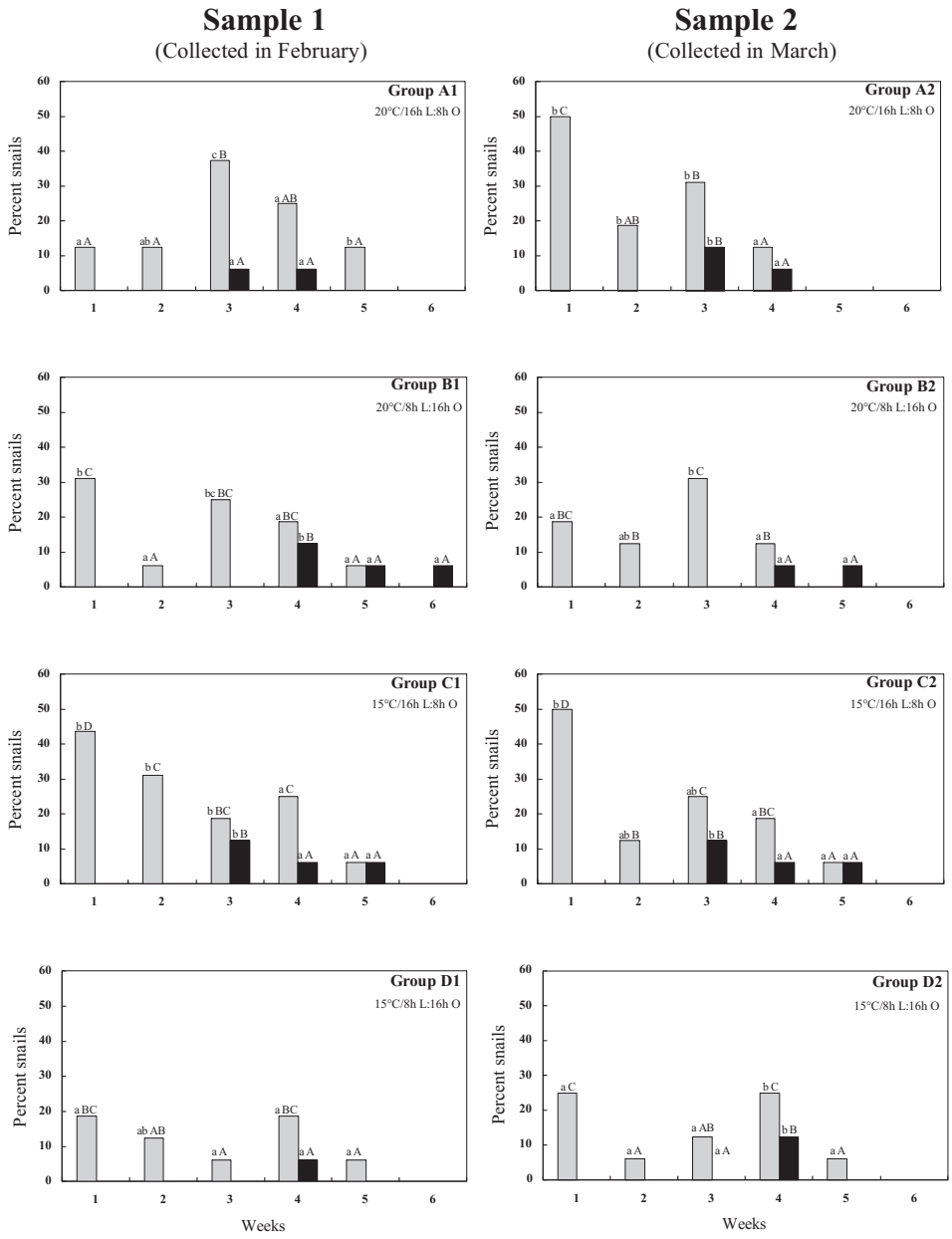


Figure 1. — Mating (grey) and egg-laying (black) rates per week after collect in *Helix aperta* snails sampled in February (Sample 1) and March (Sample 2) under four controlled combinations of temperature (°C) and photoperiod (in hours (h) of light (L) and dark (D)). Within each sample, the vertically corresponding values with different small letters are significantly different ($P < 0.05$); likewise, for matings and egg-layings separately, the capital letters compare all the values within a couple of corresponding groups taken as a whole; those percentages with different capital letters are significantly different ($P < 0.05$).

observed in Sample 3 at 20°C/16L:8D, and the shortest (1 week) in Sample 1 and Sample 2 at 15°C/8L:16D (Fig. 1 & Tab. II, line 7).

The numbers of matings and layings per snail ranged from 0-2 and 0-1 respectively for all groups (Fig. 2) except for Sample 3 in which these numbers ranged from 0-3 and 0-2 res-

TABLE II

Compared reproductive activities of Helix aperta *snails collected at different dates. Sample 1 and Sample 2 were collected at Annaba in February 2007 (during hibernation) and March 2007 (after hibernation). Sample 3 was collected at Bejaïa in September 2005 (during aestivation). For each sample, the codes A_i, B_i, C_i and D_i represent the four snail groups submitted to different combinations of temperature and photoperiod respectively; each of the four groups was of 16 snails in Sample 1 and Sample 2, and 25 snails in Sample 3. For each line, the small letters under values compare in increasing order all the twelve groups (columns); values with same small letters are not significantly different ($P > 0.05$). For each column (each group), the capital letters compare the mean weights of snails before (line 14) and after (line 15) the reproduction period; the mean values with different capital letters are significantly different ($P< 0.05$)*

Descriptive traits	Sample 1 (Collected in February 2007)				Sample 2 (Collected in March 2007)				Sample 3 (Collected in September 2005)			
	A_1 (20°C/L)[1]	B_1 (20°C/D)[2]	C_1 (15°C/L)[1]	D_1 (15°C/D)[2]	A_2 (20°C/L)[1]	B_2 (20°C/D)[2]	C_2 (15°C/L)[1]	D_2 (15°C/D)[2]	A_3 (20°C/L)[1]	B_3 (20°C/D)[2]	C_3 (15°C/L)[1]	D_3 (15°C/D)[2]
1. Percent of snails that mated (%)	87.50 b	68.75 b	87.50 b	56.25 b	87.50 b	62.50 b	81.25 b	62.50 b	92 c	48 a	84 b	32 a
2. Number of matings snail^{-1}±SD	1±0.5 abc	0.9±0.7 abc	1.25±0.7 c	0.63±0.6 a	1.12±0.6 bc	0.75±0.7 ab	1.12±0.7 bc	0.75±0.7 ab	1.52±0.7 b	0.56±0.6 a	1.28±0.7 b	0.32±0.5 a
3. Duration of mating activity (weeks)	5	5	5	5	4	4	5	5	6	4	5	2
4. Time between mating and egg-laying (days)	20	20	20	20	20	20	20	20	-	-	-	-
5. Percent of snails that laid eggs (%)	12.5 a	25 a	25 a	6.25 a	18.75 a	12.5 a	25.00 a	12.5 a	80 c	32 ab	64 b	12 a
6. Number of clutches snail^{-1} ±SD	0.12±0.3 a	0.25±0.4 a	0.25±0.4 a	0.06±0.2 a	0.19±0.4 a	0.12±0.3 a	0.25±0.4 a	0.12±0.3 a	0.84±0.5 c	0.32±0.5 ab	0.64±0.5 b	0.12±0.3 a
7. Duration of laying activity (weeks)	2	3	3	1	2	2	3	1	5	3	4	2
8. Total duration of reproduction activity (weeks)	5	6	5	5	4	5	5	5	7	6	6	5
9. Mean number of eggs ±SD clutch^{-1}	313±8.5 bcd	302.0±14.4 bc	292.7±11.4 b	323.0±0.0 bcde	247.7±4 a	237.5±6 a	241.5±6 a	237.0±14 a	347.7±27 e	344.2±31 de	340.6±19 cde	337.3±41 cde
10. Mean egg weight (mg)	16.3±0.1 a	16.1±0.3 a	16.1±0.2 a	16.2±0.3 a	17.6±0.2 c	17.5±0.3 bc	17.5±0.0 c	17.8±0.2 c	16.6±1.2 b	16.4±1.2 ab	16.5±0.9 b	16.3±1.2 ab
11. Time of incubation (days)	13	13	13	13	13	13	13	13	13	13	13	13
12. % of successful hatching	79 a	85 a	88 a	83 a	84 a	86 a	87 a	85 a	84 a	86 a	85 a	78 a
13. Mortality rate during reproduction period (%)	25 a	31.25 b	25 a	37.5 b	18.75 a	25 a	25 a	37.5 b	28 a	8 a	12 a	20 a
14. Mean weights ± SD (g) of snails at start of experiment	7.2±0.9 aA	7.3±0.9 aA	7.4±0.8 aA	7.1±0.8 aA	9.3±1.5 bA	8.7±1.0 bA	9.3±1.3 bA	9.2±0.8 bA	14.4±2.0 cdB	14.2±1.9 deB	14.4±1.9 eA	14.3±1.6 fB
15. Mean weights ± SD (g) of snails after reproduction	12.6±0.5 bcdB	12.2±1.0 bcB	12.8±1.1 cdB	13.1±1.0 deB	13.1±0.8 deB	12.9±0.7 deB	12.7±1.0 cdB	12.5±1.2 bcdB	12.2±1.3 bcA	12.1±1.2 bA	13.8±1.1 fA	11.3±1.1 aA

[1] *L: 16 hours of light and 8 hours of dark (Light days); D: 8 hours of light and 16 hours of dark (Dark days).*

pectively in Group A_3 (20°C/16hL:8hD) and from 0-1 in Group D_3 (15°C/8hL:16hD) (data not shown). In both Sample 1 and Sample 2, the modal values of numbers of matings and numbers of layings were 0 and 1 respectively (Fig. 2). In contrast, in Sample 3, the modal values of

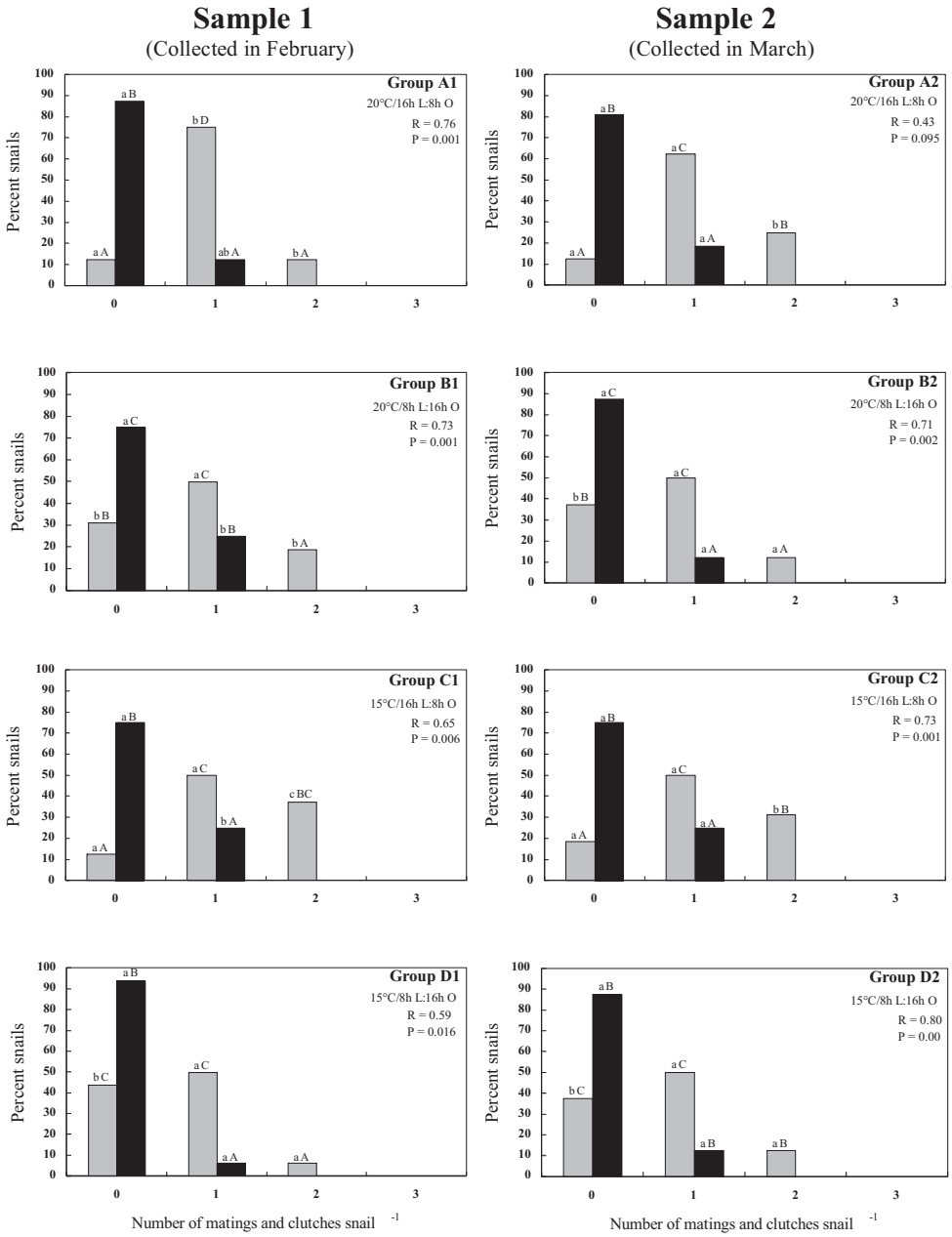


Figure 2. — Numbers of matings (grey) and clutches (black) variation in *Helix aperta* snails collected in February (left) and March (right) under four controlled combinations of temperature (°C) and photoperiod (in hours (h) of light (L) and dark (D)). Within each sample, the vertically corresponding values with small letters are significantly different ($P < 0.05$); likewise, for each pair of horizontally corresponding groups, separately for the matings and clutches, the values with different capital letters are significantly different ($P < 0.05$). R and P values mentioned in each group box are those of Pearson's correlations and their significance levels between the numbers of matings and the numbers of clutches within the group.

numbers of matings and numbers of layings were 1 and 2 respectively under long day photoperiod, and 0 under short day photoperiod (data not shown). In most cases, the numbers of matings were significantly correlated with the numbers of layings ($P < 0.05$).

Strikingly, even though the snails collected in February and March (Samples 1 and 2 respectively) showed a rather high mating activity in comparison with those collected in September (Sample 3) (Tab. II, lines 1 & 2), their laying performances were drastically lower (Tab. II, lines 5 & 6). Among the snails that had mated, the proportion of those that laid eggs was of only 11-36 % in Samples 1 and 2, and as much as 38-87 % in Sample 3. Otherwise, in Sample 1 and Sample 2, although most snails had mated at least once, most of them did not lay eggs; but in Sample 3, most snails mated and laid at least once except under short day photoperiod. The long day photoperiod was significantly ($P < 0.05$) more stimulating of mating and egg-laying activities; the best combination of temperature and photoperiod was 20°C/16L:8D and the worst was 15°C/8L:16D (Tab. II, lines 1, 2, 5 & 6).

Clutch and egg sizes

The snails collected in February and September (Sample 1 and Sample 3 respectively) laid significantly more eggs per clutch than those sampled in March ($P < 0.001$) (Tab. II, line 9). Significant differences between groups were revealed within Sample 1 and Sample 3 only, but not within Sample 2 (Tab. II, line 9). The average range of variation in eggs numbers was of 31 eggs in Sample 1 and only of 10 and 11 eggs in Sample 2 and Sample 3 respectively.

The eggs laid by snails collected in March and September were in average significantly heavier and more heterogeneous than for snails sampled in February ($P < 0.001$) (Tab. II, line 10). In addition, there was a strong negative correlation between weights of eggs and numbers of eggs per clutch ($P < 0.001$) in all groups but no significant dependence was detected between weights of snails and weights of eggs ($P = 0.473$).

Mortality rates and body weights variation

During the reproduction period, especially at low temperature and short day photoperiod, the mortality rates were higher and more heterogeneous in Sample 1 (25-37.5 %) and Sample 2 (18.75-37.5 %) than in Sample 3 (8-28 %) (Tab. II, line 13). During the five weeks following the reproduction period, the mortality was becoming higher and higher and the surviving snails were inactive or trying to burrow themselves into the soil of the egg-laying pots.

Just after the reproduction period, the mean body weights of snails very significantly increased ($P < 0.001$) for Sample 1 and Sample 2, and significantly decreased ($P < 0.05$) for Sample 3 (Tab. II, lines 14 & 15).

DISCUSSION

Matings and laying activities

It is from late December to late March in winter, and early May to late September in summer that temperature conditions did not fit the optimums of *H. aperta* snails (7-27°C) (Tab. I and data not shown). So, approximately, Sample 1 and Sample 2 were collected after two and three months of hibernation respectively; and Sample 3, after 4-5 months of aestivation. Despite these differences, all the three samples began to mate on the first week and to lay on the 3^{rd}-4^{th} week. It appears from this that hibernation or aestivation durations do not influence times of mating and egg-laying activities in *H. aperta*. In *H. aspera* (*Cornu aspersum*), for instance, after a month and a half of hibernation, the mating activity is delayed by 7 weeks; after six months of hibernation, matings take place earlier and the first clutch is laid after 7 weeks; after a year of hibernation, mating activity occurs right after the waking, and the first clutch is observed after only 3 weeks (Bonnefoy & Deray, 1984).

The snails collected during or after hibernation were very similar for their mating and egg-laying activities (Tab. II, lines 1-8). Although they had, in most cases, mated more

than those collected during aestivation, they strikingly laid far less clutches (Tab. II, lines 1, 2, 5, 6). Obviously, the problem resided in some aspect of their gonad physiology; the meteorological conditions (likely temperatures and photoperiods) during hibernation did not favour gametogenesis or egg fertilization processes; these seemed to be stimulated rather during aestivation. During hibernation of snails, neuro-endocrine modifications are induced, with negative repercussions on cytological activities (gametic multiplication and differenciation) of the ovotestis (Bouillon, 1956) depending on both temperature (Gomot *et al.*, 1986; Gomot & Deray, 1990) and photoperiod (Gomot & Griffond, 1993), the main factors controlling the sexual behaviour. The cerebral centres responsible of this kind of neuro-endocrine regulation of reproduction activity are the caudo-dorsal cells of the cerebral ganglia in *Lymnaea stagnalis* (Geraerts & Bohlken, 1976), the bag cells of the abdominal ganglia in *Aplysia caltfornica* (Kupfermann, 1967; Arch, 1976) and the dorsal bodies in *Helisoma* (Saleuddin & Khan, 1981) and *Cornu aspersum* (Saleuddin *et al*., 1983).

Our observations are in full agreement with those reported by Giusti & Andreini, (1988) on *H. aperta* snails collected from Siena (Tuscany, Italy) and kept under controlled conditions imitating those in the field for 3 consecutive years (1982-1984); it resulted that *H. aperta* snails mate preferentially from early October to mid-December. Similar observations have also been made on *Theba pisana* in Italy (Giusti & Andreini, 1988) as well as *Theba pisana* and *Cernuella virgata* in Australia (Baker, 1991).

De Vaufleury & Gimbert (2009) have noted that *H. aperta* snails, collected in September at Bazina (Tunisia), lay eggs under short-day photoperiod (6hL:18hD) only, they do not lay eggs at all under long-day (18hL:6hD) although they have mated normally; this suggests that the extreme long day photoperiod of 18hL:6hD is gametogenesis inhibitory. When acclimatized to the laboratory environment over 4 generations under conditions as in the present study, *H. aperta* snails from Bakaro (Bejaïa, Algeria) are reproductively 7-16-fold more efficient, especially under 20°C/16hL:8hD (Benbellil-Tafoughalt *et al.*, 2009). These higher reproductive performances are probably the product of a physiological plasticity of the process of reproduction as in *Cornu aspersum* (Madec, 1988).

The significant positive correlation between numbers of matings and egg-layings ($P = 0.00$-0.09) (Fig. 2) indicates that the snails that laid more clutches were those that mated more frequently. A similar observation has also been reported on laboratory *H. aperta* snails (Tafoughalt-Benbellil *et al.*, 2009), *Arianta arbustorum* (Baur, 1988; Baur & Baur, 1992) and *Cornu aspersum* (Madec *et al*., 1998, 2000).

Numbers of eggs per clutch, egg weights and rates of hatching

The snails collected in March (Sample 1) were those that laid significantly ($P < 0.05$) less eggs per clutch than those collected in February (Sample 2) and September (Sample 3) (Tab. II, line 9). Consequently, this resulted in significantly ($P < 0.05$) heavier mean egg weights in Sample 2 than in Sample 1 and, at a lesser degree, Sample 3 (Tab. II, line 10). Otherwise, there was a strong negative correlation between numbers of eggs per clutch and the mean egg weights ($P < 0.01$): the larger were the numbers of eggs per clutch, the lighter were, in average, the individual egg weights of a clutch.

H. aperta snails collected in September at Bazina (Tunisia) have shown a lower mean egg number per clutch (279), lighter mean egg weights (12.6-14.8 mg) and a shorter time of eggs incubation (12 days) (Vaufleury & Gimbert, 2009). This means that the extreme short day photoperiod (6hL:16hD) used by the authors is even more stressing than 8hL:16hD used in the present study (Tab. II, lines 11-14). However, the laboratory acclimatized *H. aperta* snails have produced far heavier mean egg weights (26-27 mg) even when the numbers of eggs per clutch are high (306-340 eggs/clutch) (Tafoughalt-Benbellil *et al*., 2009).

In spring, the Mediterranean helicid species, such as *Helix lucorum* (Staikou *et al.*, 1988) and *Helix texta* (Heller & Ittiel,1990), tend to reduce their eggs number per clutch (70-80 eggs/clutch) and compensate it by an increase in eggs weight. This is in relation with youthful mortality during aestivation because heavier eggs have higher nutritive contents, as in *Arianta arbustorum* (Baur, 1994), leading to larger youthful and better survival during the long aesti-

vation (May-September) under Mediterranean climate. In Western Europe too, where winter survival is the principal factor in the helicid populations dynamics, several authors have also noted a reduction in eggs number per clutch accompanied by an increase in eggs weights during the autumnal season (Wolda & Kreulen, 1973; Pollard, 1975; Oosterhoff, 1977; Peake, 1978; Caïn, 1983; Cowie, 1984; Madec *et al.*, 1998, 2000).

Mortality rates and body weights during the reproduction period

The higher mortality rates and lower reproductive performances under 15°C/8hD:16hD in most cases (Tab. II, lines 1-3 & 5-7) suggest that *H. aperta* snails prefer the 20°C/16hL:8hD combination of temperature and photoperiod closer to the autumnal conditions, especially overnight, their phase of activity (Tab. I).

In regard to body weights, if we assume the genetic identity of the snails, the differences between samples observed for this trait before the reproduction period can be attributed to differences in amounts of food ingested before the reproduction period (Tab. II, line 14).

The snails sampled during or after hibernation had significantly increased in body weight and those collected during aestivation had more or less sensibly decreased in weight (Tab. II, line 15). This is simply because, in the first case, the snails reproduced very little and tended more to consume food (in prevision of survival and optimized gametogenesis during aestivation ?) whereby their significant increase in weight; in the second case, the snails that had already stocked much energy occupied themselves much more to reproduce and therefore sensibly decreased in body weight.

All these arguments on mortality rates and body weights, and those gathered above regarding reproductive performances plead that *H. aperta* snails are better adapted to reproduce in autumn (decreasing days and higher temperatures) after a long aestivation stimulating gametogenesis than in spring (increasing days and lower temperatures) after hibernation inhibiting gametogenesis.

ACKNOWLEDGEMENTS

We thank very much Pr. Benyakoub Slim (University of Annaba, Algeria) for providing meteorological data of the study site in Annaba, and Mrs. Mounira Rachedi (University of El Tarf, Algeria) for collecting snails at Annaba (Northeastern Algeria) in February 2007. The first version of the manuscript has been much improved upon the orientations of the editor, Pr. Christian Érard, and above all the valuable suggestions of three anonymous reviewers.

REFERENCES

Arch, S. (1976). — Neuroendocrine regulation of egg-laying in *Aplysia californica*. *Am. Zool.,* 16: 167-175.

Aupinel, P. & Daguzan, J. (1989). — Étude du rôle de la photopériode sur l'activité métabolique des jeunes escargots Petit-gris (*Helix aspersa* Müller) et mise en évidence d'une phase photosensible. *Haliotis*, 19: 47-55.

Bailey, S.E.R. (1981). — Circannual and circadian rhythms in the snail *Helix aspersa* and the photoperiodic control of annual activity and reproduction. *J. Comp. Physiol*., 142: 89-94.

Baker, G.H. (1991). — Production of eggs and young snails by adult *Theba pisana* (Müller) and *Cernuella virgata* (Da Costa) (Mollusca, Helicidae) in laboratory cultures and field populations. *Austral. J. Zool.*, 39: 673-679.

Baur, B. (1988). — Repeated mating and female fecundity in the simultaneously hermaphrodic land snail *Arianta arbustorum. Invertebr. Reprod. Develop.*, 14: 197-204.

Baur, B. (1994). — Inter-population differences in propensity for egg cannibalism in hatchings of the land snail *Arianta arbustorum. Can. J. Zool*., 48: 851-860.

Baur, B. & Baur, A. (1992). — Effects of courtship and repeated copulation on egg production in the simultaneously hermaphrodic land snail *Arianta arbostorum. Invertebr. Reprod. Develop.,* 21: 201-206.

Bonnefoy-Claudet, R. & Deray, A. (1984). — Influence de la durée d'hibernation sur l'activité reproductrice de l'escargot *Helix aspersa* Müller. *C. R. Soc. Biol*., 178: 442-449.

Bonnefoy-Claudet, R., Deray, A. & Griffond, B. (1987). — La reproduction de l'escargot *Helix aspersa* Müller en fonction des conditions d'environnement: données synthétiques. *Haliotis*, 16: 57-67.

Bouillon, J. (1956). — Influence of temperature on the histological evolution of the ovotestis of *Cepaea nemoralis* L. *Nature*, 177: 142-143.

Caïn, A.J. (1983). — Ecology and ecogenetics of terrestrial molluscan populations. Pp 597-647 in W.D.Russel-Hunter (ed.). *The mollusca, Vol.6.* Academic Press, London.

Charrier, M. (1980). — *Contribution à la biologie et à l'écophysiologie de l'escargot Petit-gris* Helix aspersa *Müller (Gastéropode pulmoné stylommatophore).* Thèse de 3e Cycle, Université de Rennes I (France).

Cowie, R.M. (1984). — The life-cycle and productivity of the land snail *Theba pisana* (Mollusca, Helicidae). *J. Anim. Ecol*., 53: 311-325.

Daguzan, J. (1981). — Contribution à l'élevage de l'escargot Petit-gris *Helix aspersa* Müller. I. Reproduction et éclosion des jeunes en bâtiment et en conditions thermohygrométriques contrôlées. *Ann. Zootech*., 30: 249-272.

de Vaufleury, A. & Gimbert, F. (2009). — Obtention du cycle de vie complet *d'Helix aperta* Born de sites tunisiens en conditions contrôlées. Influence de la photopériode. *C. R. Biologies*, 332: 795-805.

Enée, J., Bonnefoy-Claudet, R. & Gomot, L. (1982). — Effet de la photopériode artificielle sur la reproduction de l'escargot *Helix aspersa* Müller. *C. R. Acad. Sci. Paris*, 294: 357-360.

Geraerts, W.P.M. & Bohlken, S. (1976). — The control of ovulation in the hermaphroditic freshwater snail *Lymnaea stagnalis* by the neurohormone of the caudo-dorsal cells. *Gen. Comp. Endocrinol.,* 28: 350-57.

Giusti, F. & Andreini, S. (1988). — Morphological and ethological aspects of mating in two species of the family Helicidae (Gastropoda, Pulmonata) *Theba pisana* (Müller) and *Helix aperta* (Born). *Monitore Zool. Ital*. (N.S.), 22: 331-363.

Gomot, A. (1990). — Photoperiod and temperature interaction in the determination of reproduction of the edible snail *Helix pomatia. J. Reprod. Fertil*., 90: 581-585.

Gomot-de Vaufleury, A. (2000). — Standardized growth, toxicity testing (Cu, Zn, Pb and Pentachlorophenol) with *Helix aspersa*. *Ecotoxicol. Environ. Saf*., 46: 41-50.

Gomot, L. & Deray, A. (1987). — Les escargots. *La Recherche*, 186: 302-311.

Gomot, L., Enée, J. & Laurent, J. (1982). — Influence de la photopériode sur la croissance pondérale de l'escargot *Helix aspersa* Müller en milieu contrôlé. *C. R. Acad. Sci. Paris*, 294: 749-752.

Gomot, L. & Griffond, B. (1993). — Action of epigenetic factors on the expression of hermaphroditism in the snail *Helix aspersa. Comp. Biochem. Physiol*., 104A: 195-199.

Gomot, P. & Deray, A. (1990). — The length of hibernation affects temperature-induced (25° C) spermatogenic multiplication in *Helix aspersa* Müll. *Experientia*, 46: 684-686.

Gomot, P., Griffond, B. & Gomot, L. (1986). — Effets de la température sur la spermatogénèse d'Escargots *Helix aspersa* maintenus en repos artificiel. *C. R. Acad. Sci. Paris*, 302: 27-32.

Gomot, P., Gomot, L. & Griffond, B. (1989). — Evidence for a light compensation of the inhibition of reproduction by low temperatures in the snail *Helix aspersa*. Ovotestis and albumen gland responsiveness to different conditions of photoperiods and temperatures. *Biol. Reprod*., 40: 1237-1245.

Gomot, P., Griffond, B. & Gomot, L. (1990). — Action de la température sur la synthèse d'ADN des cellules mâles et la spermatogenèse d'*Helix aspersa* en hibernation. *J. Therm. Biol*., 15: 267-280.

Griffond, B., Gomot, P. & Gomot, L. (1992). — Influence de la température sur le déroulement de l'ovogenèse chez l'escargot *Helix aspersa*. *J. Therm. Biol*., 17: 185-190.

Heller, J. & Ittiel, H. (1990). — Natural history and population dynamics of the land snail *Helix texta* in Israel (Pulmonata, Helicidae). *J. Moll. Stud*., 56: 189-204.

Hunter, R.D. & Stone, L.M. (1986). — The effect of artificial photoperiod on growth and reproduction in the land snail *Cepaea nemoralis*. *Int. J. Invertebr. Reprod. Dev*., 9: 339-344.

Jess, S. & Marks, R.J. (1998). — Effect of temperature and photoperiod on growth and reproduction of *Helix aspersa* var. *maxima*. *J. Agr. Sci*., 130: 367-372.

Kerney, M.P. & Cameron, R.A.D. (1979). — *A field guide to the land snails of Britain and NW Europe*. William Collins Sons and Co. Ltd., London.

Kupfermann, I. (1967). — Stimulation of egg-laying: possible neuroendocrine function of bag cells of abdominal ganglion of *Aplysia californica*. *Nature* (Lond), 216: 814-15.

Laurent, J., Deray, A. & Grimard, A.M. (1984). — Influence de la photopériode, du degré d'hétérogénéité de la population sur la dynamique de croissance et la maturation sexuelle de l'escargot *Helix aspersa*. *C. R. Soc. Biol*., 178: 421-441.

Madec, L. (1988). — Origine et importance des différences affectant la forme et la taille des œufs chez l'escargot « Petit-gris » (*Helix aspersa* Müller). *Haliotis*, 19: 143-152.

Madec, L., Desbuquois, C. & Coutellec-Vreto, M.A. (2000). — Phenotypic plasticity in reproductive traits: Importance in the life history of *Helix aspersa* (Mollusca, Helicidae) in a recently colonized habitat. *Biol. J. Linn. Soc*., 69: 25-39.

Madec, L., Guiller, A., Coutellec-Vreto, M.A. & Desbuquois, C. (1998). — Size-fecundity relationships in the land snail *Helix aspersa*: preliminary results on a form outside the norm. *Invertebr. Reprod. Develop*., 34: 83-90.

Oosterhoff, L.M. (1977). — Variation in growth rate as an ecological factor in the land snail *Cepaea nemoralis* (L). *Nether. J. Zool*., 27: 1-132.

Peake, J. (1978). — Distribution and ecology of the Stylommatophora. Pp. 171-217 *in:* V. Fetter & J. Peake (eds). *Pulmonates, Vol. 2A*. Academic Press, London.

POLLARD, E. (1975). — Differences in shell thickness in adult *Helix pomatia* L. from a number of localities in southern England. *Oecologia*, 21: 85-92.

SACCHI, C.F. (1955). — Il contributo dei molluschi terrestri alle ipotesi del "ponte siciliano". Elementi tirrenici ed orientali nella malacofauna del Maghreb. *Arch. Zool. Ital.*, 40: 49-181.

SACCHI, C.F. (1958). — Les mollusques terrestres dans le cadre des relations biogéographiques entre l'Afrique du Nord et l'Italie. *Vie et Milieu*, 9: 11-52.

SALEUDDIN, A.S.M., FARRELL, C.L. & GOMOT, L. (1983). — Brain extract causes amoeboid movement in vitro in oocytes in *Helix aspersa* (Mollusca). *Intern. J. Invert. Reprod.*, 6: 31-34.

SALEUDDIN, A.S.M. & KHAN, H.R. (1981). — Motility of the oocyte of *Helisoma* (Mollusca). *Eur. J. Cell. Biol.*, 26: 5-10.

SCHÜTT, H. (2001). — Die Türkische Landschnechen 1758-2000. *Acta Biologica Benrodis*, Suppl., 4: 550.

STAIKOU, A., LAZARIDOU-DIMITRIADOU, M. & FARMAKIS, N. (1988). — Aspects of the life cycle, population dynamics, growth and secondary production of the edible snail *Helix lucorum* L. in Greece. *J. Moll. Stud.*, 54: 139-155.

STATSOFT, INC. (1999). — *Statistica pour Windows, kernel version 5.5*. StatSoft France, Maisons-Alfort, France.

STEPHENS, C.I. & STEPHENS, G.C. (1966). — Photoperiodic stimulation of egg-laying in the land snail *Helix aspersa. Nature*, 212: 1582.

TAFOUGHALT-BENBELLIL, S., SAHNOUNE, M., DE VAUFLEURY, A. & MOALI, A. (2009). — Effects of temperature and photoperiod on growth and reproduction of the land snail *Helix aperta* Born (Gastropoda, Pulmonata). *Rev. Ecol. (Terre et Vie)*, 64: 207-219.

WOLDA, M. & KREULEN, D.A. (1973). — Ecology of some experimental populations of the landsnail *Cepaea nemoralis* L. II. Production and survival of eggs and juveniles. *Neth. J. Zool.*, 23: 168-188.