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Study of the varietal behaviour of five tomato cultivars *Solanum lycopersicum* to the tomato yellow leaf curl (TYLC) disease

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Abstract

Symptom scoring, dosage of viral DNA and estimation of tolerance level, were three steps successfully used to study the varietal behaviour of five tomato cultivars to the tomato yellow leaf curl (TYLC) disease in Morocco. Forty plant of each cultivar; Cherry, Tres Cantos, Campbell 33, F1 Pepite and the susceptible control Marmand VR, were inoculated mediate *Agrobacterim tumefaciens* and *Bemisia tabaci* en separate to scoring symptoms using scale (from 0 to 4) and quantification of viral DNA accumulates on the leaf tissue by molecular hybridization dot-blot 70 day post inoculation. Then, a simple mathematic formula based on symptom scoring, was used to estimate the tolerance level of each cultivar. Symptom severity increased up, was different between the two inoculation methods. Agro-inoculation was more effective and 100 % was obtained in the susceptible control. The symptoms evolution was faster and more uniform (low variability). It can be used in breeding programmes as complementary to inoculation using *Bemisia tabaci*. Moreover, the dosage of viral DNA at leaf tissues revealed the coexistence of the TYLCV and TYLCSV to plants inoculated via *Bemisia tabaci*. Considering the tolerance level and agronomic factors, F1 Pepite was the best of the cultivars tested with T (tolerance level) = 0, 54. Whereas Tres Cantos and Cherry were the most sensible with T = 0,32; T = 0,34 respectively.

Key words: TYLCV, *Agrobacterium tumefaciens, Bemisia tabaci, Solanum lycopersicum*, genetic tolerance.

Introduction

Emergence of viral diseases can cause considerable damage (Garcia-Andrés *et al.* 2007; Chua *et al.*, 2000; Hahn *et al.*, 2000). In the Mediterranean basin, two sorts of geminivirus attack the cultures of tomato (*Solanum Lycopersicum*), in the field and in greenhouses as well. It's the Tomato Yellow Leaf Curl Sardinian Virus (TYLCSV), previously known as TYLCV-Sar (Kheyr-Pour *et al.*, 1991), and Tomato Yellow Leaf Curl Virus (TYLCV) previously known as TYLCV-Is, native of Israel (Antignus & Cohen, 1994). Both species occur in Spain (Kheyr-Pour *et al.*, 1991; Moriones *et al.*, 1993; Navas-Castillo *et al.*, 1999; Sánchez-Campos *et al.*, 1999; Jordá *et al.*, 2000; Accotto *et al.*, 2000; Accotto *et al.*, 2003), wherefrom was introduced the virosis to Morocco as a consequence of intensive movements of people and agricultural products.

The complex of viruses associated with there two virus species, denominated tomato yellow leaf curl disease (TYLCD), are single-stranded DNA viruses of the of the genus *Begomovirus* (family *Geminiviridae*) that severely constrain crop production and continue to emerge world-wide (Seal *et al.*, 2006; Stanley *et al.*, 2005). These begomoviruses, transmitted naturally by the whitefly *Bemisia tabaci*

Gen. (*Hemiptera: Aleyrodidae*) in a circulative manner (Mehra *et al.*, 1994), pose a severe threat to tomato (*Solanum lycopersicom* L.) and common bean (*Phaseolus vulgaris* L.) production in many warm and temperate regions of the world (Moriones & Navas-Castillo, 2000).

The use of resistant and/or tolerant tomato cultivars is the most promising approach to control TYLCV disease (Rubio *et al.*, 2003). However, the lack of good methods to evaluate plant resistance/tolerance is a limiting factor in breeding programmes. Certainly, the selection of resistant plants cannot base itself, only, on the absence of symptoms, it must be justified by molecular analyses to determine the accumulation level of viral DNA. The mathematical approach, proposed by Rubio *et al.* (2003), completes these two criteria of selection. It consists of a formulae developed to estimate the degree of resistance or tolerance of plant to virus using virus titre (estimate by tissue-print hybridization) and symptom intensity of infected plants as parameters. An ideal evaluation of the resistance / tolerance must consider all the factors affecting the response of the plant to the viral infection including the genetic diversity of the viral population infecting the plants. Furthermore it is necessary to execute artificial infections, as a supplement to the tests using *Bemisia tabaci*. Indeed, many problems can be bound in the definition of the resistance in the natural conditions; variability in assay conditions sometimes leads to contradictory results, attributing different resistance levels to the same genetic source (Pico *et al.*, 2001). Resistance to the vector, reported in wild *S. Lycopersicum*, can mask the existence of virus resistance (Muniyappa *et al.*, 1991). Furthermore, the affinity of the insect in a given cultivar influences the concentration of inoculum from a cultivar to another. These difficulties derived from the inoculation by *Bemisia tabaci* and for the management of this vector to define the resistance to TYLCV have encouraged the development of alternative inoculation procedures. Agroinoculation uses *Agrobacterium tumefaciens* to deliver cloned viral DNA into host cells is the most used (Grimsley *et al*., 1986).

In this work, we study profoundly the varietal behaviour of five tomato cultivars to the TYLCV, to determine their levels of tolerance by calculating the coefficient of tolerance. The response of the various cultivars to the viral infection is compared with their behaviour to a natural inoculation with *Bemisia tabaci*. A both search aims to study the potential of tolerance / resistance (even relative) in the studied cultivars to the TYLCV to a later use in the breeding programmes of inter-specific hybridization with wild entries.

Materials and methods

Five commercial tomato cultivars were used: Cherry (SA, Spain), Tres Cantos (Fito, Spain), Campbell 33 (Technisem, French), Marmand VR and F1 hybrid Pepite (Vilmorin, French). Marmand VR is considered susceptible to TYLCV (Lapidot *et al.*, 2006) and was used as a positive control for natural and artificial infection.

Forty plants of each cultivar were planted in greenhouse at controlled conditions 25 ± 2°C and photoperiod 16/8. Six weeks after plantation (4 in 6 leaves), a half of plants is naturally inoculated with the vector *Bemisia tabaci* and the other half is agro-infected.

Origine of the vector *Bemisia tabaci,* the virus and the strain of *Agrobacterium tumefaciens*

Bemisia tabaci used in this work is collected in infested plants from the field in the region of Souss Massa (city of Agadir) in the South is from Morocco, presented very severe symptoms of the disease of the TYLC. The vector and the virus were maintained on plants of tomato *Lycopersicum* until the moment of the infection.

Agrobacterium tumefaciens LBA 4404 bearing a tandem repeat of the TYLCSV-Es and TYLCV-Mld were used in all assays. TYLCSV-Es is an isolate of the TYLCV-Sr (Sardinia) species and TYLCV-Mld is an isolate of the TYLCV-Is, and were provided by Dr. Moriones Enrique, Estacion Experimental La Mayora, Malaga (Spain), preparated in binary vector pBin 19. For routine inoculation, bacterial cultures were grown for 48 h at 28ºC in YEB medium supplemented with 50 mg ml-1 kanamycine. Cells were concentrated tenfold by centrifugation, and immediately used for inoculation.

Inoculation

At four-six-leaf stage (approximately 6 weeks after plantation), plants destined for the natural inoculation were caged with viruliferous *Bemisia tabaci* in a muslin cage. The density of flies is approximately between 25 and 30 unities by plant. The contact with the fly was prolonged in five days and five nights because of the weak density of the flies. The plants were, then, grown in an insect-proof greenhouse after elimination of the vector. No insecticides were used during the experimental period. Plants destined for the agro-inoculation were injected in a separated greenhouse. The injection of the two bacterial suspension (mixed agroinoculation) was into the axillary buds of the three youngest leaves (Kheyr-Pour *et al.*, 1994). Measures to prevent accidental release of *Agrobacterium* into the environment were taken. *A. tumefaciens* LBA 4404 bearing a tandem repeat of the TYLCV-Alm (Almeria, Spain) was used in all assays and was provided by Dr. Moriones Enrique.

Symptom scoring

Symptoms were observed on bi-weekly basis. Severity was scored on a scale of 0 (symptomless) to 4 (symptoms as severe as the susceptible control, including leaf yellowing, curling and severe stunting of the plant) as described in Picó *et al.* (1998).

Dot-blot hybridization

Molecular hybridization was used to test individual plants for the presence of TYLCV and TYLCSV viral DNA at 70 DPI in both assays. The leaf tissue was taken from the upper canopy of the plant at each date. DNA extraction was carried out following the procedure described by Crespi *et al.* (1991) with modifications: 150 mg of frozen tissue were crushed in 500 µl of extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA, 500 mM NaCl, 10 mM 2-β-mercaptoethanol and 1% sodium dodecyl sulfate) and incubated at 65ºC for 5 min. Then, 150 µl of 5 M potassium acetate were added and samples were incubated on ice for 10 min. After centrifugation for 10 min, DNA was precipitated from the supernatants with isopropanol and resuspended in 77 µl of distilled water. One µl of each sample, corresponding to about 1,5 mg of fresh tissue, and a ten-fold dilution of the sample were denatured with 30 mM NaOH and 1 mM EDTA for 30 min and then blotted

on nylon positively charged membranes for hybridization. DNA was fixed on the membrane by UV crosslinking. Hybridization was carried out according to 'The DIG system user's guide for filter hybridization' (Roche Molecular Biochemicals) using digoxigenin-11-dUTP and chemiluminiscent detection. Membranes were prehybridized in standard hybridization buffer plus 50% deionized formamide for at least 1 h. Subsequent hybridization was done at 42ºC overnight in fresh prehybridization solution containing 20 ng of denatured probe per ml. The probes employed (kindly supplied by E.R. Bejarano, Universidad de Málaga, Spain) represented the intergenic region of the Spanish isolates previously cited belonging to TYLCV and TYLCSV species. The probes were labelled by incorporation of digoxigenin-11-dUTP during PCR. One replicate of each membrane was hybridized with each probe, respectively. Washing steps and incubation with antibody were done according to manufacturer's instructions.

Detection and quantification of viral DNA

Detection was carried out with CSPD and direct exposition to a CCD camera (Intelligent Dark Box-II, Fujifilm, Tokyo, Japan). The amount of viral ssDNA was quantified according to a standard curve of TYLCV or TYLCSV DNA, respectively, dotted on the same membrane (ranging from 20 pg to 5 ng). Plant DNA extracted was also quantified in order to relate virus concentration to plant DNA present in each sample. Fluorimetry was employed as the method to quantify doubled-stranded DNA (Hoefer DyNA Quant 200 fluorimeter, according to manufacturer's instructions).

Results and discussion

Symptom scoring and comparison of inoculation methods

A slow rate of infection development was observed in plants inoculated with *Bemisia tabaci.* The first symptoms of the disease appeared 20 DPI, they were not uniform on all the plants (Table 1) and the time of apparition of the symptoms was different. At 70 DPI, 43 % of plants only reached the level (2 - 2,5) while the rest was slightly infected.

In the other essay (agro-inoculation plants), the evolution of the symptoms was faster, more uniform and the degree of severity bordered 3,5 and on second observation (40 JAI) (Figure 2 and Table 3). In 70 JAI, practically all the agro-inoculated plants showed symptoms; 70 % reached are the level 4 of severity while 14 % bordered the level 3,5 and the rest presents relatively light symptoms.

Generally, we can observe clearly that the characteristic symptoms of the TYLCV (Figure 1) are much severs at agro inoculated plants. The statistical comparison of the averages between plants agro inoculated and plants inoculated by the *Bemisia tabaci* show highly significant differences in the degree of severity of the symptom ($p<0,001$). It's due to the absence, in agro-inoculated plants, of plant - fly interactions, wich capable to slowing-down the infection: the TYLCV has a latency period from 17 to 20 hours in the vector whereas the infectivity can be retained until 8 days (Caciagli *et al.*, 1995).

Dosage of viral DNA

The dosage of viral DNA at the level of leaf tissues revealed the coexistence of the TYLCV and TYLCSV to plants inoculated via *Bemisia tabaci* (Figure 2) collected in the field. These two viruses are

Table 1. Symptom scoring of five cultivars of tomato grown under natural and artificial inoculation.

Cultivars (N [a])	DPI [b]	% Inf.	Score ([d])	Cultivars (N)	DPI [c]	% Inf.	Score ([d])
Cherry (10)	25	70	1	**Cherry (32)**	25	3	1
	40	100	1 (50) – 2 (50)		40	43	1 (16) – 3 (28)
	55	100	2 (30) – 3 (70)		55	75	3 (50) – 4 (25)
	70	100	2 (30) – 3 (70)		70	87,5	3 (53) – 4 (34,5)
Tres cantos (10)	25	80	1	**Tres cantos (31)**	25	16	2
	40	80	1 (70) –2 (10)		40	64,5	3 (48) – 4 (16,5)
	55	100	1 (70) – 2 (30)		55	80,6	3 (48,6) – 4 (32)
	70	100	2 (50) – 3 (50)		70	100	3 (45) – 4 (55)
Marmande VR (8)	25	62	1 (50) – 2 (12)	**Marmande VR (20)**	25	5	1
	40	87	1 (75) – 2 (12)		40	80	3 (45) – 4 (35)
	55	87	1		55	95	3 (45) – 4 (50)
	70	87	1		70	100	3 (40) – 4 (60)
Campbell VR (6)	25	83	1	**Campbell VR (30)**	25	50	1
	40	100	1		40	86,6	1 (70) – 3 (16,6)
	55	100	2 (50) – 3 (67)		55	100	2 (80) – 3 (20)
	70	100	2 (83) – 3 (17)		70	100	3 (66) – 4 (34)
Pepite F1 (8)	25	0	0	**Pepite F1 (17)**	25	0	0
	40	0	0		40	17	1
	55	0	0		55	30	1
	70	75	1		70	41	1

[a]: Number of tested plants. [b]: Days Post Inoculation under inoculation with *Bemisia tabaci*. [c]: Days Post Inoculation under agroinoculation. [d]: % of plants with this score of the total of plants.

therefore involved in epidemics in Agadir's area. The necessary to note, it is the bigger variability of the values observed for plants naturally inoculated in comparison with plants agro inoculated, as show in the standard deviation. Indeed, in plant inoculated with *Bemisia tabaci*, this value is 6,301 ηg of viral DNA/mg of total DNA, while it is 3,282 ηg/mg at the agroinoculated ones. This shows that the agro infection is more homogeneous for routine inoculations than the inoculation by *B. tabaci*. In spite of this difference of variability, we did not reveal, statistically, differences between the compared averages (p = 0,11).

From the symptom scoring and dosage of viral DNA, we can conclude that the agroinoculation method is more effective than the inoculation mediate *Bemisia tabaci* for the tomato plants inoculation in laboratory. But it's extremely essential complete the test by natural inoculation to study the eventual interactions between the plant and the vector.

Figure 1. TYLCV symptoms: (A) Yellowing and curling of leafs (B) dwarfing of infected plants (C) abortion of flowers (D) in comparison with no infected plants.

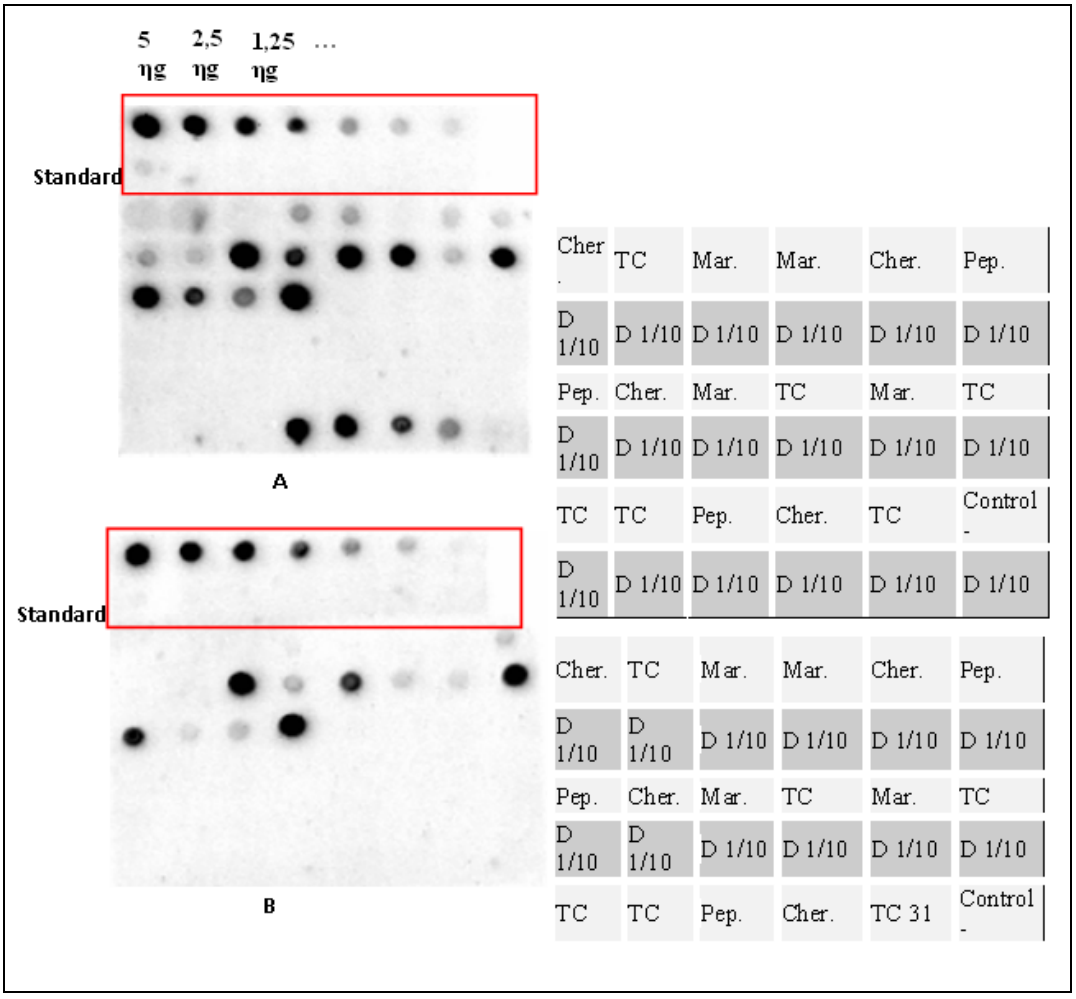

Figure 2. Dot-blot hybridization of tomato plant total ADN, resulting from four genotypes, inoculated with the whitefly *Bemisia tabaci*. (A) specific standard to TYLCV. (B) specific standard to TYLCSV. Cher.: Cherry ; TC: Tres cantos ; Mar. : Marmande VR; Pep : F1 Pepite ; D 1/10 : Dilution 1/10.

Procedure to evaluate plant tolerance to virus infection

The lack of good methods to evaluate plant resistance/tolerance is a limiting factor in breeding programmes. Resistance is considered a host characteristic hindering virus infection, whereas tolerance is considered a host characteristic, which allows it to support systemic viral infection, while developing milder symptoms than more sensitive hosts. The formula, developed by Rubio *et al.* (2003) can be used to estimate the degree of resistance and tolerance of plants to virus infection using virus titre (estimated by tissue-print hybridization) and symptom intensity of infected plants as parameters. The lack of some tissue-print hybridization data restricts the use of formulae to evaluate only the relative tolerance of the five tomato cultivars to a TYLC virus population from Agadir (Morocco), and Spain.

The tolerance level of a cultivar *x* (tolerance index, T_x) was calculated as the average of the tolerance indexes of individual infected plants (T_i). T_i was estimated using the relative symptom intensity of a plant in comparison with

the maximum symptom intensity observed in a sensitive cultivar:

$$T_x = (1/P_x)\sum_{i=1}^{i=P_x} T_i \qquad \text{(Equation 1)}$$

Where $T_i = 1-(S_i/S_m)$, P_x is the number of infected plants of cultivar *x*, S_i is the symptom intensity of plant *i*, and S_m is the maximum symptom intensity.

The values obtained with these formulae range from 0 for no tolerance, to 1 for complete tolerance.

Evaluation of tolerance of tomato cultivars to a TYLC virus populations

The formula was applied to evaluate the tolerance of tomato cultivars Cherry, Tres Cantos, Campbell 33 and F1 Pepite to the TYLC virus populations in comparison with the susceptible/sensitive cultivar Marmande VR. Tolerance levels are presented in Table 3. The index T_x (see above) was only 0,23 for Marmande VR, low for Tres cantos (0,32) and Cherry (0,34) and moderate for Campbell 33 (0,42) and Pepite (0,54) (Table 3). The Glm test showed that these differences were significant. Considering agronomic factors such as production and fruit diameter, colour and aspect, the best cultivar was Pepite. Data demonstrated en table 3 indicate that is (Pepite) also the most tolerant to TYLCV, whereas Tres Cantos and Cherry were the most sensible.

Table 3. Tolerance to tomato yellow leaf curl virus (TYLCV) of five tomato cultivars indicated by tolerance index value.

Cultivars	N	P	I	T
Cherry	32	28	0,87	0,34 ± 0,10
Tres Cantos	31	31	1	0,32 ± 0,27
Marmande	20	20	1	0,23 ± 0,19
Campbell 33	30	30	1	0,42 ± 0,32
Pepite	17	7	0,41	0,54 ± 0,24

N, Number of plants analysed; P, number of plants infected by TYLCV (symptomatic); I ratio of infected plants, $I = P/N$; T, tolerance index values and standard error (estimated from Table 4 as indicated in equation 1).

Table 4. Symptom intensity of individual plants of five tomato cultivars grown under natural *Tomato yellow leaf curl virus* pressure 70 DPI

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cherry	3	3	4	0	4	4	4	4	4	4	1	0	4	0	0	4
Tres Cantos	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3
Marmande VR	4	4	4	4	4	4	4	4	4	4	4	4	3	3	4	4
Campbell 33	4	3	3	4	4	4	2	4	3	4	4	4	4	4	1	1
Pepite	2	3	0	0	0	3	0	0	0	0	0	1	1	1	0	0

17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
4	4	4	3	3	3	3	3	3	2	3	3	3	2	3	1
3	3	1	1	1	1	2	1	3	4	3	3	2	4	2	
4	3	2	1												
2	2	2	2	2	2	4	2	2	4	4	3	3	1		
1															

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Volatile compounds from four species of Moroccan truffles

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Abstract

In the present study, an experimental design has been used to optimize the extraction of volatile compounds from Moroccan truffles aroma (*Tuber oligospermum, Terfezia. Arenaria, Terfezia leptoderma* and *Tirmania nivea*) by using a dichloromethane extraction under an inert atmosphere followed by simultaneous steam distillation - dichloromethane extraction. The extracted compounds have been analyzed by gas chromatography with a flame ionization detector. Of the 44 volatile compounds detected, any one of these compounds was observed from all 4 species. *T.oligospermum* and *T. Arenaria* are richer in volatile products than *T. nivea* and *T. leptoderma*. The major volatile compound of *T. oligospermum, T. arenaria, T. nivea* and *T. leptoderma* were benzaldehyde, nonanal, 2-octenal and 2-methyl-1-propanol respectively. Itch one, account for almost 17, 26, 29 and 31 % of the totals areas of there chromatograms respective.

Keywords: Aroma, Truffle, *Terfezia, Tirmania, oligospermum, Arenaria, leptoderma*, *nivea*,

Introduction

Truffles are mushrooms Ascomycetous, under class of Discomycetous pertaining to the order of Tuberales. This order is represented in Morocco by some species belonging to the kinds: *Tuber*, *Terfezia*, *Delastria*, *Picoa* and *Tirmania* (Khabar *et al.*, 2001). These fungus are underground mushrooms which grow in symbiosis with roots of certain trees like the oak (*Quercus sp.*), the hazel tree (*Corylus sp.*), the pine (*Pinus sp.*) (Reyna, 2000) or numerous herbaceous plants, mainly of the genus *Helianthemum* (Khabar *et al.*, 2001) with which they exchange metabolites, mineral salts and ions (France *et al.*, 1983; Bonfante & Perotto, 1992; Read, 1995; Miranda *et al.*, 1997). The tubers develop with a depth from 5 to 20 cm; what makes their gathering (the calvage) always random. Their research and their harvest are done primarily using an animal like the dog, the pig, the fly with truffles (genus *Suillia*) or thanks to the mark cracking of the ground crust under the pressure of the mushroom (Harki, 1996). Some species of the genus *Tuber* are known internationally for their gastronomically qualities and their economic importance (i.e. black Perigord truffle (*T. melanosporum* Vitt.) (economic value more than €1000 kg^{-1}) and the white truffle of Italy (*Tuber magnatum* Pico.). In Morocco, 8 truffle species are listed (Khabar *et al.*, 2001). They are commonly called "Terfass". Four of them are particularly appreciated and very required (*Tuber oligospermum,Terfezia Arenaria, Terfezia leptoderma* and *Tirmania nivea*). They are the subject of an important trade at the edge of the roads and on the central markets of certain areas (economic value more than €50 kg^{-1}). Few scientific research tasks were devoted to Moroccan truffles. The studies carried out are of a nature taxonomic and floristic (Malençon, 1973; Chatin, 1891a, 1891b) or cytological and ultrastructural (Khabar, 1988; Khabar *et al.*, 1994).

To differentiate from the truffle species in particular those whose morphological characteristics are similar,

the molecular techniques of biology were used to find specific molecular markers for each truffle species (Mabru *et al.*, 2001; Amicucci *et al.*, 2002; Paolocci *et al.*, 2004). But these techniques require equipment and quite specific expertise. Recently, the most used analytical techniques consist to extract concentrate and analyze the aroma volatile organic compounds of the aroma of truffles and to analyze by capillary gas chromatography (GC) and GC/mass spectrometry (Fabio *et al.*, 1995; Diaz *et al.*, 2002; Diaz *et al.*, 2003; Falasconi *et al.*, 2005; Gioacchini *et al.*, 2005; March *et al.*, 2006).

The objective of the present research has been to fully characterize aroma of Moroccan truffles of different species. Also, the investigation should be extended to an exploration of the differences among the volatile organic compounds from each species of truffle over a geographical area.

Materials and methods

Fungus material

The truffles studied are collected in three different areas from Morocco in March 2004: *Terfezia. Arenaria* and *Terfezia leptoderma in* the forest of Mamora between Rabat and Kénitra (west of Morocoo), *Tirmania nivea* in the area of Bouâarfa (east of Morocco) and *Tuber oligospermum* in area of Missour (North-eastern of Morocco). The fresh samples were washed with distilled water and the peridium was removed. They were freeze-dried and stored in a freezer at -25°C until processing. Five truffles were used for etch species. Their size was 12 ± 2g.

Aroma truffles extraction and analysis

Aroma truffles were extracted using the method of Bouseta & Collin (1995). The concentrated extracts obtained were analyzed by Gas chromatography (GC), model Hewlett-Packard 5890, equipped by a model of automatic injector simple with type Hewlett-Packard 7673, a flame ionization detector, and a Shimadzu CR4A integrator. A Column EC-WAX (Alltech) with 30m x 0.25 mm internal diameter and 0.25 µm film thickness was used. The oven temperature was programmed to rise from 30 to 85 °C at 5 °C/min, then to 145 °C at 1 °C/min, and to 250°C at 3 °C/min. The carrier gas was helium at a flow rate of 1.5 mL/min. The injector temperature was maintained to 3 °C above the oven temperature. The detector temperature was 275 °C. Mass spectrometry analysis was carried out using an HP 5988 quadruple mass spectrometer. Electron Impact mass spectra were recorded at 70 eV. Compounds were tentatively identified by comparison of the spectra with those in a mass spectrometry library and with data found in the literature.

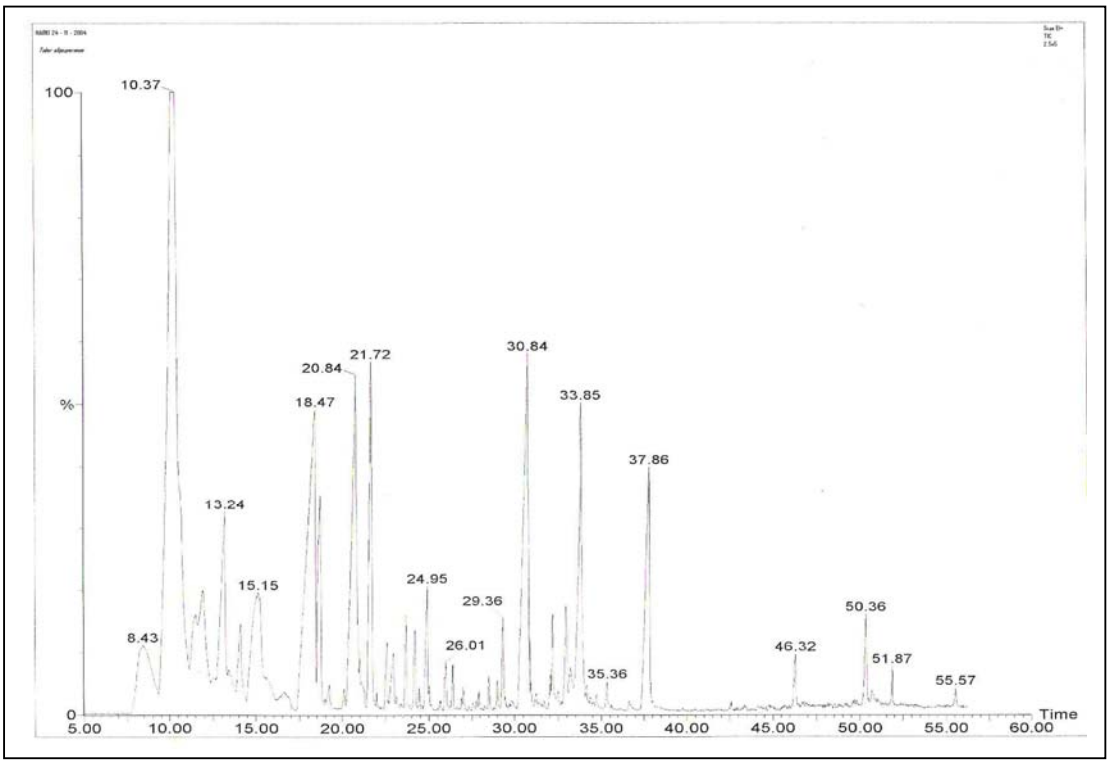
Results and Discussion

The volatile compounds from four species of truffles were analyzed by gas chromatography and mass spectrometry. A representative gas chromatogram of a truffle simultaneous extraction-distillation sample extract is shown in Figure 1. Peak identifications and relative amounts of volatile compounds, expressed in area (%) were listed in Table 1.

A total of 44 volatiles were detected and quantified in the different species of truffles. Forty of these were identified by comparison of their mass spectral data with those from authentic compounds and/or mass spectra suggested by the NIST database and GC retention indices (Table 1).

Each truffle species has its own volatile products except for propanal, 2 undecanone, 2,4-nonadienal, 1,2-dimethoxy-4-(2-propenyl)-benzene and 1-methyl-4-(phenylmethyl)benzene detected at the same time at 3 out of 4 species studied. The four species examined exhibited between 7 and 29 volatile compounds. *T. leptoderma* exhibiting the lowest number (7) and *T. oligospermum* exhibiting the highest number (29).

Figure 1. Gas chromatogram of an extract obtained by simultaneous extraction-distillation of a truffle sample.



Twenty-seven of 29 compounds detected were identified at *T. oligospermum*. The majority products are benzaldehyde, 3-methy-l-butanol, ethylbenzene and 2-methyl-1-propanol with a relative percentage 17, 16, 14 and 13 respectively. These 4 products only account for with them 60% of the totality of the products detected at this species. On the other hand, the major compounds of *T. arenaria* are : nonanal, 2, 4-nonadienal, octanal and β-caryophyllene which account for almost 74% of the total aroma. Twenty seven products were detected and 23 identified in *T. arenaria*. *T. nivea* comes in third position with 10 compounds identified. 2-methyl-1-propanol, 1,3-pentadiene and ethylbenzene are the majority product with a total percentage over 68%. All the products of this specie have a time of retention lower than 30.7 minutes. Lastly, *T. leptoderma* which presents only 7 products of which 3 are majority: 2-octenal (29 %), phenylacetaldehyde (26 %) and 2-undecanone (23 %). With opposition to the products of *T. nivea*, the products of *T. leptoderma* leave only beyond 28.2 mn.

In the present work, other compounds such as acetaldehyde, propanal, 1-octen-3-one, 3-octanone and naphthalene have also been detected. Such compounds have been previously described in *T. melanosporum* (black Truffle) and *T. aestivum* (summer truffle) Diaz *et al.* (2003). Two of them, 1-octen-3-one and 3-octanone, have been described as responsible of the characteristic mushroom odour of such fungi.

However, specific volatiles of *T. melanosporum* such as dimethylsulfide, and 2-methylbutanol (Ney, 1989; Talou *et al.*, 1989) were not detected in extract samples of studied Moroccan truffles. Each studied truffle species has its own chart of volatile products. This difference can be considered associated to the origin (factor such as growing conditions, ecology, etc.).

Table 1: Identified compounds listed in order of increasing retention time of the four truffle species studied.

No.	RT(mn)	Compound	Area (%)			
			T. oligospermum	***T. arenaria***	***T. leptoderma***	***T. nivea***
1	8,2	1, 3-pentadiene	1,0638	-	-	19.6543
2	11.2	acetaldehyde	0.9641	-	-	0.9832
3	11,5	propanal	1.2510	0,3206	-	1.5941
4	12,8	2-propanone	-	-	-	8.4360
5	13,2	2- methyl-butanal	0.7651	-	-	1.3969
6	14,6	3- methyl-butanal	0.0324	-	-	-
7	15,1	hexanal	1.3342	-	-	-
8	18,3	2-methyl-1-propanol	13,4052	-	-	30.7098
9	19.0	2-methyl-2-butanal	0.8320	-	-	-
10	20,6	ethylbenzene	14,2929	-	-	17.5490
11	21,6	3-methyl-1-butanol	16.2374	-	-	2,0337
12	22.0	5-methyl-2-heptanone	0,0607	-	-	-
13	22,8	6-dodecanol	0.0743	-	-	1,9134
14	23.1	1,2,4-trimethylbenzene	0,0333	-	-	-
15	23,7	octanal	0.0421	14,6529	-	-
16	24,9	3-hydroxy-2-butanone	1,0756	-	-	-
17	25,8	nonanal	-	25,5673	-	-
18	26,0	1-hexanol	0,2367	4,1248	-	-
19	27,4	3-octanone	0.2401	2,1711	-	-
20	28,2	2-octenal	-	0.8763	29.3422	-
21	29.0	decanal	-	0.8531	-	-
22	29,7	1-octen-3-one	0,2675	4,6468	-	-
23	30.0	1-heptanol	-	2,7345	-	-
24	30,7	benzaldehyde	16.9820	0.3490	-	6. 5478
25	31.5	2-undecanone	0,0474	0.2679	23,4367	-
26	31, 7	phenylacetaldehyde	-	-	25.6329	-
27	32.5	2-propenoic acid	0.0653	-	7.6544	-
28	32,7	2,4-nonadienal	0.0321	19,2096	7.8123	-
29	33,7	β-caryophyllene	12,8437	14.5469	-	-
30	34,2	dodecanal	-	0.9433	-	-
31	34.6	naphtalene	-	0.8774	-	-
32	35,3	1,3-dimethoxybenzene	0.0142	-	-	-
33	37,7	2,4-decadienal	11.5410	0.6653	-	-
34	39.6	2,5-dimethoxytoluene	-	0.4043	-	-
35	40,4	3,4-dimethoxytoluene	-	0.7010	-	-
36	41,6	unknown	-	0,0896	-	-
37	42,3	2-methoxy-4ethyl-6-methylphenol	-	0,0754	-	-
38	43.8	phenylethanol	-	0.0553	-	-
39	44,6	phenol	-	0,0876	-	-
40	46,5	unknown	1,0242	0 .0123	-	-
41	50,4	1,2-dimethoxy-4-(2-propenyl)-benzene	1.4562	4,0547	0.0657	-
42	50.8	unknown	-	0.0553	-	-
43	51,7	1-methyl-4-(phenylmethyl)benzene	0.0238	1.0165	0.0567	-
44	55,4	unknown	0.0134	0.3654		

Conclusion

The methods used in this study have permitted the identification of a total of 40 compounds in the volatile fractions from 4 species of truffle from Morocco. The Moroccan truffles studied present few volatile chemicals compared to *T. melanosporum* (black Perigord). This report is normal because the collected Moroccan truffles have a low and discrete odour whereas the black truffle on the contrary, with a strong and pleasant odour. It should be noted that the results presented here are somewhat preliminary. With the proven analytical technique, the investigation should be extended to an exploration of the impact of the different stage of truffle maturity, truffle condition, state of hydration, storage, etc., on the volatility profiles of the truffles examined here. In addition, the investigation should be extended to an exploration of the differences among the volatile organic compounds from each species of truffle over a geographical area.

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Antiporters: role in salinity tolerance A REVIEW

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Abstract

Soil salinity is a most serious environmental problem around the world. Soil salinity is an important limiting factor for plant growth and crop productivity. Although sodium is used by plants in very small amounts, high levels of Na+ are toxic and can decrease the K+ uptake by plants. Na+ extrusion from cells and vacuolar compartmentation of this cation are essential mechanisms used by all living organisms to deal with high salinity. In both yeast and plants cation/proton antiporters at the vacuolar and plasma membrane contribute to sodium extrusion from the cells and salt tolerance. Here in this review we will compare the different families of Na+ transport systems in Plants and yeast.

Keywords: Cation transporters, pH regulation, salt tolerance, sodium, yeast, plants

Introduction

Soil salinity is a significant limiting factor for agricultural production. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers & Yeo, 1995). High concentrations of NaCl may cause both hyperionic and hyperosmotic stress effects, which lead to a decline of turgor, disordered metabolism, and the inhibition of uptake of essential ions, as well as other problems in plant cells (Kim *et al.*, 2007).

Plants have several mechanisms to respond and adapt to salt stress, by changing gene expression patterns, metabolic activity and ion and water transport to minimize stress damage and to e-establish ion homeostasis (Hasegawa *et al*., 2000).

In plants and yeast, an important mechanism to overcome salt stress is the exclusion of Na+ from the cytoplasm, by the operation of Na/H antiporters at the plasma membrane or tonoplast (Figures 1 and 2). Several studies have shown that under saline conditions, Na1 influx into root cells occurs via Na1 permeable transporters (Amtmann *et al.*, 1997; Roberts & Tester, 1997; Tyerman *et al.*, 1997), which in turn elevates the cytoplasmic sodium concentration and causes toxicity (Kingsbury & Epstein, 1986).

In order to be able to improve salt tolerance of crop plants, a basic understanding of salt tolerance mechanisms is needed. In this respect, the yeast *Saccharomyces cerevisiae* has been used, first as a model system to study yeast Na+ transport systems, and later to functionally characterize plant ion transporters involved in Na+ transport. In this review we discuss the Na transport systems of Plants and yeasts.

Update on Na+ Transporters in Plants

Na+ ATPases

In *Saccharomyces cerevisiae* (Figure 2), the main system acting in Na+ extrusion from the cells is a Na+-ATPase encoded by the *ENA1* gene (Haro *et al*., 1991; Wieland *et al*., 1995, Horie & Schroeder, 2004). This powerfull transport system permits yeast to grow at NaCl concentrations of more than 0.5 M. In

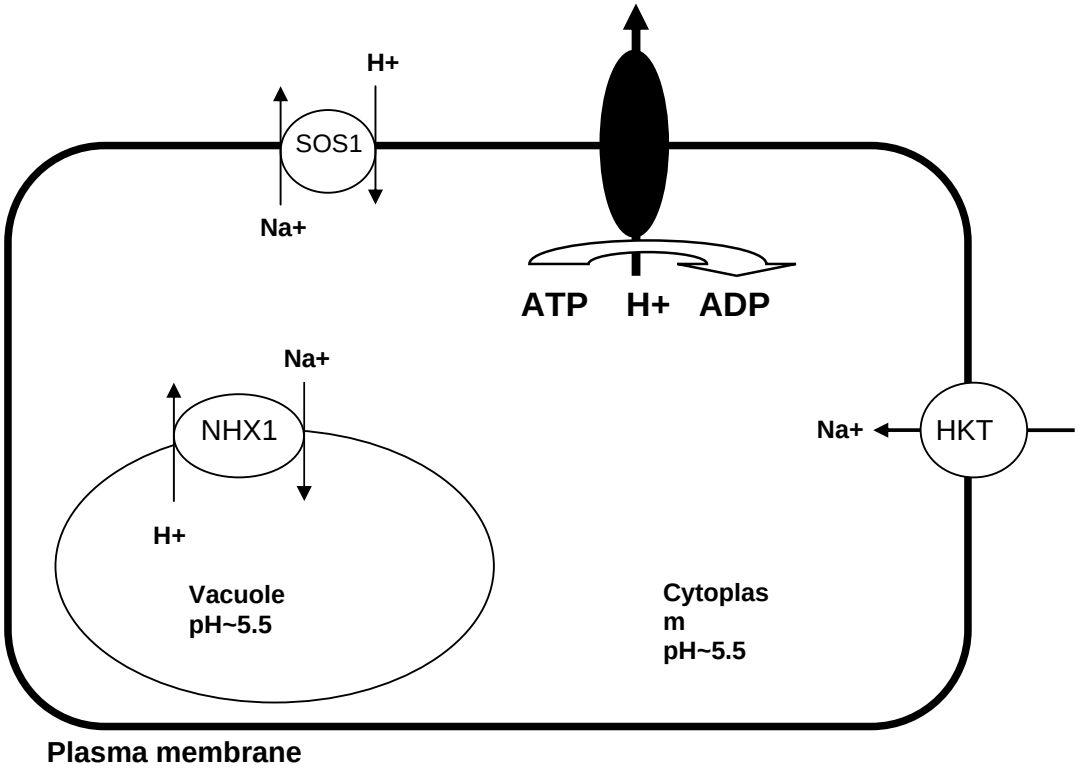

Figure 1. Sodium transport systems in Plants. CHX and KEA transporters remain largely unknown.

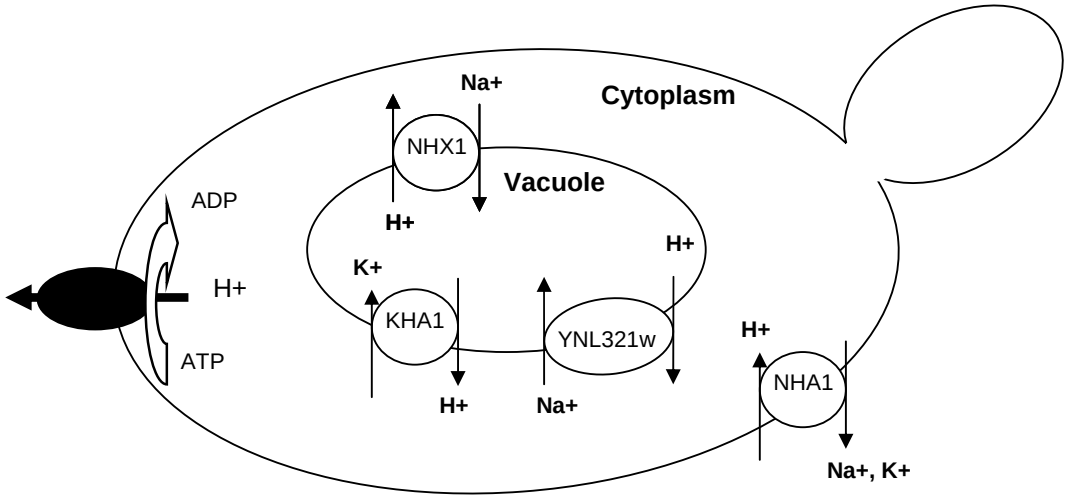

Figure 2. Na+ transport systems in yeast.

higher plants, no Na+-ATPases similar to the ENA1 gene could be identified, indicating that plants rely on other Na+ extrusion mechanisms. However, Benito & Rodríguez-Navarro, (2003) isolated ENA1 homologs from the moss *Physcomitrella patens* (*PpENA1*, *PpENA2A*). The expression of the *PpENA1* cDNA in the highly sodium sensitive yeast mutant, *ena1-4 nha1*, complemented the salt-sensitive phenotype, whereas *PpENA2A* did not. This suggests that Na^+-extruding pumps existed in primitive land plants but these genes may have been lost in higher plants during evolution.

Cation/proton antiporters

In many cases, cations are transported against their electrochemical gradient by proton coupled transporters rather than by primary ion pumps. In this way, the plasma membrane H+-ATPases (P-ATPase), vacuolar H+-ATPases (V-ATPase) and plant H+-PPase participate in salinity stress tolerance by energizing active Na+ extrusion from the cytosol and compartmentation within various endomembrane bound compartments (*e.g.*, vacuole, endoplasmic reticulum, golgi, chloroplasts and vacuole), respectively (Cushman, 2001). In yeast several Na+/H+ transport systems have been identified (Figure 2), but their effect on salt tolerance is only minor. The prevacuolar/endosomal (K+,Na+)/H+ exchanger NHX1 is primarily involved in vesicle trafficking by regulating cytoplasmic and endosomal pH (Brett *et al.*, 2005), but has also a secondary role in Na+ tolerance and osmotic tolerance, by sequestration of Na+ in vacuoles (Nass *et al.*, 1997; Nass & Rao, 1999). The plasma membrane (K+,Na+)/H+ antiporter NHA1 is suggested to exhibit electrogenic transport of K+ or Na+ out of the cell (Ohgaki *et al.*, 2005), and also contributes to salt tolerance (Bañuelos et al 1998). A third antiporter present in yeast is the KHA1 gene (Figure 2). Although originally proposed to reside on the plasma membrane, it is now believed that the KHA1 gene has a function similar to NHX1, and resides on internal membranes. Disruption of the gene renders cells more sensitive to alkaline pH and hygromycin (Maresova & Sychrova, 2005). It was observed that yeast with disruptions in all these proposed Na+ antiport systems still has the capacity to accumulate Na+ in vacuoles (Hirata *et al.*, 2002). Recently, the yeast gene YNL321w of the CaCA family (Figure 2), which shares homology to the *VCX1* Calcium/H+ antiporter was shown to be able to transport Na+, and was suggested to be responsible for this vacuolar Na+ accumulation (Cagnac *et al.*, 2007).

In plants many more Cation/proton antiporters can be identified (Figure 1). Based on the classification made by Saier (1999), Mäser *et al.* (2001) classified the different antiporters in the Arabidopsis genome in the following families: CaCA, CCC, CPA1, CPA2 and NhaD. The CPA1, CPA2 and NhaD families are proposed to harbour many Na+/H+ antiporters. The CPA1 family includes the antiporters of the NHX1 and SOS families that have been characterized in some detail. The large CPA2 subfamily has 33 members that include 28 CHX proteins thought to mediate cation/H+ exchange and six homologues of the K+/H+ antiporter *AtKEA1*, that share some homology to the bacterial KefB and KefC proteins.

The salt tolerance locus SOS1 from Arabidopsis has been shown to encode a putative plasma membrane Na+ /H + antiporter with homology to bacterial antiporters of the NhaP subfamily (Shi *et al.*, 2002). Although no SOS1 homologs can be found in the yeast genome, it appears to be the most important determinant of salt tolerance in plants (Figure 1). When expressed in a yeast mutant deficient in endogenous Na+ transporters, SOS1 was able to reduce Na+ accumulation and improve salt tolerance of the mutant cells (Shi et al., 2002). SOS1 activity is regulated by a complex composed of the SOS2 kinase and the SOS3 Ca2+ binding protein in vivo (Quintero *et al.*, 2000).

Recently our group demonstrated that SlSOS1 antiporter is not only essential in maintaining ion homeostasis under salinity, but also critical for the partitioning of Na^+ between plant organs (Figure 1). The ability of tomato plants to retain Na^+ in the stems, thus preventing Na^+ from reaching the photosynthetic tissues, is largely dependent on the function of SlSOS1 (Olías *et al.*, 2009)

The Plant NHX antiporters can be subdivided in a vacuolar clade, exclusive for plants (Figure 1), and an endosomal clade, present in plants, fungi and animals

(Brett *et al.*, 2005; Pardo *et al.*, 2006). The first NHX protein that was described in plants is *AtNHX1*, member of the vacuolar clade of NHX proteins (Gaxiola *et al.*, 1999; Quintero *et al.*, 2000; Yokoi *et al.*, 2002). In yeast *AtNHX1* was capable to complement the phenotypes of salt and hygromycin sensitivity caused by *ScNHX1* disruption (Gaxiola *et al.*, 1999; Quintero *et al.*, 2000). It was also shown that overexpression of this protein in various plants improves salt tolerance (Apse *et al.*, 1999; Zhang & Blumwald, 2001; Zhang *et al.*, 2001; Ohta *et al.*, 2002; Xue *et al.*, 2004; He *et al.*, 2005). It was however shown that the encoded protein also catalyzes K^+/H^+ exchange with similar activity (Venema *et al.*, 2002). Furthermore, T-DNA insertional mutants of *AtNHX1* show reduced leaf area and cell size, which also indicates that the protein is involved in Na^+ or K^+ accumulation inside vacuoles for maintenance of cell turgor to drive cell expansion (Apse *et al.*, 2003). Notably, the *AtNHX1* gene shows very high expression in stomatal cells, suggesting that the protein is involved in the high K^+ accumulation in these cells (Shi & Zhu, 2002).

The *Solanum lycopersicon LeNHX2* protein and *Arabidopsis thaliana AtNHX5* protein constitute the first NHX members of the endosomal clade of antiporters in plants (Yokoi *et al.*, 2002; Venema *et al.*, 2003). In yeast the *LeNHX2* protein cofractionated with Golgi and prevacuolar membrane markers, and catalyzes K^+/H^+ antiport (Venema *et al.*, 2003). Based on localization and ion specificity, it was proposed that endosomal NHX isoforms are essential to set the pH of endosomal compartments, which is believed to be fundamental for proper functioning of vesicle sorting in the secretory and endocytic membrane system (Pardo *et al.*, 2006). Acidification of these compartments would depend on K^+/H^+ exchange to prevent accumulation of toxic Na^+ ions (Pardo *et al.*, 2006).

The CHX transporters, along with the related KEA subfamily, constitute the CPA2 family of cation/proton antiporters. The function of individual members of the large CHX family remains largely unknown but three CHX isoforms, *AtCHX17*, *AtCHX20* and *AtCH23*, have been shown to affect K+ homeostasis and the control of chloroplast pH (Cellier *et al.*, 2004; Song *et al.*, 2004; Sze *et al.*, 2004; Padmanaban, 2007). It was shown that in yeast, CHX17 and CHX20 can complement disruption of the KHA1 gene, restoring growth at alkaline pH in the presence of low K+ (Maresova & Sychrova, 2006; Padmanaban *et al.*, 2007).

The KEA genes in Arabidopsis have not been characterized so far Especially *AtKEA1* to *AtKEA3* show homology to bacterial KefB and KefC transporters that are activated by the detoxification reaction of GSH with electrophiles, in order to restore intracellular pH, via K+ efflux (Booth *et al.*, 2003).

There are two members of the NhaD subfamily in Arabidopsis (NHD1 and NHD2) that have similarity to Na+/H+ antiporters found in bacteria, but that are currently uncharacterized (Pardo et al 2006). The PeNhaD1 isoform of poplar restores growth of a salt sensitive E coli strain in the presence of salt (Ottow *et al.*, 2005). Furthermore, NhaD isoforms from Physcomitrella patens were localized to chloroplasts, complemented salt sensitive bacterial strains and stimulated K+ uptake in K+ influx mutants (Barrero-Gil *et al.*, 2007).

Conclusions

Although yeast has been used as a model system to understand plant salt tolerance, important differences in the basic set of ion transporters involved can be found. In yeast, the predominant system responsible for salt tolerance is ENA1, whilst in plants sodium transport is mediated by a large number of plasma membrane and vacuolar antiporters. Nevertheless, many of the plant proteins

can be expressed in yeast, conferring salt tolerance. As expression in bacteria of most of these transporters is very toxic, only yeast expression provides the possibility to perform functional studies on these low abundant proteins that would be otherwise difficult to perform.

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Quantitative analysis of bacteria isolated from American cockroaches (*Periplaneta americana L.*) and Houseflies (*Musca domestica L.*) collected in six districts of Tangier

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Abstract

The feeding mechanisms and filthy breeding habits of American cockroaches and Houseflies make them potential vectors and transmitters of human pathogens. In this study, *Periplaneta americana* and *Musca domestica* were collected from six urban districts in Tangier, so as to isolate and count *Staphylococcus* and *Enterobacteriaceae* from their external body. On the one hand, our results indicate that there were significant differences between the American cockroaches and houseflies in these loads of bacteria. On the other hand, there were also significant differences between the six selected districts and the Bani Makada district recorded the higher concentrations of bacteria from their houseflies and cockroaches. While the lowest numbers of these bacteria were found in Val fleuri, Roi fahd and Place Mozart. These findings; all proportion considered , show that *Musca domestica* carries more bacteria than *Periplaneta americana* and suggest the role of American cockroaches and houseflies as biological indicator of sanitation conditions of each district.

Key words: *Periplaneta americana*, *Musca domestica*, vectors, *Enterobacteriaceae*, biological indicator, sanitation.

Introduction

Periplaneta americana and *Musca domestica* live in close association with humans and are by far the most common species in and around houses, in urban areas and villages with poor sanitation conditions (Greenberg, 1973). Owing to their association with human environments both American cockroaches and houseflies may acquire and disseminate human pathogens (Fotedar *et al.*, 1991; 1992; 1993; Grubel & Cave, 1998). Because of their omnivorous habits of feeding and indiscriminate deposition of faecal materials, it was demonstrated that cockroaches are the ideal agents for the transmission of microorganisms (Roth & Willis, 1957; Oothman *et al.*, 1977; Cloarec *et al.*, 1992; Rivault *et al.*, 1993). Moreover, several species of bacteria of public health significance have been isolated from the cockroaches (*Periplaneta americana*) such as *Staphylococcus aureus*, *Streptococcus* species, *Klebsiella* species, *Pseudomonas aeroginosa*, *Salmonella* species, *Escherichia coli*, etc. (Cruden & Markovetz, 1987; Fotedar *et al.*, 1991; Rivault *et al.*, 1993; Pai *et al.*, 2003; 2005). The American cockroach is 30 to 50 mm long and is intimately associated with human sewage and sewer facilities from which it will enter bathrooms and basements (Brenner et al., 1987). It may live for well over a year. The females produce a sclerotized ootheca containing 16 young (Bell & Adiyodi, 1981). After hatching, nymphs will undergo between 6 to 14 molts (Bell & Adiyodi, 1981) over a period of several months (Barcay, 2004) depending on environmental conditions.

Houseflies have been suspected to be a reservoir and vector for pathogens; *Shigella* spp., *Vibrio* spp., *E. coli*, *Staphylococcus aureus*, *Campylobacter* spp., *yersinia enterocolitica*, *Pseudomonas* spp., *Enterococcus* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp. and

Acinetobacter spp. (Greenberg, 1971; Echeverria *et al.*, 1983; Fotedar *et al.*, 1992). They have a worldwide distribution extending from the sub polar region to the tropics, being present in Asia, Africa, Australia, the Americas and Europe (Grubel & Cave, 1998). A typical female housefly deposits 75-150 eggs on a variety of decomposing organic materials found in rubbish dumps, household garbage and waste foods from kitchens. The eggs hatch into maggots with feed on almost any substrate and thrive only in the presence of live microorganisms (Greenberg, 1965; Grubel & Cave, 1998). At about the fifth day, the maggot stops feeding and enters the pupae stage. After another 5 days adult houseflies emerge. During its lifetime, a housefly can travel up to 32 kilometers (Schoof & Siverly, 1954), although the usual dispersal remains within a radius of 3 kilometers (Levine & Levine, 1991).

The city of Tangier records a high demographic growth and a persistent rhythm of urbanization which create the formation of insalubrious and under-equipped districts. Moreover, in its various districts, there are noticeable differences in density, town planning and social level (Bouamama *et al.*, 2007). However, the potential for bacterial transfer by American cockroaches and houseflies has been demonstrated in a qualitative rather than quantitative manner. Moreover, the idea of direct relationship between body size of insect and capability of contamination and transmission of pathogens has been supported by several researches around the world (Mariluis *et al.*, 1989; Brown 1997; Graczyk *et al.*, 1999; 2000; 2005; Fischer *et al.*, 2001; Maldonado & Centeno, 2003). In the present study, the numbers of housefly and American cockroach bacteria can carry on their body were determined and compared to quantify the potential capability of transporting pathogens of each insect and in each site. Therefore, the concentrations of bacteria carried by insects were compared between the six selected districts of Tangier.

Materials and Methods

Insect collection sites

The city of Tangier has an estimated urban population of 703614 and 151755 households. Cockroaches and flies were collected from 6 selected districts of Tangier according to their social-economic conditions (kind of population, urbanization and social level). The districts were: Bendiban (BD), Banimakada (BM), Castilla (CA), Val fleuri (VAL), Place Mozart (PM) and Charf (CF). Banimakada and Bendiban are the popular districts of the city and they are disadvantaged and under-equipped owing to high density of population and inadequate waste disposal and treatment network. Place Mozart and Charf benefit by favorable social-economic situation. Val fleuri and Castilla were situated between these low categories of districts.

Collection and identification of cockroaches and flies

600 houseflies (100 per district) and 60 American cockroaches (10 per district) were collected from the six selected sites, between March and October 2006. 10 others American cockroaches and 660 houseflies were collected only from the district Bendiban and during the same time period.

Flies were caught with sterilized nets from the garbage heaps and open defecating grounds in each district and from 9:00 to 13:00 am when the flies are active.

The cockroaches were caught at night from houses of the selected districts, directly by hand using a sterilized gallon container.

Cockroaches and flies trapped were placed in sterile test tubes and were subsequently taken to the laboratory and stored in the refrigerator at 4°C until identification and processing for bacteria examination. Identification was made by examining the insect under a low power microscope and following standard taxonomic keys.

Processing external body of insect for bacteria isolation

For cockroaches bacteria isolation, 5 ml of sterile normal saline was added to the tube containing one cockroach and vortexed for 2 min to wash-off any bacteria from its external body. However, flies were pooled in batches of 10 houseflies. Each pool of flies was shaken thoroughly in sterile saline (5ml) for 2 min. In the second set of the experiments, flies were pooled in batches of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 houseflies per batch to wash their external surface as described previously. The experiences were repeated 10 times in each site and for the 2 species of insects. The suspension washings were then serially diluted and inoculated on MacConkey agar and Chapman agar. Plates were incubated 24h at 37°C, and colonies with morphological and biochemical characteristics of *Enterobacteriaceae* and *Staphylococcus* were counted.

Statistical analysis

Results of enumeration of the bacteria were analyzed using STATISTICA 6.0. The average numbers of UFC were compared by ANOVA/MANOVA using Tukey post hoc test. $P<0.05$ was considered to be statistically significant.

Results

Report load of bacteria isolated from insect / insect size

Figures 1 and 2 show the difference in the concentrations of bacteria (*Enterobacteriaceae* and *Staphylococcus*) carried by one cockroach and those by ***n*** flies (with *n*=1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 flies analyzed per batch).

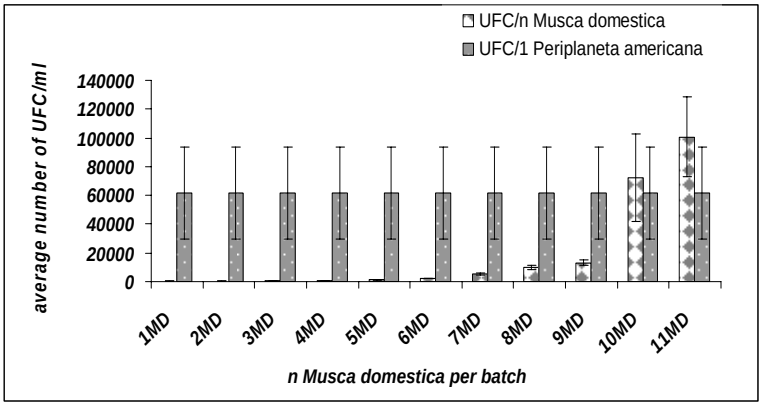

Figure 1. The average number of *Enterobacteriaceae* isolated from one cockroach compared with that in n houseflies (*n* MD). With "*n*" the number of flies analyzed per batch ($1 \leq n \leq 11$). At n=10 and 11 houseflies there is no significant difference between the loads of bacteria isolated from one American cockroach and houseflies ($p>0.05$).

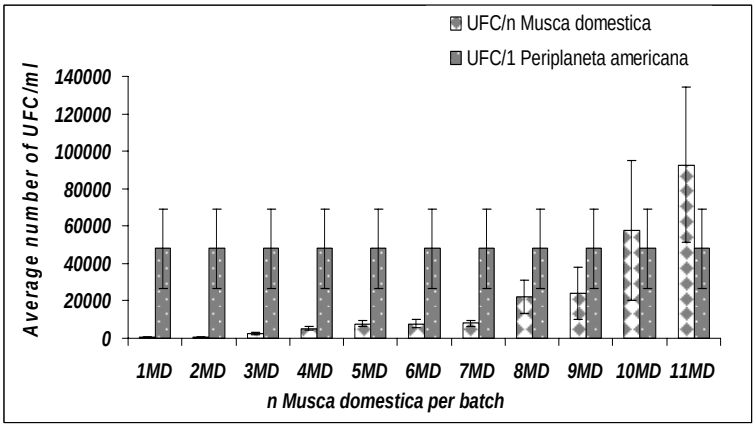

Figure 2. The average number of *Staphylococcus* isolated from one cockroach compared with that in n houseflies (*n* MD). With "*n*" the number of flies analyzed per batch ($1 \leq n \leq 11$). At n=8, 9, 10 and 11 houseflies there is no significant difference between the loads of bacteria isolated from one American cockroach and houseflies ($p>0.05$).

For *Enterobacteriaceae* (Figure 1), their loads in one American cockroach were significantly higher than *n* houseflies ($p<0.01$: *n*=1, 2, 3, 4 and 5; $p<0.05$: *n*=6, 7, 8 and 9). At *n*=10 and 11 there was no significant difference

between the loads of these bacteria in one American cockroach and *n* houseflies ($p>0.05$), and *Musca domestica* carries more bacteria than *Periplaneta americana*. However, the loads of *Staphylococcus* in the same site (Figure 2), were found to be significantly higher in one American cockroach than in *n* houseflies ($p<0.01$: $n=1, 2, 3, 4, 5, 6$; $p<0.05$: $n=7$). There was no significant difference between these loads at $n=8, 9, 10$ and 11 ($p>0.05$). *Musca domestica* carries more bacteria than *Periplaneta Americana* at $n=10, 11$.

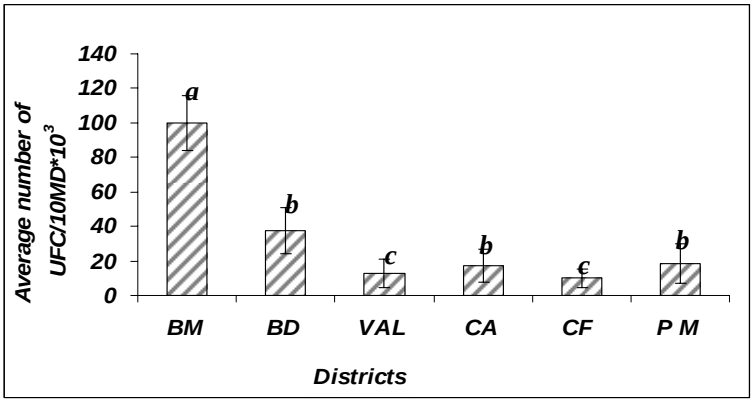

Figure 3. The average number of *Enterobacteriaceae* isolated from houseflies collected in the six districts of Tangier. The averages followed by the same letters are not significantly different according to the Tukey post hoc test.

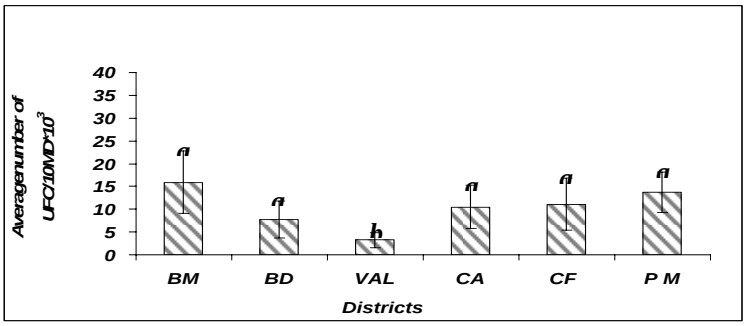

Figure 4. The average number of *Staphylococcus* isolated from houseflies collected in the six districts of Tangier. The averages followed by the same letters are not significantly different according to the Tukey post hoc test.

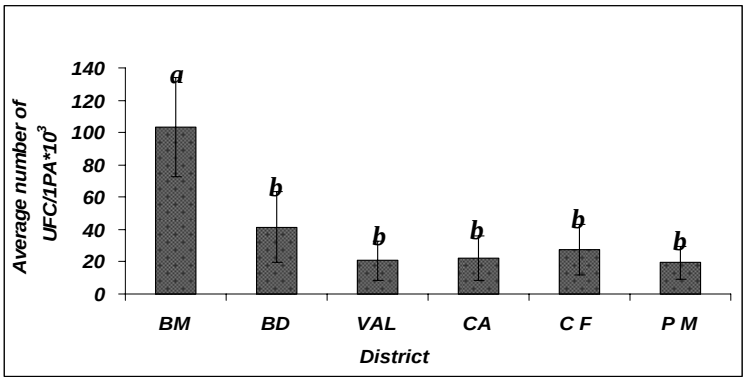

Figure 5. The average number of *Enterobacteriaceae* isolated from American cockroaches collected in the six districts of Tangier. The averages followed by the same letters are not significantly different according to the Tukey post hoc test.

Comparison between the loads of bacteria in different districts for two species of insect

Figure 3 show that the concentrations of *Enterobacteriaceae* carried by flies coming from Banimakada (BM) were very significantly higher than those in the others districts ($p<0.001$). There was no significant difference between these concentrations in the rest of the others districts. Furthermore in figure 4, there was no significant difference in the loads of *Staphylococcus* between (Banimakada and Bendiban) and (Charf and Place Mozart) ($p>0.05$). Though, between the districts Val fleuri and Castilla, there was a much higher significant difference in the loads of these bacteria.

The low numbers of UFC were recorded in Val fleuri ($p<0.001$).

The average number of *Enterobacteriaceae* was found to be significantly higher in *Periplaneta americana* coming from Banimakada ($p<0.001$), Figure 5. There was no significant difference in the amounts of these bacteria between the others districts. In the figure 6, there was no significant difference in the quantities of *Staphylococcus* carried by *Periplaneta americana* between (Banimakada and Bendiban), (Val fleuri and Castilla) and (Charf and Place Mozart). However, the amounts of these bacteria were significantly higher in Banimakada and Bendiban than those in Val fleuri, Charf and Place Mozart.

Discussion

Periplaneta americana and *Musca domestica* are the most common ones in Africa because of the favorable environmental and climatic conditions (Boulesteix *et al.*, 2005). However, most studies report only qualitative data on contamination by cockroaches and flies. Our results provided quantitative data on bacterial harboring by the houseflies and the American cockroaches in six selected districts of Tangier. In the first set of experiments, the loads of bacteria recovered from one cockroach were significantly higher than n houseflies ($1\leq n \leq 11$). However, this difference between the loads of bacteria in *Periplaneta americana* and *Musca domestica* became no significant at n=10 houseflies per batch (in case of *Enterobacteriaceae*) or at n=8 houseflies per batch (in case of Staphylococcus), and *Musca domestica* carries more bacteria than *Periplaneta americana*. Furthermore, adult houseflies measure 4-7 mm in length while adult American cockroaches 30-50 mm, and the cockroaches have a voluminal surface which can reach at least 10 times that of the domestic flies. These findings may be related not only to theirs sizes but may also depend on the association of these insects with unsanitary conditions of the environment and their omnivorous habits of feedings. Indeed, *Musca domestica* flies move and settle on several surfaces compared to *Periplaneta Americana*.

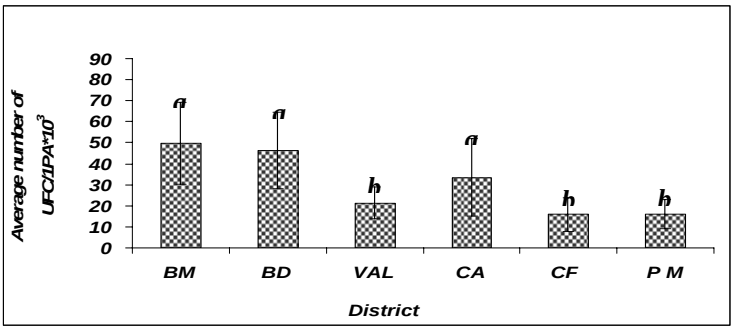

Figure 6. The average number of *Staphylococcus* isolated from american cockroaches collected in the six districts of Tangier. The averages followed by the same letters are not significantly different according to the Tukey post hoc test.

In the second part of this study, we noted that Banimakada recorded the higher concentrations of bacteria from their houseflies and cockroaches. While the low numbers of these bacteria were found in Val fleuri, Charf and Place Mozart. There was no significant difference between the rest of the districts (Figures 3, 4, 5 and 6). These results may be due to insalubrious conditions in Banimakada and favorable social-economic factors in Val fleuri, Charf and Place Mozart. The rest of the districts had relatively the same social-economic factors. We have found in similar but qualitative study that houseflies and American cockroaches caught in Banimakada carried pathogenic bacteria more often than those caught in other districts (Bouamama *et al.*, 2007).

In summary, our study demonstrated that houseflies and American cockroaches carry different loads of bacteria in the six selected districts of Tangier and may indicate the sanitation conditions of site.

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Isolation and Identification of Bacterial Strains from "EL HALASSA" Phosphate Deposit (Morocco)

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Abstract

A total of 75 bacterial strains have been isolated from 3 major phosphatic layers of EL HALASSA deposit at different levels. Pure cultures of the bacterial strains were subjected to a range of biochemical plate and tube tests including the use of the commercially available miniaturized API 20E Kit (BioMérieux). Computer-assisted numerical taxonomic analysis was carried out using simple matching and Jaccard types of similarity coefficient. The generated dendrogram yielded nine phena defined at the similarity range of 70% to greater than 90%. Two strains of *Pseudomonas* were regrouped in phenon 1, two strains of *Leclercia adecarboxylata* in phenon 2, two strains of *Aeromonas* in phenon3, five strains of *chromobacterium violaceum* were regrouped in phenon 4 and three strains of *Aeromonas claviae* in phenon 5. The other organisms were identified as *Sphingomonas paucimobis*, *Pasteurella* spp., *Acinetobacter* spp., *Achromobacter* spp., *Enterobacter agglomerens* and two strains of *bacillus.* Other three cocci gram positive strains remained unidentified. The unexpectedly high bacterial diversity of the strains isolated from the phosphate deposit samples is a surprinsing finding in such environment. This bacterial flora involves various metabolic pathways, and are either aerobic and/or facultative anaerobic with oxidation and/or fermentation of the organic matter present in the phosphatic layers. The Chemoautotrophic bacteria such as *alcaligenes* derive their energy from the oxidation of the mineral compounds present in the mining environment. In fact, this bacterial community diversity emerging out of this study emphasizes a wide-spreading ecological interest toward the solubilisation of inorganic phosphate and the bioremediation processes.

Key words: Phosphatic layers, bacterial diversity, taxonomy, EL HALASSA deposit.

Introduction

Since The tremendous variety of microorganisms represent the richest repertoire of molecular and chemical diversity on earth (Torsvik & Ovreas, 2002; Bhattacharya *et al.*, 2003), there has been a growing interest in studying the diversity of indigenous microorganisms in various environments such as soils (Torsvik & Ovreas, 2002; Kumar *et al.*, 2004) marine sediments (Hunter-Cevera *et al.*, 2005; Li & Qin, 2005) and volcanic environments (Staudigel *et al.*, 2008). The exploration of microbial diversity has been spurred by the fact that microorganisms underlie basic ecosystem processes as well as maintain elegant relationships between themselves and higher organisms. They perform numerous functions essential for the biosphere that include nutrient cycling and environmental detoxification (Watanabe *et al.*, 2002). Furthermore, the microbial world constitutes a huge and almost unexplained reservoir of resources likely to provide novel organisms, products and processes (Dubey *et al.*, 2005). A careful exploration of new habitats might continue to be useful.

The biological diversity of the Maghreb region is one of the richest in the world. Due to its vast geographic area and the variable climate shaped by the country's varied topography, Morocco presents many unexplored environments (e.g. the rhizospheric soils of endemic plants like *Argania spinosa* L., soils from

the desert or from snowy peaks of the Atlas, seawater from bays such as the Essaouira city, where the temperature is around 20 °C all year long). Thus, Moroccan phosphate mines remain an unexplored ecological niche eventually hosting a population of microorganisms with interesting metabolic characteristics. In fact, Moroccan phosphate deposits are worldwide known. Morocco has ¾ of the world phosphate reserves; it is the first exporter in the world and the second producer after the USA (Jasinski, 2002). Unfortunately, even though their need is obvious, microbiological researches on phosphate deposits are rare. Iddar *et al.* (2002) have studied the elevation of inorganic phosphate concentrations on primary metabolism of *bacillus cereus.* This strain was isolated from a phosphatic layer containing more than 65% w/w phosphorus, belonging to the basin phosphate of Khouribga (Morocco). They revealed a phosphate-stimulated $NAD(P)^+$-dependent GAPDH in *B. cereus,* which indicates that this bacterium can modulate its primary carbon metabolism according to phosphate availability. Moroccan phosphate mines are also sources of *Actinobacteria* showing abilities to solubilize insoluble natural phosphate rock (Hamdali *et al.*, 2008a). These strains are related to *streptomyces griseus* and *Micromonospora antiaca* and were isolated from three different phosphate mines centres in Morocco: Benguerir, Khouribga and Youssoufia. They have also shown multiple plant growth properties under laboratory conditions (Hamdali *et al.*, 2008b).

The intracellular phosphate metabolism in microorganisms is closely related to the protein synthesis which is stimulated or inhibited by the concentration of orthophosphate (Pi) (Pourriot & Meybeck, 1995). The micro-flora that can be met in the phosphate deposits shall be essentially formed of bacteria. The chemistry of the Moroccan phosphate deposits allows presuming that electron donors must be diverse. In fact the organic matter present in the phosphate layers with different rate (Khaddor *et al.*, 1997) may be the carbon source for the indigenous bacteria via fermentation and/or oxidation reactions. The mineral species of this environment can also be an energy source for the lithotrophic bacteria.

The aim of this study was to investigate the bacterial diversity in the EL HALASSA phosphate layers samples. Strains were subject to a set of 40 phenotypic tests using various culture media to determine their biochemical and physiological characteristics. The API 20E (BioMérieux) identification system was also applied. Such study will greatly enhance the understanding of the microbial diversity and its vital role in such environment.

Materials and methods

The sampling site and procedure

El HALASSA phosphate deposit site (32°40'60N, 6°49'60W) is within the phosphate basin of the Khouribga region about 12 Km south-west of Khouribga city (Figure 1a). The climate of the phosphate plateau is essentially arid. Rainfall is from November to May and is usually below 400 mm. Vegetation is of sparse dwarf palm trees. This sampling site consists of phosphate reserve deposit made up of 3 major phosphatic layers (C1, C2 and C3; Figure 1b). These layers were sampled, according to the accessibility, in aseptic conditions using sterile bags at six different positions (P1, P2, P3, P4, P5 and P6), to have at least 9 composite samples. They were then transported to our laboratory at a temperature of 4°C where they were sieved under sterile conditions (2 mm nominal pore size) and analyzed within the following 48h.

Isolation of Bacterial strains

Approximately ten grams of the sieved phosphate sample were placed into a sterile tared 250 mL Erlenmeyer flask. The flask was weighed, and the sample weight was

calculated. A volume of sterile physiological water (9g/L NaCl) equivalent to nine times the sample weight was added, and the flask was shaken for 30 min. This solution was then decimally diluted in sterile physiological water (10^{-1} to 10^{-5}). Aliquots of the resulting solutions were plated in Petri dishes, on the Yeast Starch Agar medium (YSA). It contained 10g/L Soluble starch, 4g/L yeast extract, 0,5g/L K_2HPO_4, 0,5g/L $MgSO_4$ x $7H_2O$ (Cooney & Emerson, 1964). This medium was shown to give high bacterial numbers from phosphate sample in our previous preliminary studies (not published data). After incubation at 30°C for up to ten days, the bacterial strains were identified and isolated according to the phenotypic characteristics described by Prescott *et al.* (2002). The individual isolates were purified at least by three successive plating and the resulting isolates were transferred to nutrient Agar, incubated at 37°C for 24 to 48 hours, stored on nutrient broth and frozen at -22°C with 15% v/v glycerol.

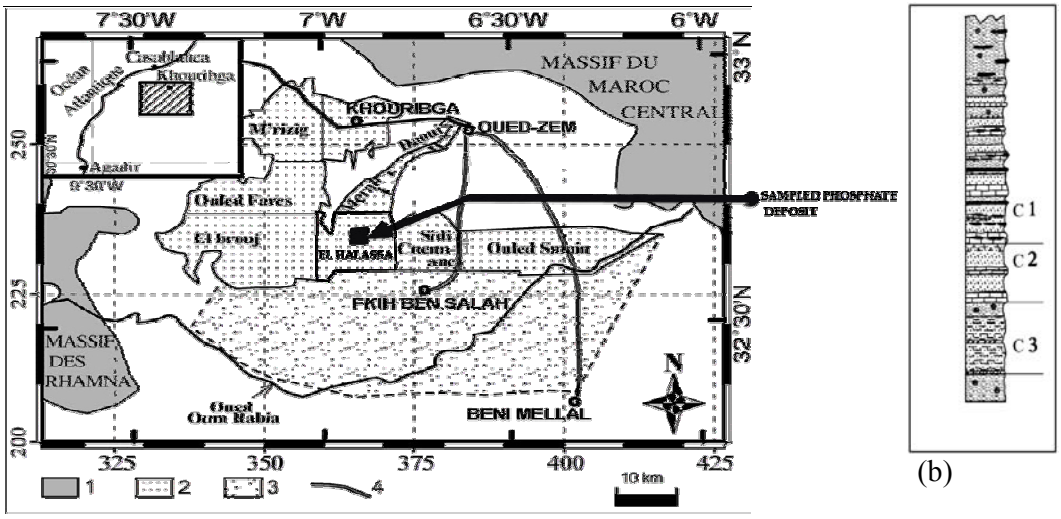

Figure 1. Presentation of the studied area: (a): Geographic location of the studied area in the sedimentary basin of Oulad Abdoun; (b): a section showing the position of the sampled phosphatic layers. 1: Hercynian massif. 2: Phosphatic area. 3: Probable extension of phosphatic mineralization. 4: Roads

Morphological and biochemical characterization

The growth medium Agar was inoculated with the individual isolates in Petri dishes to narrow down the microbial suspects. It distinguishes bacteria from one another based on its form, elevation, margin, pigmentation and size. The morphology of individual cells has been studied with the light microscope (x100) to define the motility, the bacterial cell shapes and arrangements. Gram staining was made with young bacterial cells following the well known procedure (Beveridge, 2001).

Pure cultures of the bacterial strains were subjected to standard biochemical plate and tube tests according to the procedures of Smibert & Krieg (1981). The oxidase test was performed according to Kovacs (1956) and the catalase was revealed using H_2O_2 10% (v/v). The Voges-Proskaur reaction, methyl red and the bacterial growth on both Simmons citrate agar and mannitol motility medium were performed according to Skerman (1967). The beef liver was used to distinguish aerobic and anaerobic bacteria, and peptone water allowed the detection of the indole production. We used the

Kligker-Hajna agar to figure out the ability to ferment lactose and Glucose with or without gas production.

The Mossel agar medium was used for the detection and enumeration of *Bacillus cereus*. It inhibits almost all contaminating micro-flora in order to favor the growth of this gram positive bacillus. Likewise, the Chapman agar medium which is a selective medium for the isolation of staphylococci was used to identify the *staphylococcus* genus.

The API 20E biochemical gallery test (BioMérieux) was performed following the manufacturer's instructions, by using bacteria as inocula suspended in 5ml of 0,85% sterile physiological water. After 18h to 24h incubation at 37°C reagents were added and the seven-digit profile number generated.

The bacteria were identified following the schemes of Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984 ; Sneath *et al.*, 1986) and the API analytical profile index.

Numerical taxonomic analysis

The data were converted into a binary format and analysed using the Jaccard coefficient (Sj) (Sneath, 1957). Simple matching similarity coefficients were used to generate the similarity matrix and dendrogram was generated by the Lance and Williams flexible method based on the unweighted pair group mean averaging (UPGMA) (Lance & Williams, 1967) using the StatistiXL for MS Excel software.

Results and discussion

This paper aimed to describe indigenous bacterial strains isolated from Moroccan phosphate deposit samples. Table 1 summarized some properties of the studied samples. Geologically, the phosphate deposit starts by Maastrichtian phosphatic marls overlain by uncemented phosphatic layers and limestones containing many bony debris (Ghoubert & Salvan, 1949).

Palaeocene is mainly composed of uncemented phosphates; its submittal part corresponds to a level of limestones formed of coprolites and silex nodules (Salvan, 1960). Eocene corresponds to an alternation of uncemented phosphates levels, phosphatic marly limestones, discontinuous horizons of silex and silto-pelitic levels. The whole sequence is characterized by a hard rock structure. We can observe a low moisture content (from 2,17% to 18,07%) and low fine-grained fractions. The organic matter present in phosphate samples (from 1,99% to 3,26%) may originate from the marine environment and took place during the burying period (Khaddor *et al.*, 1997; Amit

Table 1. Physical properties and bacterial count of the studied phosphate deposit samples.

Samples		Amt (%) of [a]		Moisture [b] content %	pH[c]	%OM [d]	Bacterial count[e] (CFU x 10^5)
		Sand	Silt + Clay				
P1C3		88,89	11,1	14,21	7,42	2,89	0,57
P2	C1	96,26	3,68	18,07	7,91	2,42	1,7
	C2	96	3,8	11,25	7,55	2,84	2,07
	C3	85,05	6,11	16,62	6,97	2,86	1,41
P3	C2	97,87	2,17	5,44	7,49	2,48	0,97
	C3	92,49	6,82	10,99	7,42	3,02	0,57
P4C2		95,25	5,42	3,07	7,88	1,99	1,4
P5C3		91,42	8,72	7,94	8,02	3,26	2,46
P6C3		90,26	9,61	6,7	7,37	3,18	1,93

(a): Assessment of soil particle size by sieving to separate sand fractions and fine-grained fractions (silt + clay). (b): Moisture contents were determined by drying at 110°C for 48h. (c): pH was determined in a slurry (5 parts distilled water, 1 part sample). (d): Organic matter contents were determined by Loss-on-ignition at 430 °C as recommended by Davies (1974). (e): colony forming units counts for total bacteria on the Yeast Starch Agar medium.

& Bein, 1982). This organic matter shall be the basis of the biological activity in this environment.

A total of 75 bacterial strains were isolated from the phosphate deposit samples and examined in this study. In most cases, direct spread plates of the phosphate suspension samples were rapidly overgrown by non pigmented strains, the pigmented colonies usually appeared after several days of incubation. Results demonstrated relatively lower CFU counts of the total bacteria (ranged from 0,57 to 2,46x10^5 CFU/g) compared to the soil samples (ranged from 4x10^6 to 2x10^9 /g dry soil) (Whitman *et al.*, 1998). Hamdali *et al.* (2008a) censused highly number of bacteria in phosphatic soils from Benguerir (67,3 x10^5 CFU/g); Youssoufia (55,3 x10^5 CFU/g) and Khouribga (42,4 x10^5 CFU/g) compared to our study. This is most probably due to the samples collection as they have sampled the extracted rock phosphate stockpiles from the studied mines which can be contaminated with exogenous bacteria.

The isolated strains were subjected to a set of 40 phenotypic tests. As a result, the shared biochemical and physiological characteristics could be taken into consideration in our numerical analysis. A simplified dendrogram constructed on the basis of the results of our tests is shown in figure 2. The clustering by the Lance and Williams's flexible method yielded nine phena defined at the similarity level ranged from 75% to greater than 90% (Figure 2.). The Phena contained at least a total number of 26 strains among the 75 isolated strains. The phenon 1 was composed of 2 motile strains isolated from the samples P2C3 and P4C2. The strains showed an oxidative metabolism with the production of oxidase and catalase. They were able to hydrolyse gelatine and they demonstrated the ability to use the amygdaline as carbon and energy source. These characteristics and the API results matched the genus description of *Pseudomonas*. The phenon 2 was composed of 2 strains isolated from the samples P4C2 and P3C3. The strains

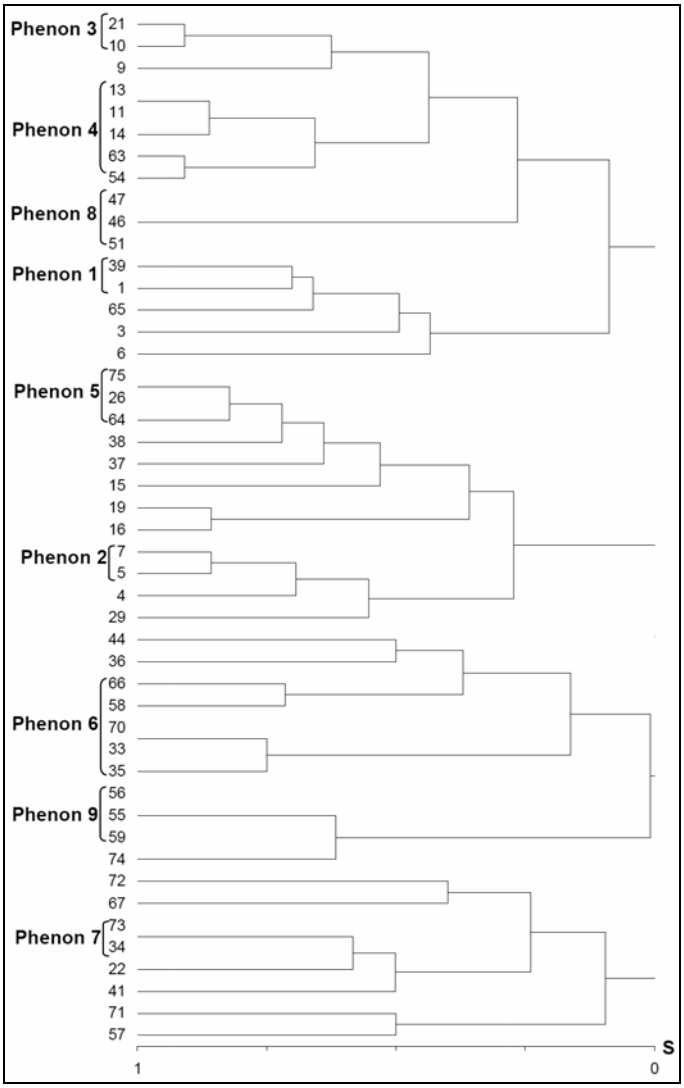

Figure 2. Dendrogram showing the isolated bacterial strains from the phosphate samples based on simple matching coefficients and clustering by the Lance and Williams's flexible method based on the unweighted pair group mean averaging (UPGMA). S, similitude value.

exhibited a fermentative and oxidative metabolism and they demonstrated the ability to use various compounds as carbon source. These trains were formally identified as *Leclercia adecarboxylata.* The phenon 3 contained also 2 strains isolated from the samples P3C2 and P3C3. The strains were gram negative facultative anaerobic rod showing oxidative and fermentative metabolism. The characteristics described in table 2 matched the genus description of *Aeromonas*. The phenon 4 was composed of 5 strains isolated from various samples (P6C3; P2C2; P3C2; P3C3). They are facultative anaerobic with the ability to ferment the glucose. The colonies were smooth, low convex with dark violet metallic sheen. They were identified as *chromobacterium violaceum*. The phenon 5 was composed of 3 strains. According to the characteristics of the table 2 they were identified as *Aeromonas caviae*. The phenon 6 contained 4 strains isolated from various samples. The strains were strictly aerobic, oxidase and catalase positive. They demonstrated the ability to use a few compounds as carbon and energy source. According to the characteristics of the table 2, they were identified as *Sphingomonas paucimobilis* and *Pasteurella* spp. The phenon 7 was composed of 2 strains isolated from the samples P2C3 and P5C3. The strains were gram negative aerobic rods, oxidase negative and they belong to the genus *Alcaligenes*. The phenon 8 contained 3 strains with a very high similarity level. They are Facultative anaerobic exhibiting a fermentative metabolism and oxidative metabolism. The strains demonstrated the ability to use the amygdalin as carbon and energy source and they were identified as *Aeromonas salmonicida* and *Pseudomonas pseudomallei.* The last cluster (phenon 9) was composed of 3 strains. They produced NO_2 and were able to use various compounds as carbon and energy source. They were identified as *Achromobacter* spp.

The characteristics of all phena and individual strains isolated in this study are presented in the table 2. We isolated two strains by the Mossel agar medium which are a Gram positive bacilli belonging to the *bacillus* genus. Three other Gram positive cocci did not grown in the Chapman agar medium and hence they remained unidentified using our identification scheme.

The main finding of the herein study lies on the identification of the bacterial strains isolated off our phosphate deposit samples. Their distribution is shown in the table 3. This distribution suggested unexpectedly high bacterial diversity in the studied deposit. Members of the *Aeromonas* genus have been isolated from many phosphate samples in our study. This genus is known to be an ubiquitous aquatic bacterium that causes serious infections in both cold- and warm-blooded animals, including humans (Holt *et al.*, 1994; Kühn *et al.*, 1997; Sha *et al.*, 2002). Nevertheless, some authors reported the isolation of *Aeromonas* from soil samples (Ajaz *et al.*, 2005), and also from archeological sites (Southern, 2008). The adaptation to terrestrial conditions is likely to have provided the driving force for *Aeromonas* species to invade such environment. In fact the phosphate deposits of the Khouribga basin is a complex of warm and shallow-marine platforms characterized by intense phosphatic sedimentation along the southern margin of the Tethys during the Late Cretaceous and Early Palaeogene (Lucas & Prévôt-Lucas, 1996). Furthermore, this phosphate basin is characterized by its richness in marine vertebrate, marine and littoral reptiles and birds (Arambourg, 1952). Further studies are recommended in order to elucidate local conditions of oxygen, organic matter and nutriments.

Table 4 presented the metabolism diversity of the identified strains isolated from the phosphatic layers. Among the metabolism pathways, most of the

Table 2. Summarized morphological and physiological features of the phena and individual strains isolated from the phosphate samples

	Phena and Strains																														
	Phenon 1	**3**	**4**	**Phenon 2**	**6**	**9**	**Phenon 3**	**Phenon 4**	**15**	**16**	**19**	**22**	**Phenon 5**	**29**	**Phenon 6**	**Phenon 7**	**35**	**36**	**37**	**38**	**65**	**41**	**44**	**Phenon 8**	**Phenon 9**	**57**	**67**	**71**	**72**	**74**
Phenotypic Characteristics																															
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pigmentation[a]	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	P	NP	Y	NP	NP	Y	NP	NP	NP	NP	NP	Y	Pu	NP	Y	NP
Motility	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Physiological Characteristics																															
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Oxidase	+	+	+	±	-	+	+	±	-	+	+	-	±	-	+	±	-	+	-	-	+	-	+	+	+	-	+	-	-	+
ONPG	-	-	+	+	-	+	+	-	+	+	+	+	+	+	±	-	-	-	+	+	-	+	-	-	-	-	-	+	+	-
O_2 utilization[b]	a	a	a	aan	a	a	aan	aan	a	aan	aan	a	a	a	a	a	a	a	a	a	a	a	a	aan	a	a	a	a	a	a
ADH	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-
LDC	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
URE	-	-	-	-	+	-	-	-	+	+	-	-	-	+	±	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IND	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	-	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-
GEL	+	-	-	+	+	+	+	+	-	-	+	-	+	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+	-	-
GLU fermentation	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
LAC fermentation	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_2	-	+	-	±	-	+	±	+	+	+	+	-	+	-	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-	+
N_2		-	-	-	-	-	-	-	-	-	-		-		-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Carbon substrates utilization																														
GLU	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	-
MAN	-	-	+	+	-	-	-	-	+	+	+	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
SAC	±	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
MEL	-	-	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+
AMY	+	+	+	+	+	-	-	±	-	+	+	-	+	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-
ARA	-	-	+	+	-	-	-	-	+	+	+	-	+	+	±	-	-	+	-	+	-	-	-	-	+	-	-	-	-	+
CIT	±	-	-	-	+	-	-	±	-	+	+	-	±	-	-	-	-	+	-	-	+	-	+	-	+	-	+	-	-	+

[a]colonial pigmentation: NP, non pigmented ; P, Pink ; Y, Yellow ; Pu, Purple. [b]Oxygen requirement: a, Aerobic ; aan, facultative anaerobic. ONPG, o-nitrophenyl-□-D-galactosidase ; ADH, arginine dihydrolase ; LDC, lysine decarboxylase ; TDA, Tryptophan deaminase ; IND, indole production ; VP, acetoin production ; GEL, gelatinase ; GLU, glucose ; LAC, Lactose ; MAN, Mannitol ; INO, Inositol ; SOR, Sorbitol ; RHA, rhamnose ; SAC, Sucrose ; MEL, Melbiose ; AMY, Amygdalin ; ARA, Arabinose ; CIT, Citrate

Table 3. Origin of the bacterial strains identified and their distribution.

Samples		Genus and species identified
P1C3		*Aeromonas hydrophila, A. caviae, Yersinia pseudotuberculosis.*
P2	**C1**	*Pseudomonas* spp., *P. aeroginosa, P. fluorescens, P. putida, Sphingomonas paucimobilis, Alcaligenes, Enterobacter agglomerans, Moraxella* spp., *Serratia plymuthica, S. rubidaea, Erwinia, Acinetobacter* spp., *Flavobacterium oryzihabitans, Chrysomonas luteola.*
	C2	*Aeromonas salmonicida, Pseudomonas pseudomallei, Chromobacterium violaceum.*
	C3	*Aeromonas salmonicida, Pseudomonas* spp., *Pseudomonas fluorescens, P. putida, P. aeroginosa, P. pseudomallei, Sphingomonas paucimobilis.*
P3	**C2**	*Aeromonas sobria, A. hydrophila, A. caviae, Yersinia enterolitica, Y. aldovae, Enterobacter agglomerans, E. amnigenus, E. cloacae, E. sakazakii, gram+Cocci, Bacillus, Flavobacterium menigosepticum, Vibrio hollisae.*
	C3	*Aeromonas sobria, A. hydrophila, A. caviae, Pseudomonas cepacia, Acinetobacter, Leclercia adecarboxylata, Chromobacterium violaceum, gram+cocci.*
P4C2		*Pseudomonas* spp., *P. fluorescens, P. putida, P. capacia, Enterobacter agglomerens, Erwinia, Leclercia adecarboxylata.*
P5C3		*Aeromonas hydrophila, A. caviae, Pseudomonas* spp., *P. fluorescens, P. putida, P. aeroginosa, Sphingomonas paucimobilis, Alcaligenes* spp., *Yersinia pestis, Moraxella* spp., *Achromobacter* spp., *Sphingomonas multivorum, Shigella* spp.
P6C3		*Aeromonas caviae, A. hydrophila, Pseudomonas* spp., *P. capacia, Sphingomonas paucimobilis, Achromobacter* spp., *Chromobacterium violaceum.*

identified bacteria are chemoorganotrophic expect *Alcaligenes* which can be chemolithotroph. The isolates were largely aerobic and facultative anaerobic probably respire and/or ferment the organic matter present in the phosphate deposit.

To the best of our knowledge, there is no similar study in the literature to date. Therefore, our results were in accordance with the environment nature of the phosphate deposit which is porous, confined environment characterized by the presence of mineral bodies and little organic materials (Benalioulhaj & Trichet, 1992). In fact, *Acinetobacter* spp., identified in the sample P2C1, can accumulate inorganic polyphosphate in the aerobic condition (Kornberg, 1995). This specie can also release phosphate in the environment simultaneously to the degradation of the accumulated polyphosphates under the anaerobic conditions (Dick *et al.*, 1995; Macaskie & Dick, 1993).

On the other hand, the bacterial diversity demonstrated in this study, is involved in numerous ecological processes. Indeed, some of the identified bacterial strains demonstrated capacity to solubilise inorganic phosphate. Kim *et al.* (1997) showed that *Enterobacter agglomerans* was significantly able to solubilise the hydroxyapatite and to hydrolyse the organic phosphorus. Other strains belonging to the genus *Bacillus*, *Flavobacterium*, *Chromobacterium*, *Enterobacter* and *Pseudomonas* are shown to be able to solubilise phosphate by releasing organic acids (Sulliasih & Widawati, 2005; Guang-Can *et al.*, 2008). Furthermore, some of the species identified in our phosphate samples seem to intervene in the bioremediation process. *Leclercia adecarboxylata*, formally identified in the samples P4C2 and P3C3, exhibited a great ability in the degradation of polycyclic aromatic hydrocarbons (Sarma *et al.*, 2004). Other strains of *Pseudomonas* spp. were able to grow just by using TNT (Trinitritoluene), 2,4-dinitrotoluene and 2-nitrotoluene as sole nitrogen source (Duque *et al.*, 1993). The ability of these strains to use the toluene can be involved in the depollution of soil contaminated with such compounds. Strains belonging to *Pseudomonas* and

Table 4. The metabolic diversity of the bacteria isolated from phosphate samples based on the Bergey's Manual of Systematic Bacteriology.

Genus and species	Metabolism basis
Pseudomonas spp., *P. fluorescens, P. putida, P. cepacia; P. aeroginosa; P. Pseudomallei.*	Strict aerobes, chemoorganotrophs
Erwinia	facultative anaerobes, with glucose fermentation
Enterobacter, E. agglomerans; E. colacae, E. amnigenus, E. Sakazakii.	facultative anaerobes, with glucose fermentation
Leclercia, L. Adecarboxylata.	Facultative anaerobe, chemoorganotroph, metabolism based on respiration and fermentation
Acinetobacter spp.	Strict aerobe, metabolism strictly by respiration
Aeromonas, A. sobria; A. hydrophila; A. caviae; A. salmonicida.	Facultative anaerobes, chemoorganotrophs
Chromobacterium, C. violaceum.	Facultative anaerobe, with glucose fermentation
Yersinia, Y. enterolitica, Y. aldovae; Y. pseudotuberculosis, Y. pestis.	Facultative anaerobes, chemoorganotrophs, metabolism based on respiration and fermentation
Flavobacterium, F. meningosepticum; F. oryzihabitans.	Strict aerobes, metabolism based on respiration
Sphingomonas, S. paucimobilis.	aerobe,metabolism strictly by respiration
Alcaligenes spp.	Aerobe, generating energy in a number of ways, can be chemolithotrophs
Serratia, S. plymuthica; S. rubidae.	Facultative anaerobes, anaerobic reduction of nitrate and chlorate
Achromobacter spp.	Obligatory aerobe, chemoorganotroph non fermenter
Sphingobacterium, S. multivorum.	Strict aerobe, Chemoorganotroph, metabolism based exclusively on respiration
Shigella spp.	Facultative aerobe

Moraxella genus were also involved in the denitrification process in the presence of various aromatic compounds as sole carbon source (Taylor *et al.*, 1970; Williams & Evans, 1975). The above examples were cited in order to point out the call of further deepen specific studies.

Even though this study is biased, these biases are commonly recognized by the culture-dependant based studies of the bacterial diversity (Hill *et al.*, 2000; Zvyagintsev *et al.*, 2002). Hence, this study can led us to further research based on the new technology approaches for the study of microbial diversity in the phosphate deposit.

Conclusion

This basic microbiological study is the first report of the bacterial diversity in the Moroccan phosphatic layers belonging to the EL HALASSA deposit. Identification of the bacterial strains isolated from our samples displayed an unexpected diversity composed of various genus such us *Pseudomonas, Aeromonas, Enterobacter, Alcaligenes* ….

Based on the Bergey's Manual of Systematic Bacteriology these identified bacteria used various metabolic pathways, they are generally aerobic and/or facultative anaerobic with oxidation and/or fermentation of the organic matter present in the phosphatic layers. The mineral species present in this mining environment can also be an energy source for the chemolithotrophic bacteria such as *Alcaligenes.*

In this study, we can not concluded any specific strains related to a given phosphate layer, since the 3 major phosphatic layers C1, C2 and C3 was sampled at different positions. Hence, we suggest increasing the samples number and thus we can certify the real distribution of bacterial strains in the phosphatic layers. Compared to studies, which investigate the soil bacterial diversity, the identified bacteria presented a great ecological interest. Their metabolic pathways let them

to grow in a mining environment where the organic matter and other carbon sources are limited. Furthermore, some of the identified bacterial strains in this study can solubilise inorganic phosphate; others seem to intervene in the bioremediation process.

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This study gave an appreciation of the bacterial diversity in the Moroccan phosphate deposit and can led to further research proposals that will adopt polyphasic approach in the investigation of the overall microbial diversity.

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Carboxymethyl cellulase Production by Moroccan *Bacillus* Isolates

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Abstract

Bacteria producing carboxymethylcellulase (CMCase) are isolated from different Moroccan ecosystems. Molecular identification showed that these strains belong to Bacillus genera (*Bacillus licheniformis*, *B. subtilis* and *Bacillus* spp.). Furthermore, the cellulase production was studied according to pH, temperature, incubation time and the source of carbon. Thus, at 60 ° C the enzyme activity was approximately 50% compared to that determined at 37 ° C. The optimal pH was at 7.0 for *Bacillus licheniformis* and *B. subtilis* and 6.0 for *Bacillus* spp. In addition, this activity was of 73%, 75% and 66% for *Bacillus licheniformis*, *B. subtilis* and *Bacillus* spp. respectively at pH 8.0, compared to that determined at pH 7.0. Finally, the carboxymethyl cellulose (CMC) was more assimilated than cellulose (avicell) by the three isolates of Bacillus.

Abreviations: CMC : Carboxymethylcellulose. CMCase **:** Carboxymethylcellulase. UI : International Unit.

Keywords: *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus* spp., Carboxymethylcellulase (CMCase).

Introduction

Cellulose is the most abundant renewable natural product in the biosphere (Whitaker, 1990; Solomon *et al.*, 1997).This polymer of β-1,4- linked glucose units, is a major polysaccharide constituent of plant cell walls. Therefore, it has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon source. The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulases; endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) (Yi *et al.*, 1999).

Carboxymethyl cellulose (CMC) is one of the most important water-soluble derivatives of cellulose that is formed by its reaction with sodium hydroxide and chloroacetic acid (Togrul & Arslan, 2003; Biswal *et al.*, 2004). Among all the polysaccharides, CMC is easily available and is widely used in many industrial sectors such as food, textiles, paper, adhesives, paints, pharmaceutics, cosmetics, detergents, mineral processing and oil well drilling operation (Biswal *et al.*, 2004; Wang & Somasundaran, 2005).

Since biomass is abundant and reasonably inexpensive, the key to its successful commercialization is the development of efficient and economical conversion methods such as enzymatic hydrolysis.

Cellulase production is influenced by a lot of factors including the type of strain used, the culture's conditions and substrate's types (Lynd *et al.*, 2002).The relationship between these variables has a marked effect on the ultimate production of the cellulase

enzyme complex (Szetela *et al.*, 1981). In this context, the first step in the present report was, to select microorganisms over producing cellulase. Then some properties such as optimum temperature and pH, effect of substrate and cellulolytic activity of isolates were investigated with a perspective to identify potential sources of industrial enzymes.

Materials and methods

Isolation of strains

Several samples (soil, water, …) were collected from different Moroccan ecological niches. These samples were treated independently as follows: soil Samples were dissolved in 36 ml of sterile physiological water and shaken for 2 h. The supernatant was then recovered and various dilutions (10^{-1} to 10^{-7}) were realised. Then, an aliquot from each dilution was inoculated on LB, YPG and Malt extract agar medium.

Plate Screening

For plate screening, Caboxymethylcellulose-Agar (CMC-Agar) medium was used. This medium consist of: 1.00% (w/v) CMC, 0.65% (w/v) NaNO3, 0.65% (w/v) K2HPO4, 0.03% (w/v) Yeast extract, 0.65% (w/v) KCl, 0.3% (w/v) MgSO4, 0.065% (w/v) glucose, 1.7% (w/v) agar (18) . Plates inoculated with isolated strains were incubated at 37ºC for two days. For cellulolytic activity observations, plates were stained with 1% Congo red dye for 0.5-1 h followed by destaining with 1 M NaCl solution for 15-20 min.

Preparation of the extract

At the end of the incubation period, three strains were separately picked from the culture medium and centrifuged at 4000 rev/min for 20 min at 4°C. The cell-free supernatant was considered as the crude enzyme and was analysed for its protein content and enzymatic activity.

Enzyme assays

Carboxymethyl cellulase (CMCase) activity was assayed in a reactional mixture (0.5 ml) containing 1% (w/v) CMC solution, 50mM acetate buffer, pH 5.0, and appropriately diluted enzyme solution. After 30 min incubation at 50 ◦C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method (Miller, 1959). One unit (U) of CMCase activity is defined as the amount of enzyme which produces 1 µmole reducing sugar as glucose per min in the reaction mixture under the specified conditions.

Determination of cells proteins content

Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Specific activity = Activity (U/ml) /Protein mg/ml

Effect of temperature and pH on CMCase activity

The optimal temperature for the hydrolysis activity of carboxymethylcellulose (CMC) was determined by measuring this activity at different temperatures ranging from 37 to 60 °C.

The influence of pH on the enzyme activity was determined by measuring this activity at different pH values ranging from 4 to 8.

Effect of carbon sources on cellulase production

Two carbon sources: CMC and avicell (Sigma-Aldrich) were evaluated for their effect on cellulose production.

Identification of the bacterial strains

To identify the bacterial isolate, a molecular approach based on the amplification and sequencing of the 16S rRNA gene was used. This methodology is currently the most used for bacterial

phylogeny (Woese *et al.*, 1990). For PCR amplification, universal primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RS16 (5'-TACGGCTACCT TGTTACG AC TT- 3') were used to amplify the 16S rDNA (Weisberg *et al.*, 1991). The amplification reaction was performed in a final volume of 50 µl containing 50 µmol of each primer, 200 µM each dNTP, 0.5 units Taq DNA polymerase and 3 µl of DNA sample in 1x Taq polymerase buffer.

The mixture was first denatured at 94 °C for 5 min. Then, 35 cycles of PCR were performed by denaturation at 94 °C for 30 s, primers annealing at 55 °C for 45 s, and primer extension at 72 °C for 90 s. At the end of the last cycle, the mixture was incubated at 72 °C for 10 min.

Results and discussions

Isolation, screening and molecular identification of strains

85 microorganism's were isolated and purified from the thermal station Moulay Yacoub (water, soil, …).Water of Moulay Yacoub is very clear, has a strong smell (hydrogen sulfide), and is highly salty and sulphurous. The selection of the three most producing cellulase was based on the diameter of the clearing zone surrounding the small well on the plate where the strains were screened (Table 1). The table 1 shows the relative index of enzymatic activity of the isolates, which was recorded as clear zone ratios = clear zone diameter / colony diameter (Bradner *et al.*, 1999). This zone on the CMC agar plates was indicated as clear orange halos after staining with 1% Congo red solution. Subsequently, the molecular identification was realised, the result showed that the strains exhibiting the high CMCase activity correspond to *Bacillus licheniformis*, *B. subtilis* and *Bacillus* spp. These genera are known to produce CMCase activity (Grau *et al.*, 1961; Baird *et al.*, 1990).

CMCase activity was measured during bacterial growth in the modified medium, in which the enzymatic activity increased simultaneously with incubation time during the first 96h. Hence, the maximal activity occurred in the late exponential growth phase, following 3 or 4 days of cultivation depending on the isolate (roughly 0.4 UI). Figure 1 demonstrates that the maximal CMCase activity was recorded over a period of 72 h as measured by the ability of the crude enzyme to degrade CMC. CMCase activity was 0.38 UI for *Bacillus subtilis*, 0.35 UI for *Bacillus* spp. and 0.40 UI for *B. licheniformis*, which represent the highest level of production, observed after 72 h of incubation. It can be also observed that cultivation beyond five days dramatically reduced CMCase activity in all isolates. These results corroborated with several works (Robson & Chambliss, 1984; Kawai *et al.*, 1988). They authors show that the maximal production in other *Bacillus* strains is in general only achieved after 2 or 3 days.

Table 1. Relative index of enzymatic activity of the three isolates and the biotope of their isolation.

Isolates	Biotope	Relative index of enzymatic activity (mm)
Bacillus licheniformis	Water	20
Bacillus subtilis	Water	8
Bacillus spp.	Soil	18
Erwinia chrysanthemi strain 3937 VIII	(LCB-CNRS-Marseille-FRANCE)	6

Enzyme specific activity for the three studied isolates is shown in figure 2, and compared with the one in *Erwinia Chrysanthemi*. Results showed that in optimized conditions, high enzyme specific activity of *Bacillus licheniformis* (7.8 U/mg)

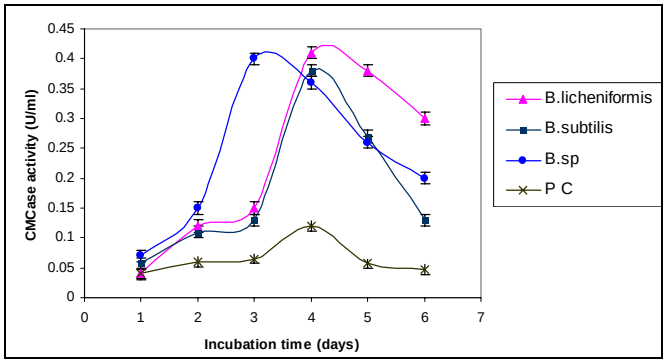

Figure 1. Kinetic of CMCase production for the three strains studied (*Bacillus* spp., *B. subtilis* and *B. licheniformis*) and *Erwinia chrysanthemi (P.C).* The maximum of CMCase activity was 0.40 UI for strains *B. licheniformis* and 0.38 UI for *B. subtilis* after 72 h of incubation.

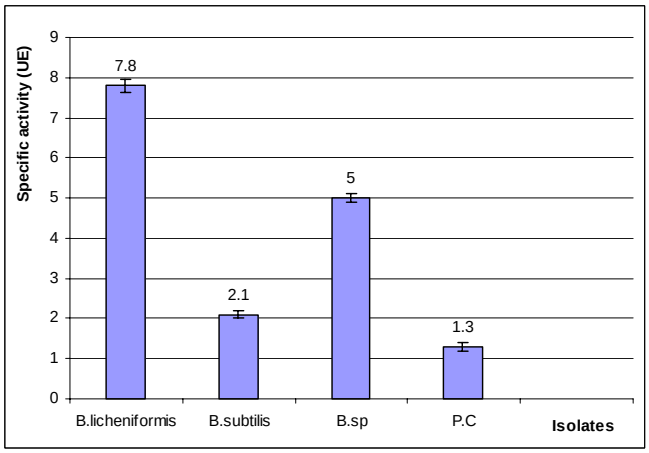

Figure 2. CMCase specific activity for three isolates and *Erwinia chrysanthemi (P.C).*The maximum enzyme specific activity (7.8 U/mg) for *Bacillus licheniformis* was observed after 72h of incubation.

and *Bacillus* spp. (5 U/mg) indicate that these isolates are over producers of CMCase.

Effect of temperature and pH on CMCase activity

Different microorganisms vary in their optimal incubation temperature, medium pH and incubation period for production of hydrolytic enzymes (Habb *et al.*, 1990). The effect of temperature on the activity of crude cellulases was determined at various temperatures ranging from 37 °C to 60 °C at pH 7.0 (Figure 3). This activity is inversely proportional to temperature because the enzymes are inactivated by high temperature. The crude enzyme preparation hydrolyzed CMC and was active over a broad temperature range, from 37°C to 60°C.

The enzyme showed a good activity between 45°C to 50 °C for the three species of Bacillus studied. The enzyme retained 51%, 52% and 50% activity for *Bacillus licheniformis*, *B. subtilis* and *Bacillus* spp. respectively at 60°C. Although the physiological changes induced by high temperatures during enzyme production are not very clear, it has been suggested that at high temperatures, microorganisms may synthesize reduced number of proteins that are probably essential for growth and other physiological processes including enzyme production (Gawade *et al.*, 1999).

On the other hand, studies of the effect of the buffer pH on enzyme catalyzed reactions are essential because hydrogen concentration in the reaction system affects the ionisation groups of the enzyme and influences the ionisation state of the substrate. For effective interaction between the substrate and enzyme, the ionisable groups of both the substrate and the active site of the enzyme must be in a suitable conformation. The crude enzyme preparation hydrolyzed CMC and was active over a broad pH range, from 4 to 9; the maximum activity was observed over a very wide pH range (Figure 4). The enzyme of the isolate *Bacillus licheniformis* shows two major activity peaks at pH 5.0 and 7.0. This result is probably due to the presence of two isoenzymes or subunits in the

enzyme preparation. For *Bacillus subtilis*,

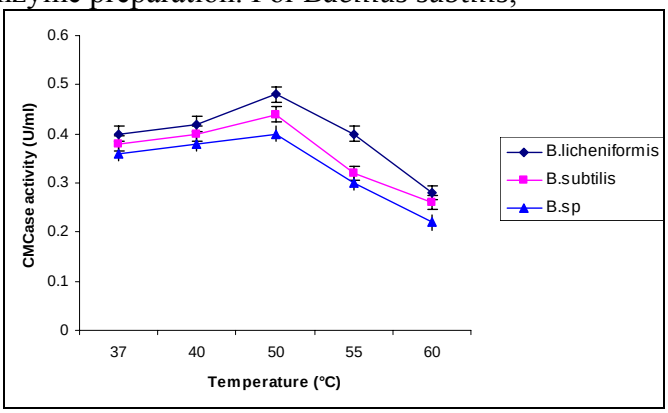

Figure 3. The optimal temperature range of CMCase. The crude enzyme preparation hydrolyzed CMC and was active over a broad temperature range, from 37°C to 60°C; the maximum activity was observed at 50°C for almost strains.

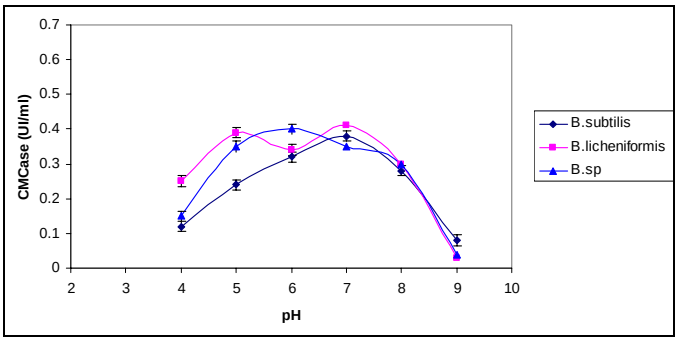

Figure 4. Effect of pH on CMCase activity. The crude enzyme preparation hydrolyzed CMC and was active over a broad pH range, from 4 to 9; the maximum activity was observed over a very wide pH range.

CMCase shows its optimum activity at pH 7.0 and its activity fell to 68% when pH ranged from 7.0 to 8.0. The enzyme retained 73%, 75% and 66% activity for *Bacillus licheniformis*, *B. subtilis* and *Bacillus* spp. respectively at pH 8.0. Only *Bacillus sp* shows its optimum activity at pH 6.0. However in pH 4.0 the enzyme remained partially active. In a previous study, it has been shown that the CMCase in *Bacillus* spp. is alkaline, which make it suitable for use as an effective laundry detergent additive (Khyami-Horani *et al.*, 1994).

Effect of carbon source on Cellulase production

The substrate specificity of the crude cellulase was determined by performing the enzyme assay with two different substrates (Figure 5). Cultures were

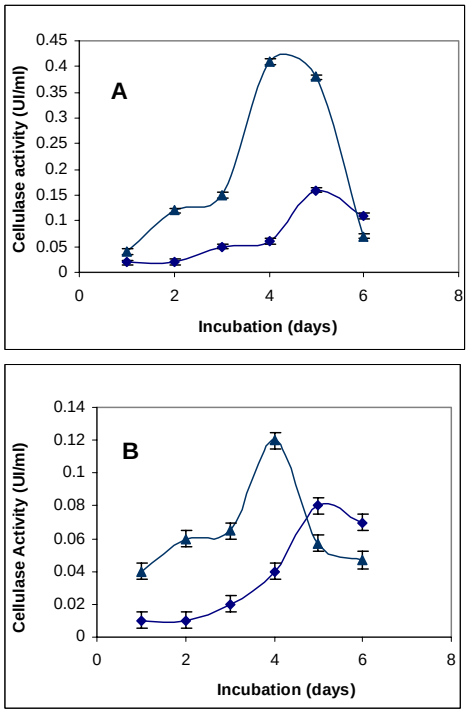

Figure 5. Cellulase of *Bacillus licheniformis* (A) and *Erwinia chrysanthemi* (B) grown on medium containing CMC (▲) Avicell (■) (the results represent the average of two assays).

grown in CMC medium (pH 7.0) containing 1% (w/v) of various carbon sources at 37°C for a period of 144 h for both *Bacillus licheniformis* and *Erwinia chrysanthemi* strains. The crude cellulase degraded avicell and CMC. The rate of CMC degradation was higher than avicell in this study. Maximum activity for the *Bacillus licheniformis* (0.41 UI) was detected in cultures that contained 1% (w/v) CMC as the growth carbon source after 72h of incubation; however, in the medium containing avicell as carbon source the maximum activity was 0.12 UI after 96h of incubation. These observations

suggest that the cellulase produced by our isolates may be used in processes operated at moderate temperatures and pH.

Cellulase amounts that were produced in other studies were different, probably because of the influence of the substrate (carbon source) on the growth of cellulolytic organisms (Mandels & Reese, 1985; Zhu *et al.*, 1988; Lakshmikant & Mathur, 1990). These observations suggest that the cellulase produced by our isolates may be used in processes operated at moderate temperatures and pH which may include preparation of baked cereal food products, saccharification of agro-residues and clarification of fruit juices.

In the perspective of this study, the cellulase from the most producer isolate will be used in extraction of fruit oil. Moreover, purification and characterisation of this enzyme would open the way for further industrial investigations

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Prevalence and Levels of Specific Ig-E to white egg's, gliadin's and peanut's proteins among Moroccan children in Fez-Meknes region

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Abstract

The aim of the present study was to estimate the prevalence of allergy and concentration of allergen-specific immunoglobulin E (IgE) among Moroccan children in Fez-Meknes region. Patients have been recruited from hospital university Centre of Fez and Meknes hospitals. All of them have completed a questionnaire before taking blood samples. These later were used to measure total and allergen specific IgE concentrations to proteins of egg's white (EWP), peanuts (PP) and gliadins. The mean age of students participating in the study was 7.3 years (1 month to 15 years). Children under 7 years represent 50.6% and adolescents (> 10 years) 33.3%. Evaluation of total IgE indicates an average of 39.8 UI/ml with 3.2 % higher than 80 UI/ml and 39.7% were between 30 and 80 UI/ml. Allergen-specific IgE measurement indicates more positive values for gliadins (46.9% up to 2UI/ml) than egg's white (29.6%) and peanut's proteins (22.2%). According to predictive values published by Sampson (2001), prevalence of allergy indicates that 14.3%of children were allergic to egg's white proteins, 4.1% were allergic to gliadins and 2.7% allergic to peanut's proteins.

Key words: Food allergy, children, egg's white, gliadins, peanuts, specific IgE

Introduction

Food allergy corresponds to clinical manifestations related to sensitization to food proteins, most often IgE mediated. The determination of IgE is a valuable tool for the diagnosis of patients with established or suspected allergic diseases. Clinical expression of food allergy is very varied such as eczema, asthma, rhinitis, urticaria, and choc anaphylactic potentially leading to death.

Number of studies has shown that food allergy is taking an important part in common life. Numerous allergists believe that the prevalence of food allergy is rising (Bhombal *et al.*, 2006). Many reasons are behind the propagation of the of food allergy in industrialized societies, for instance food diversification and the evolution of related technologies. Allergy is considered as a very important public health problem and is estimated by WHO as the fourth disease in the world. A remarkable increase of food allergy is actually full established. The prevalence of food allergy in the general population is estimated between 6 and 8% for children (Sampson, 1997; Dutau, 2003). Egg and peanut allergy is the most common IgE- mediated food allergies for children (Eggesbo *et al.*, 2001; Sampson, 2004).

This study assessed the presence of serum IgE specific to food allergens (egg's white, gliadins and peanuts) for children. Our objective is to evaluate food allergy for children in the region of Fez-Meknes. Then, we will determinate the main foods components that are concerned by allergy. This subject hasn't been much explored by researchers, because of the lack of numerous epidemiologic data, particularly in Arab countries.

Materiel and methods

Patients

The study material comprised 81 infants. The subjects were recruited from the Hospital-University Center of Fez (HUCF) and from Meknes hospitals. As part of the study protocol, a questionnaire was performed. Subjects were asked whether they had allergic reactions to food, and if so, the type of reaction was recorded in detail for ten possible food allergens. Then sera were collected for IgE determination.

Gliadin extraction

For gliadin extraction, wheat flour (100 mg) was sequentially extracted according to a modified Osborne procedure (Lokhart & Bean, 1995). The albumin was extracted from flour firstly with deionized water. Then globulin was separated from pellet by 0.5 M of NaCl. Finally, gliadins were isolated from restant pellet after solubilisation in 70% aqueous ethanol. For every extraction, mixture was vortexed every 10 min during 30 min then centrifuged 5 min at 2000 rpm. This operation was repeated three times for the three proteins. Supernatant was pooled, diluted at 1:10 in PBS buffer and stored at –20°C until use.

Egg's white extraction

Whole chicken egg's white was homogenized by stirring for 5 min, then suspended at 1:10 in NaCl 0.9% before centrifugation to eliminate insoluble data. Supernatant was then diluted at 1:5 in NaCl 0.9% and stored at –20°C.

Peanut's proteins extraction

Proteins of peanuts were extracted according to Brown method (Brown, 1944) using chloroform for defatting. Once this preparation filtered, the powder is dried at 50°C for one hour. Then this defatted powder is mixed with 0.5 M NaCl in PBS and centrifuged 15 min at 3000 rpm. Finally, the supernatant was filtered and stored at –20°C until use.

Determination of serum IgE

The serum total IgE concentration and specific IgE were measured for gliadins, egg's white proteins, and peanut's proteins. Total serum IgE was deteminated by ELISA. 100µl per well of sera were deposite on 96 microplates then incubated for overnight at 4°C. After washing, 300µl of 0.25% Bovine serum albumin were added to every well before incubation with anti-IgE peroxydase conjugate for 60 min at 37°C. Anti-IgE fixed was revealed by adding for 15 min 50µl of 0.05% OPD (O-Phenylenediamine). Reaction was stopped by adding 50µl of 50mM HCl. Then, absorbance was measured at 450 nm.

Specific IgE was determinated using same procedure for total IgE except that microplates were precoated with specific food proteins. Precoating was performed by adding 100µl of 1mg/ml of food proteins per well and incubating microplates overnight at 4°C. Micrplates were then washed and stored at –20°C until use.

Specific IgE has been measured without previous patient sensitization or oral challenge.

Ethics

This study and patients recruitment was approved by ethical committee of Hospital-University Center of Fez.

Results

This study comprised thirty-four children (37 boys, 44 girls) recruited from the Hospital-University Center of Fez and from Meknes hospitals. Forty-seven were from Meknes, three girls and one boy were from Taounate, one boy from Taza and the rest from Fez city. Only one girl presented urticaria without any reported allergy. Referring to questionnaire, children and their parents didn't report any food allergy.

Patients ranged in age from 1 month to 15 years with a median age of 7.3 years (Table 1) and a sex ratio of 0.84 (Male/female). Children under 7 years represented 50.6% (n=41). Adolescents (>10 years) represented 33.3% with a median age of 12.5 years.

Sera were analyzed for total IgE and food allergen–specific IgE antibodies. Sera of thirty-one children evaluated for total IgE indicates an average of 39.8UI/ml. Values ranged from 2.5UI/ml to 128.8UI/ml. This higher value was recorded for a girl aged of three years. Thirty nine percent of total IgE values were between 30 and 80UI/ml. These high values were recorded for twelve boys and thirteen girls (Table 2).

For specific IgE values, Egg's white proteins represented the major allergen to which 29.6 % of children possess positive IgE levels. Eight children (five girls and three boys) represented IgE values up to 7 UI/ml and the higher value was recorded in a boy (8 years) serum with 49.2 UI/ml.

For other allergens, 46.9% of sera analyzed were positive for gliadins with two girls (9 and 15 years) and one boy (6 years) presenting a specific IgE higher than 25 UI/ml. Higher value was recorded for the girl of 9 years with 51.9 UI/ml. Five children (three girls and two boys) possess values up to 15 UI/ml. For peanut's proteins, 22.2% of specific IgE are positive with two girls (6 and 8 years) showing a higher IgE value of 17.8 and 19.8UI/ml respectively.

Table 1. Description of the study population.

	Children	**Boys**	**Girls**
n (%)	81	37 (45.7%)	44 (54.3%)
< 5 years n (%)	29 (35.8%)	16 (43.2%)	13 (29.5%)
Median age (Years)	7.3	6.5	7.9

Table 2. Distribution of total IgE measured in 70 children.

Total IgE (UI/ml)	**Children**	**Boys**	**Girls**
>80	2 (3.2%)	0	2 (5.5%)
30-80	25 (39.7%)	12 (44.4%)	13 (36.1%)
<30	36 (57.1%)	14 (51.9%)	22 (61.1%)

Discussion

The aim of this study is to evaluate food allergy in Moroccan children and particularly in Fez-Meknes region. Children recruited from HUC of Fez and Meknes hospitals. They has not been challenged or sensitized by allergens. They have been questioned and their sera analyzed for total and specific IgE for gliadins, egg's white and peanut's proteins by ELISA.

Results of total IgE measurements showed that 39.1% of the children presented values up to 30 UI/ml. These levels indicate that these infants were suffering of allergy probably not related to food only since we observed only 7.5% of food allergy (Table 3). These IgE levels were probably related to respiratory allergies.

Specific IgE analysis for three allergens gliadins, egg's white and peanuts showed that 70.4 % presented positive IgE values. Higher level was recorded for gliadins with 46.9 %. From those positive results, three persons presented a value up to 25 UI/ml. According to predictive allergy value published by Sampson (2001), this data indicate that those persons were allergic to gliadins supposing that prevalence of gliadin allergy was about 4.1 %.

For egg's white proteins, positive results recorded were 29.6%. According to Sampson (1997, 2001) a level of 7UI/ml of specific IgE to egg's white is a predictive value for allergy. This conclusion has been supported later by Rancé *et al.* (2003). On these conclusions, in our study, we found that 14.3 %of children were allergic to egg's white proteins. Our results are in accordance with

Table 3. Specific IgE measured in children sera. Values indicate means (±SEM) of positive specific-IgE (n) with maximum values observed in boys and girls.

	Children	Boys	Girls
Specific Gliadin IgE	10.5 ± 1.7 (n=38) (51.9 UI/ml)	9.5 ± 2.1 (n=19) (40.5UI/ml)	11.5 ± 2.7 (n=19) (51.9 UI/ml)
Specific Egg-white IgE	9.0 ± 2.0 (n=24) (49.2 UI/ml)	11.6 ± 4.3 (n=11) (49.2 UI/ml)	7.1 ± 1.4 (n=13) (15.5 UI/ml)
Specific peanut IgE	7.5 ± 1.3 (n=18) (19.8 UI/ml)	5.3 ± 1.7 (n=8) (13.9 UI/ml)	9.3 ± 1.8 (n=10) (19.8 UI/ml)

the study published by Ghadi *et al.* (2007) concerning sensitization of atopic children in Marrakech from Morocco. These authors observed that from the food allergens used, egg's white was the major allergen to which children were more sensitive.

The results of the latest allergen studied, peanuts, showed 22.2% of positive values with two girls which were probably allergic to peanuts since specific IgE was up than predictive value, which is of 15UI/ml (Sampson, 1997, 2001; Sporik *et al.*, 2000). This indicates that prevalence of food allergy to peanuts was about 2.7%.

In conclusion, from specific IgE measurements, we could suppose that food allergy in Fez and Meknes region was important for egg's white with probably about 14.3 % of allergic children followed by gliadins and peanut's proteins.

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Profile of Intestinal Protozoan and helminthic infections in the Provincial Hospital Center of Kenitra city (Morocco)

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Abstract

This retrospective epidemiological investigation, the first in Kenitra (Gharb area), was designed to specify the global prevalence of intestinal parasitism to the Provincial Hospital of EL IDRISSI Kenitra in 1996-2005. 606 examinations positive of 4285 presents a parasitism index of 14.15%. The analysis of data recorded shows that the Specific Parasitism Index (IPSp) of each parasite has experienced an annual and monthly irregular evolution. This evolution was marked by recording higher rates of especialy species of: *Entamoeba histolytica* (26.4%), *Giardia intestinalis* (22.71%) and *Entamoeba coli* (22.11%) in the case of protozoa and *Ascaris lumbricoides* (11.87%) in the case of helminths. 10.72% subjects were concerned by polyparasitism. In view of these results, it seams necessary to support measures to reduce the parasitism by intestinal protozoa and prevent the spread of helminth.

Key-words: Intestinal parasitism, Epidemiology, Provincial Hospital, Kenitra (Morocco).

Introduction

The prevalence of intestinal parasite is particularly high in certain populations because of weather conditions and especially precarious hygienic. In addition, the real problem with these parasites is the large number of asymptomatic carriers involved in the perpetuation of these parasites.

According to estimates by the World Health Organization (WHO) for the year 2002, an estimated 3.5 billion number of subjects infected with digestive parasites and 450 million the number of patients (WHO, 2001).

The effectiveness of control methods depends in part on good knowledge of the distribution of these parasitic infections (Menan *et al.*, 1997). Also a retrospective epidemiological study over a period of 10 years (1996-2005) was conducted at the Provincial Hospital EL IDRISSI of Kenitra (Morocco) to determine the prevalence of intestinal parasites, specific parasitic index to different parasitic species which are encountered, and to assess their progress during these ten years.

Materials and methods

From 1996 to 2005, the Medical Laboratory of the Provincial Hospital Center EL IDRISSI of Kenitra has conducted 4285 Stools Parasitological Examination (SPE).

Samples

They come from patients aged from 0 to 76 years. They are:

- ✓ Patients hospitalized or followed in the departments of gastroenterology and general medicine; pediatrics services, surgical and specialties services.
- ✓ External consultant Patients in health centers and clinics in the region.

Methods

Each collection has been a direct microscopic examination between blade and slide, after dilution by physiological water on stools freshly issued. The search for protozoa was made systematically by staining Lugol.

Calculation of parasitic index

- ✓ Simple Parasitic Index "I.P.S."; The Simple Parasitic Index, which is the percentage of subjects with parasites compared to the total SPE made.
- ✓ Specific Parasitic Index "I.P.Sp."; The Specific parasitic Index is the percentage of subjects parasitized by a species or group of parasites compared to the total positive SPE.

Statistical data

The data collected were typed on Excel software to calculate other parameters needed for analysis, after verification and validation of data, statistical analysis was done on specialized software of epidemiology (Epi-Info6).

Results

Simple Parasitic Index by sex

The number of female parasitized subjects (320 is an IPS = 15.68%) is higher than that of male parasitized subjects (286 or GPI = 12.76%). The reduced gap calculated shows that the difference between the two sexes is very significant (Σ reduced = 2.74).

Simple Parasitic Index by age

485 samples have been tested positive in patients aged less than 18 years, they are particularly children with a percentage of 80.03%. The rest of the population is considered adult subjects with a higher age to 18 years (121 adults or 19.96%). In children, the IPS is to 16.39%, while in adults, it is 9.14%. The reduced gap calculated shows that the difference between these two age groups is highly significant (Σ reduced = 6.29).

Simple Parasitic Index by years

The IPS varies from one year to another with an average of 14.15%, which means that one in four has a species or a number of parasites species. The highest rate was recorded in 1999 (18.10%) and 1996 (17.48%) while the lowest was in 2002 (9.10%). Statistical analysis based on the gap reduced calculated shows that the difference is highly significant between the following years: 1996-2002 (Σ reduced = 3947); 1999-2002 (Σ reduced = 4097). On the other hand, there is a very significant difference between the years 1996-2005 (Σ reduced = 2958); 1996-2005 (Σ reduced = 2603); 1998-2002 (Σ reduced = 2693); 1999-2001 (Σ reduced = 3131); 1999-2005 (Σ reduced = 2748) and 2002-2005 ((Σ reduced = 2760). So it is significant between the following years: 1997-2002 (Σ reduced = 2513); 2000-2002 (Σ reduced = 2476) and 2002-2003 (Σ reduced = 2034).

The annual evolution of intestinal parasitosis cases of protozoa and helminth parasites between 1996 and 2005 CHP of Kenitra (Table 1).

The monthly changes cases of intestinal parasites protozoa between 1996 and 2005 CHP of Kenitra (Figure 1).

The monthly changes cases of intestinal parasites to helminths between 1996 and 2005 CHP of Kenitra (Figure 2).

An analysis of reported cases for each species parasites found during this study period shows that:

- ✓ Protozoa / Amoeba:
  - o *Entamoeba histolytica*: the number of cases recorded this species varies from one month to another, two peaks amoebiasis E. histolytica have been marked, the month of August with 19 cases and the month of March with 18 cases. The trophozoites minuta forms of this species have been found with a very small number (4 cases) only during the months of March, April, May and November.

Table 1. Evolution of the parasites prevalence between 1996 and 2005 in the Provincial Hospital of Kenitra (Morocco). * % compared to the examined cases.

	Years										Total	
	1996	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**Nb.**	**%***
SPE charged	595	489	422	514	515	533	473	281	236	227	4285	-
SPE positive	104	70	63	93	73	60	43	39	38	23	606	14,15
Protozoa / Amoeba												
Entamoeba histolytica (cys	34	14	18	30	10	14	12	8	13	7	160	3,74
E. histolytica (minuta)	1	1	2	0	0	0	0	0	0	0	4	0,10
Entamoeba coli	16	15	14	25	26	15	12	10	8	8	149	3,48
Endolimax nana	1	1	2	0	0	0	0	0	0	0	4	0,10
Protozoa / Flagella												
Giardia intestinalis	19	21	17	21	14	22	9	13	12	5	153	3,58
Trichomonas intestinalis	8	5	2	4	4	1	4	4	2	3	37	0,87
Chilomastix mesnilii	4	0	0	0	0	0	0	0	0	0	4	0,10
Total (Protozoa)	**83**	**57**	**55**	**80**	**54**	**52**	**37**	**35**	**35**	**23**	**511**	**11,93**
Helminth / Nematoda												
Ascaris lumbricoides	15	12	7	11	17	10	5	2	1	0	80	1,87
Trichuris trichiura	12	7	5	6	6	0	1	1	0	0	38	0,89
Enterobius vermicularis	0	1	3	0	1	2	4	2	0	1	14	0,33
Anguillules	1	0	0	0	2	0	0	0	0	0	3	0,08
Helminth / Cestoda												
Hymenolepis nana	3	2	2	3	3	2	1	1	1	0	18	0,43
Taenia saginata	0	1	1	1	0	0	1	0	1	0	5	0,12
Total (Helminth)	**31**	**23**	**18**	**21**	**29**	**14**	**12**	**6**	**3**	**1**	**158**	**3,69**
Total (Protozoa & Helminth)	**114**	**80**	**73**	**101**	**83**	**66**	**49**	**41**	**38**	**24**	**669**	**15,62**

- *Entamoeba coli*: 19 cases were reported during the month of July against 4 cases during the month of January during the study period.
- *Entamoeba nana*: 2 cases were recorded during the month of March 1 against single case registered during April and June.

Broadly speaking, the highest number of amoeba was notified during the month of August with 35 cases against 14 cases during the month of January (Figure 1).

- ✓ Protozoa / Flagella:
  - *Giardia intestinalis:* this species has been found frequently during the months of February, April, May and June with a peak during the month of July (19 cases). The least number of cases of this species was recorded in the month of August with 7 cases.
  - *Trichomonas intestinalis*: 6 cases were reported during the month of May. We note the complete absence of this species during the month of January.
  - *Chilomastix mesnilii*: 2 cases were recorded in May. The other two reported cases were divided between August and October.

Broadly speaking, flagellates are very common between April and July with a peak of 25 cases during the May me (Figure 1).

- ✓ Helminths / Nematoda:
  - *Ascaris lumbricoides*: 15 cases were recorded during the month of December against 2 cases were reported in March.
  - *Trichuris trichiura*: 6 cases were reported during the months of June and December. The lowest number of cases was recorded during the months of July and November (1 case).
  - *Enterobius vermicularis*: 3 cases were reported during the months of January, April and July, 1 single case during the months of

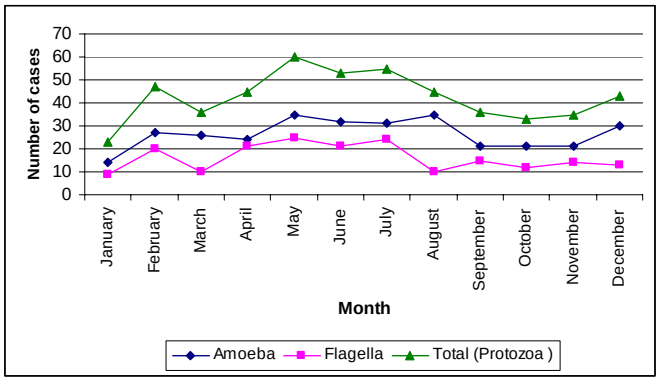

Figure 1. Monthly evolution of protozoa cases between 1996 and 2005 in the Provincial Hospital of Kenitra (Morocco).

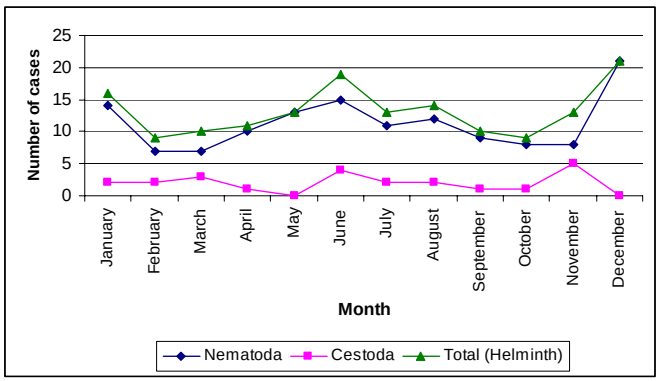

Figure 2. Monthly evolution of helminth cases between 1996 and 2005 in the Provincial Hospital of Kénitra (Morocco).

March, June, August, October and November. No cases of *Enterobius vermicularis* was recorded during the month of February, May, September and December.

- Anguillules to *Stronyloides stercoralis*: 3 cases were registered during the month of August, October and November.

The species of nematodes are found frequently during the months of December (21 cases) against 7 cases recorded during the months of February and March (Figure 2).

✓ Helminths / Cestoda:

- *Hymenolepis nana:* 3 cases were reported respectively during the month of March, June and November, 2 cases during the months of July and August, only 1 case during the months of January, February, April, September and October. So we note 0 cases in May and December.
- *Taenia saginta:* 2 cases were registered in November. The months of January, February and June were marked by recording a single case. While other months are marked by the absence of this species in the stool examined during this retrospective study.

Cestodes species are found frequently during the month of November with 5 cases. The months of May and December are marked by the absence of such kinds of intestinal parasites (Figure 2).

Discussion

In light of the results obtained in our retrospective investigation, we reached a number of findings about the epidemiological profile of intestinal parasites and protozoa to helminths in patients hospitalized or external at the Provincial Hospital of Kenitra (Hospital El Idrissi).

The methods and techniques of concentrations used during examinations parasitological stool at the Medical Analysis Laboratory at the Hospital "Idrissi" will only rarely highlight pathogenic forms and protozoa vegetative shapes. In addition, specific methods for finding Enterobiasis (*Enterobius vermicularis*, parasite quite common among children because of its particular cycle) were not used (Scotch-test Graham). The prevalence parasitic calculated are certainly below the actual

prevalence we should find in this area of study.

According to this study, we note that the number of stool parasitological examinations (SPE) requested between 1996 and 2005 appears to decrease over time. This decline could be due to the installation of other medical analysis laboratories in the study area (public and private). At Kenitra, the frequency of parasitic infections is about 50% in a private laboratory outside the hospital (Lebbar, 1997). On the other hand, some parasites (Ascariasis, Enterobiasis, Trichuriasis, Taeniasis, …) are often treated solely on the symptomatic argument and does not always subject to a diagnosis at the specialised laboratory. However, Hajfani (1976) has been an increase in the number of examinations coprologiques charged between 1973 and 1975 at CHU Rabat (Morocco). An epidemiological study was conducted at the hospital in Mahajanga (Madagascar) showed that many helminthiasis can go unnoticed due to irregular laying eggs, a small infestation, an immature worms or to a predominance of males (Buchy, 2003).

The decrease in the number of tests performed is accompanied by a significant decrease in positive reviews. In Martinique, a comparison of the prevalence of major digestive parasitism was made between the years 1968, 1972 (results of the institute pastor of Martinique) and 1995 (results of the Laboratory of Hygiene Department of Martinique). This study shows that the highlight, namely the collapse of the prevalence of intestinal parasites, was initiated in the early years 70. According to Magnaval (1998), the event includes an explanatory combined action of improving the general level of hygiene, an amplification of pressure drug linked to the emergence of anthelminthic manageable and effective on these parasites. Other works done by Goalkeeper et al. in public laboratories of Martinique between 1988 and 1995 have confirmed its results (Gardien *et al.*, 1997).

Of the 4285 SPE performed between 1996 and 2005, 606 were positive or 14.15%. The rate of parasitism is relatively representative, because it involves a targeted population, since the stool parasitological analysis is not made in a systematic way in all subjects hospitalized, but especially interested in those showing a sign of digestive appeal.

In addition, the rate may be deemed to be underestimated since a fancy human population, mainly from the region of Gharb or environmental conditions, hygiene and eating habits differ from those of other regions of Morocco. The rate of parasitism calculated in this retrospective study is lower than those found in El Mohammedia (Khales, 1998); Casablanca (Laraqui, 1978; Jemaaoui, 1998); Oujda (Harrou, 1993); Marrakech (Içam, 1991); Rabat (Rhrari, 1991; Tligui *et al.*, 2002); Sidi Yahia (Melyani, 1983) and Knitra (Lahlou, 1983). This difference in rates found is probably related to geographical variations and complexity of socio-economic factors between the different mentioned regions.

The protozoa are much more frequent than helminth parasites because they are only the ¼ parasites found while ¾ remaining are represented by parasitic protozoa. Among intestinal parasites found, the amoeba up more than half, followed by flogged and helminths. These are generally *Ascaris lumbricoides*, while other verminoses due both to nematodes as cestodes are much rarer. Cases outstanding anguillulose to *Stronyloides stercoralis* were recorded (1 case in 1996 and 2 cases in 2000). Thus, the protozooses, diseases of dirty hands, danger and fecal contaminated food constitute the vast majority of cases. We also note the low index of orally transmitted helminths and a representation minimal or no helminths transmitted through transcutaneous including anguilluloses. These results are comparable to those obtained by Faye *et*

al. (1998), Dialoo *et al.* (1979) and Diwara (1984).

The three species of amoeba are found by descending order of frequency: *Entamoeba histolytica, Entamoeba coli* and *Endolimax nana*. In the group of flagellates, 3 species have been found: *Giardia intestinalis, Trichomonas intestinalis* and *Chilomastix mesnilii*. The amoebae parasites are the most numerous (317 cases is a prevalence of 7.40%). The species *Entamoeba histolytica* has emerged as the most pathogenic parasite dominant in the stool examined (cystic form) with a Parasitic Specific Index 26.40% and is found in almost ¼ stool positive. It is also found in the form non-pathogenic *Entamoeba histolytica minuta* with a prevalence of 0.66%.

A prevalence of 2 to 3% for *E. histolytica* was observed among children hospitalized in Libreville (Gabon) a cause of acute diarrhea (Gendrel, 2003). In Tunisia, the prevalence of this case is 5.03% (Ayadi *et al.*, 1991). By cons, no holder of *E. histolytica* was detected in Senegal (Faye *et al.*, 1998). In addition, Salem *et al.* (1994) has reported a very high prevalence of 22.6%. A Burkina Faso, the authors have reported a prevalence of 10.6% for *E. histolytica* (Diano *et al.*, 2004).

In the second position, are *Giardia intestinalis* (IPSp = 22.71%) and *Entamoeba coli* (IPSp = 22.11%). The *Trichomonas intestinalis* comes in third position with a IPSp of 5.49%. The species occasionally seen as pests or pathogens absolutely not: *Endolimax nana* and *Chilomastix mesnilii* have equal IPSp (IPSp = 0.60%). In Ivory Coast, Adou-Bryn *et al.* (2001) have reported a prevalence of 22.4% for E. coli and 4.8% for *Endolimax nana* with a complete absence of the *E. histolytica.*

In addition, Assale *et al.* (1985) have reported a prevalence of 29.2% for E. coli, 4.2% for *E. hitolytica* and 4.2 per *Endolimax nana*. In Tunisia, Ayadi *et al.* (1991) have found a frequency of 1.32% for *E. histolytica,* 1.22% for *E. coli* and 2.74% for *Endolimax nana*. While the work of Bachta *et al.* (1990) in the Algérois show 7.25% (*E. histolytica*), 7.58% (*E. coli*) and 9.36% (*E. nana*). In Brazil, it is 8.8% (Santos *et al.*, 1995). In Niger, it is 21.8% for *E. coli*, 10.11% for *E. histolytica* and 2.7% for *E. Nana*. In Madagascar, *E. histolytica* and *E. nana* have the same prevalence (12.5%), *E. coli*, it is 31.9% (Buchy, 2003).

As for helminthiases, they are dominated by Ascaridiase whose IPSp of the species *Ascaris lumbricoides* east of 11.87% of parasites found. This species is alone nearly ½ of all helminths found. Other helminthiases are relatively rare since it was found that:

- ✓ 38 cases of Trichuriasis to *Trichuris trichiura*.
- ✓ 18 cases of Hymenolepiasis to *Hymenolepis nana.*
- ✓ 14 cases of Enterobiasis to *Enterobius vermicularis.*
- ✓ 5 cases of Tanaisis to *Taenia saginata.*
- ✓ 3 cases of Anguilluloses to *Stronyloides stercoralis.*

The prevalence of 11.87% attributed to Ascariasis approximates that found in Aboisso in the south of Ivory Coast (Menan *et al.*, 1997). In Gabon, high frequencies above 50% have been reported (Garin e*t al.*, 1987) while no case of Ascariasis have been detected in the Central African Republic (Rippert *et al.*, 1987). In Africa intertropical, prevalence of this parasite is variable. It seems to be very common in wet and rainy forest region, which allows an easier spread of eggs (Menan *et al.*, 1997).

A Sfax (Tunisia), Ayadi *et al.* (1991) have reported a total of 115 cases of helminths with a prevalence of 12.45% for Enterobiasis, 1.88% for *Hymenolepis nana*, 0.12% *Taena saginata* and total absence of *Ascaris lumbricoides* and *Trichuris trichiura.*

The analysis of data recorded shows that IPSp all species of parasites is changing irregular annual and monthly. For all protozoa found (Amibea and Flagella), we note that the number of cases, the highest of these intestinal parasites was recorded during the period between March and July with a peak of 60 cases during the month May. As for helminthiases, the highest number of cases was reported during the month of December (21 cases) followed by the month of June with 19 cases. Low cases were recorded during the months of February and October with 9 cases per month.

During the study period spanning between 1996 and 2005, the number of intestinal parasitism cases with protozoa or helminths varies monthly during this retrospective investigation:

- ✓ The highest number of cases was notified during the month of May with 73 cases and June with 72 cases.
- ✓ The lowest number of cases was recorded during the month of January (39 cases).

According to Tligui et al. (2002), this can be explained by several factors: the change in temperature and humidity that promote maturation of parasites in the external environment, and changing eating habits with an increase the consumption of water and raw foods (fruits, vegetables, salads, ...). In addition, several studies including those made by Renault *et al.* (1962) in Kenitra city and Squat in Rabat and Fez show an intestinal disease outbreak in summer and autumn-estivo (Seqat, 1974).

This evolution was marked by recording higher rates of particular species: *Entamoeba histolytica* (shape and cystic minuta), and *Giardia intestinalis Entamoeba coli* in the case of protozoa and species *Ascaris lumbricoides* in the case of helminths.

By sex, IPSp of Entamoeba histolytica is higher among female subjects (IPSp = 12.46%) compared with males (IPSp = 11.27%). The same was observed for Entamoeba coli. As against this index is higher among male subjects (IPSp = 12.46%) than among females (IPSp = 10.23%) in the case of Giardia intestinalis. For other species of protozoa, there are approximately IPSp similar. Thus, no cases of amoeba due to Endolimax nana have been reported among male subjects. For helminths, IPSp the highest was recorded among male subjects (IPSp = 6.08%) in this case Ascaris lumbricoides. In the case of Trichuris trichiura, there are IPSp almost similar in both sexes. Whereas in the case of oxyuroses, this index is higher among female subjects than that recorded among male subjects. In a study conducted in the region of Mahajanga, West coast of Madagascar, all encountered intestinal parasites are more common among women than among men (Buchy, 2003).

Concerning the degree of polyparasitisme, Polyparasitisme index (PPI) calculated in this investigation is to 1.47%. The PPI is close to that recorded by Içam (1991) in Marrakech (PPI = 1.52%). The PPI fancy 10.72% (65/606) of subjects with parasites. Data analysis of monoparasitisme and polyparasitisme shows that the monoparasitisme represents 89.27% of positive cases, biparasitisme represents 9.73% and finally triparasitisme represents 0.5% of these positive cases. In most cases found, subjects polyparasites suffering from biparasitisme protozoal (5.12% SPE positive) and biparasitisme Joint (2.80% SPE positive). The study of different forms of polyparasitisme shows: 31 cases of pure biparasitism protozoal; 2 cases of pure triparasitism protozoal; 11 cases of pure biparasitism to helminths; 2 cases of pure triparasitisme to helminths; 17 cases of mixed biparasitisme (protozoa + helminthes) and 2 cases of mixed triparasitisme.

The qualitative study of polyparasitisme shows that the combination of two pure protozoa is the most common Entamoeba histolytica +

Entamoeba coli with 31 cases or 6.06% of positive cases followed by mixed associations with 17 cases or 2.54 % Of which 6 cases are represented by the association Ascaris lumbricoides + Giardia intestinalis.

The three associations protozoa are less frequent (2 cases or 0.39% of positive cases). As association's pure helminths, there are 11 cases or 6.96% of positive cases. The association is the most common Ascaris lumbricoides + Trichuris trichiura with 8 cases or 5.06% of positive cases. The triparasitisme was rarely found in this study, among helminths pure, it represents only 1.26% of positive cases with 2 cases while in the case of mixed associations, triparasitisme represents only 0.29% positive cases.

It is noteworthy that the presence of parasitic association shows the very low level of hygiene health, food and fecal and the adverse living conditions of these subjects polyparasitized (Tligui *et al.*, 2002). The predominance of species of protozoa is because the parasites concerned often have similar kinds of infestations.

Conclusion

Most of these parasites species identified are non-pathogenic. They reflect the conditions of life and the surrounding environment of the population and they are also witnessing an imperfect hygiene which confirms that its carriers are at risk. Indeed, because of a mode of infestation probably identical, these subjects are more likely to host, alongside non-pathogenic parasites, other species pathogenic parasites, either simultaneously or subsequently.

Acknowledgments

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A comparison of lead toxicity using physiological and enzymatic parameters on spinach (*Spinacia oleracea* L.) and wheat *(Triticum aestivum* L.) growth

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Abstract

The effects of lead (Pb) stress on plant growth and on the activity of antioxidant enzymes and lipid peroxidation were studied in two species, spinach (*Spinacia oleracea L.*) and wheat (*Triticum aestivum L.*), grown under hydroponical conditions in the absence or in the presence of various concentrations (1.5, 3, and 15 mM) of lead nitrate. Leaves and roots of control and Pb-stressed plants were harvested after one month of germination. The Tolerance Index (T.I. – to be defined) measured on leaves and roots of both plants decreased with the increase of Pb concentrations. In every case, T.I. was significantly higher in spinach than in wheat. The activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)] was increased in leaves and roots by lead treatment in a dose-related manner, but this increase was reduced in roots with the highest Pb concentration. Ascorbate peroxidase (APX) activity increased in spinach, but remained unchanged in wheat. The relative increase in enzyme activities demonstrated that spinach is more tolerant to Pb than wheat. Lipid peroxidation was enhanced with all levels of Pb in stressed wheat, whereas in spinach it increased only with the highest Pb concentration. These results indicate that under Pb-stress spinach is more resistant than wheat, and the possible mechanisms of these differences are discussed.

Key words: Lead, Spinach, Wheat, Tolerance index, Lipid peroxidation, antioxidative enzymes.

Introduction

Lead (Pb) exists in many forms in natural sources throughout the world. According to the USA Environmental Protection Agency, Pb is one of the most common heavy metal contaminants in aquatic and terrestrial ecosystems and can have adverse effects on growth and metabolism of plants, due to its direct release into the atmosphere (Watanabe, 1997). The effect of lead depends on the concentration, type of salts, soil properties and plant species involved (Patra *et al.*, 2004). In general, effects are more pronounced at higher concentrations and durations. In some cases, low concentrations stimulate metabolic processes and enzymes involved, such as hydrolytic enzymes as well as peroxidase, acid phosphatase, and α-amylase (Patra *et al*., 2004). There have been many reports of Pb toxicity in plants (Choudhury & Panda, 2005), including disturbance of mitosis (Wierzbicka, 1998; Jiang & Liu, 2000), inhibition of root and shoot growth (Liu *et al.*, 2009), induction of leaf chlorosis (Pandey *et al.*, 2007), reduction of photosynthesis (Xiao *et al*., 2008) and inhibition or activation of several enzyme activities (Verma & Dubey, 2003; Sharma & Dubey, 2005; Liu *et al*., 2009).

Despite the worldwide severity of Pb contamination, it remains unclear how the Pb's concentration induce a reduction in plant growth under conditions similar to those experienced in 'typical' soil solutions. Malkowski *et al.* (2002) found

that Pb at 10, 100 or 1000 µM reduced the growth of maize (*Zea mays* L.). Similarly, Fodor *et al*. (1996) reported that 10 mM Pb was toxic to cucumber (*Cucumis sativus* L.), and Wozny & Jerczynska (1991) found 10 mM Pb to be toxic to bean (*Phaseolus vulgaris* L.). However, Godbold & Kettner (1991) reported that as little as 0.1 mM Pb caused a reduction in the root elongation of Norway spruce (*Picea abies* L.) seedlings.

Lead and most heavy metals can induce oxidative stress by generating free radicals and toxic oxygen species (Hegedüs *et al.*, 2001). These species react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability. The deleterious effects resulting from the cellular oxidative state may be alleviated by enzymatic and nonenzymatic antioxidant machinery of the plant that vary at various cellular and subcellular levels in different plants.

Plants use a diverse array of enzymes like superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.1), as well as low molecular weight antioxidants like cysteine, nonprotein thiol, and ascorbic acid to scavenge different types of reactive oxygen species (ROS), thereby protecting against potential cell injury and tissue dysfunction (Halliwell, 1987). SOD is a key antioxidative enzyme that catalyzes disproportionation of superoxide anion ($O_2^{\cdot -}$) to H_2O_2 and O_2. Catalase localized in peroxisomes, scavenges H_2O_2 by converting it to H_2O and O_2. Peroxidase reduces H_2O_2 or peroxides (ROOR') using several reductants, of phenolic compounds. POD is also the key enzyme in lignin biosynthesis and participates in the formation of radicals of lignin units before their polymerization (Gaspar *et al.*, 1991). APX appears to play a pivotal role in scavenging ROS and maintaining the level of antioxidant ascorbate (Verma & Dubey, 2003).

Wheat is grown on 17% of all crop areas and represents the staple food for 40% of the world's population (Maccaferri *et al.*, 2009). Spinach is an important dietary vegetable with a high antioxidant capacity, principally involving flavonoids, that constitute the major water-soluble polyphenols found in this species (Herrmann, 1995).

Wheat and spinach are two important agricultural species of the North of Morocco. We previously investigated the effects of lead exposure on wheat seedlings (Lamhamdi *et al.*, 2011). Whether wheat and spinach display different resistance levels to lead exposure is not known, and this prompted us to engage a comparative study. We have presently investigated the effects of Pb stress on growth, lipid peroxidation, SOD, CAT, APX, and POD antioxidant enzymes activities in leaves and roots of spinach and wheat.

Materials and methods

Plant growth, Pb treatment and index of tolerance evaluation

A variety of wheat (*Triticum aestivum* L. cv. Achtar) was provided by the National Institute of Agronomical Research (INRA), Tangier, Morocco, and spinach (*Spinacia oleracea L.*, var. "Géant d'hiver", purchased from Truffaut, France). Prior to germination, seeds were surface-sterilized with 5% (v/v) sodium hypochlorite for 10 min and rinsed several times with distilled and sterilized water. The seeds were then germinated in Petri dishes containing two sheets of Whatman no. 1 filter paper moistened initially with 6 ml of distilled and sterilized water. After germination, when cotyledons had fully emerged (after 6 days for spinach, and 4 days for wheat), the seedlings were grown in 13 x 100 mm glass test-tubes (7 ml capacity) containing 5 ml Hoagland's solution (pH 5.5) (Hoagland & Arnon, 1950), at 25°C in a 16-h light/8-h dark photoperiod at 45 $\mu mol.m^{-2}.s^{-1}$ from cool

white fluorescent tubes. Fertiliser solution was changed twice a week.

Different concentrations of $Pb(NO_3)_2$ (0, 1.5, 3 and 15 mM) were added 25 days after the onset of germination, and at 30 days plantlet shoot and root length were evaluated, then harvested for analysis of lipid peroxidation products and SOD, APX, POD and CAT activities. Each treatment was set up in 15 replicates; ten replicates for tolerance index evaluation, and five for biochemical analyses.

The tolerance index was evaluated separately on both parts (shoots and roots) of seedlings by the application of this formula:

$$\textit{Tolerance index} = \frac{\textit{Elongation of treated plants}}{\textit{Elongation of control plants}} \times 100$$

Enzymes extraction

All biochemical analyses were performed at 4 °C; 1 g of fresh leaves or roots were extracted in 3 ml of 100 mM sodium phosphate buffer (pH 7) including 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 9,000g for 20 min, and the supernatant was used for the enzymatic assays. Proteins were determined according to Bradford (1976) using bovine serum albumin as the standard protein (data not shown).

Enzyme assays

Superoxide dismutase (SOD) activity was determined by the method of Beauchamp & Fridovich (1971) by following the photoreduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2 µM riboflavin and 100 µL of the supernatant. Riboflavin was added as the last component and the reaction was initiated by placing the tubes under two 15 W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. The photoreduction of NBT (production of blue formazan) was measured at 560 nm. One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as SOD units per mg of protein.

Catalase activity (CAT) was measured according the method of Beer & Sizer (1952), with minor modifications. The reaction mixture (1 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 20 mM H_2O_2 and 50 µl enzyme extract. The reaction was started by addition of the extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 $L.mol^{-1}.cm^{-1}$) and the results expressed as CAT units per min and mg of protein.

Peroxidase (POD) activity was measured following the change of absorbance at 470 nm due to guaiacol oxidation. The activity was assayed for 3 min in a reaction solution (1 mL final volume) composed of 100 mM Na-phosphate buffer (pH 7.0), 0.6 mM guaiacol, 10 mM H_2O_2 and 50 µL of crude extract, as described in Zhang *et al.* (1995). APX activity was measured by the decrease of ascorbate absorbance at 290 nm. The reaction mixture contained 50 mM of Hepes buffer (pH 7.6), 0.25 mM ascorbate and 0.1 mM H_2O_2 (Houssain & Asada, 1984).

Lipid peroxidation

Malonedialdehyde (MDA) is one final product of lipid peroxidation and has been used as an index for the status of lipid peroxidation. Thiobarbituric acid reactive substances (TBARS) representing the lipid peroxidation products were extracted by homogenization of 0.2 g of plant material in 5 mL of a solution containing 20% tri-chloroacetic acid and 0.5% 2-thiobarbituric acid. The mixture was heated at 95 °C for 30 min and the reaction was arrested by quickly transferring the mixture to an ice

bath. The cooled mixture was centrifuged at 5,000 g for 10 min at 25 °C and the absorbance of the supernatant at 532 and 600 nm was recorded. After subtracting the nonspecific turbidity at 600 nm, the MDA concentration was determined by its molar extinction coefficient 155 $L.mmol^{-1}.cm^{-1}$ (Kosugi & Kikugawa, 1985).

Statistical analyses

In all experiments three replicates were performed for each sample, and each treatment was examined with two parallel samples. Data presented here are the means±SD. One-way analysis of variance (ANOVA) post hoc testing was carried out using the Tukey's test. A significant level of 0.05 was used for all statistical tests, to examine any difference between the two plant species under lead stress in terms of index of tolerance, lipid peroxidation and enzymatic activities.

Results

Tolerance index (T.I.)

Figure 1 shows the T.I. of leaves (Figure 1A) and roots (Figure 1B) exposed to Pb stress compared to the control. The T.I. decreases for wheat and spinach with increased Pb stress, indicating a concentration-dependent growth inhibition. There was a direct relationship between the severity of the response and the increasing metal concentrations (37.8% and 56.4% in leaves of wheat and spinach respectively, and 31.8% and 52.3% in roots of wheat and spinach respectively at 15 mM). Moreover, the T.I. was significantly higher in spinach than in wheat.

Superoxide dismutase activity

SOD can eliminate O_2^-, reduce peroxidation of membrane lipids and maintain cell membrane integrity. SOD activity in spinach leaves increased with increasing Pb stress. In wheat leaves SOD activity peaked at 3 mM but then decreased at 15 mM. In all treatments of both plants SOD activity was still significantly higher than the controls (Figure 2A). In roots, SOD activity increased significantly at

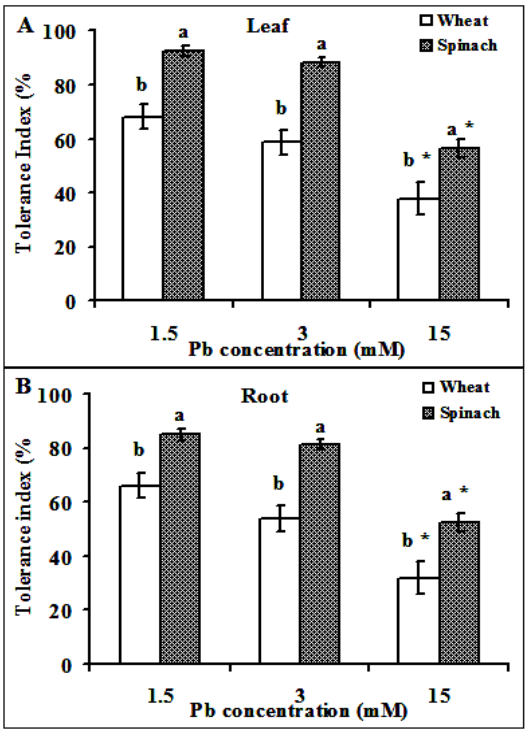

Figure 1. Effect of lead on tolerance index in leaves (A) and roots (B) of wheat and spinach. Results are the mean of 10 replicates ± SD. * indicates significant differences between the same plant (*P<0.05). Different letters indicate significant differences between the two species in each treatment.

3 mM and then decreased at 15 mM in both species, but the change was more pronounced in spinach (Figure 2B). SOD activity of leaves and roots was significantly different between the two species.

Catalase activity

CAT can eliminate H_2O_2. CAT activity in leaves of both species remained nearly the same as control levels at 1.5 and 3 mM, but at 15 mM its activity increased significantly (to *ca.* 165% in wheat and 128% in spinach) (Figure 3A). In the roots, CAT activity of wheat peaked at 1.5 mM and then deacresed to control levels at 3 and 15 mM. In spinach, CAT activity slightly increased at 1.5 mM, significantly increased at 3 mM (approximately 140% of the control) and then decreases to achieve the control levels at 15 mM (Figure 3B). CAT activity was

significantly higher in leaves and roots of spinach as compared to wheat.

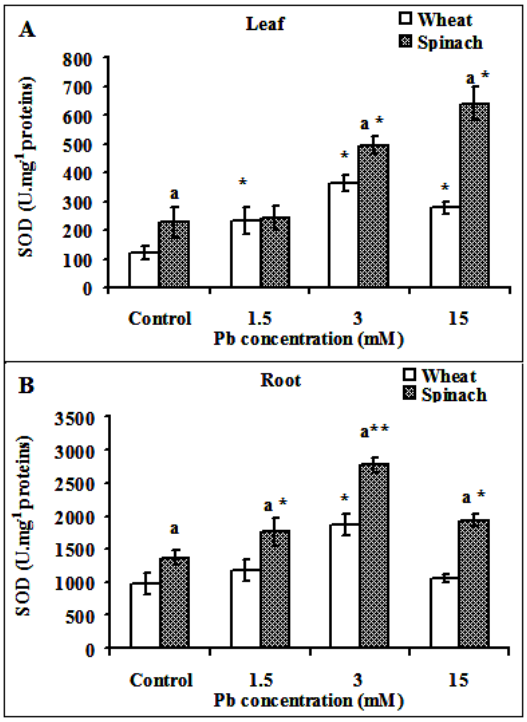

Figure 2. Effect of lead on SOD activities in leaves (A) and roots (B) wheat and spinach. Results are the mean of five replicates ± SD. * indicates significant differences between the treatments and the control of the same plant (*P<0.05; **P<0.01). Different letters indicate significant differences between the two species in each treatment.

Peroxidase activity

POD plays a role in decreasing H_2O_2 accumulation, reducing MDA resulting from peroxidation of membrane lipids and maintaining cell membrane integrity. POD activity was significantly higher in spinach than in wheat. POD activity in spinach leaves slightly increased at 1.5 mM Pb, and this increase became more important at higher Pb concentrations. POD activity increased significantly at 3 mM and then decreased at 15 mM in wheat leaves (Figure 4A). In the roots POD activity increased (180% at 1.5 mM in spinach, and 200% at 3 mM in wheat, as compared with the controls) then decreased in both species at 15 mM. The changes were more pronounced in spinach at 1.5 mM (Figure 4B).

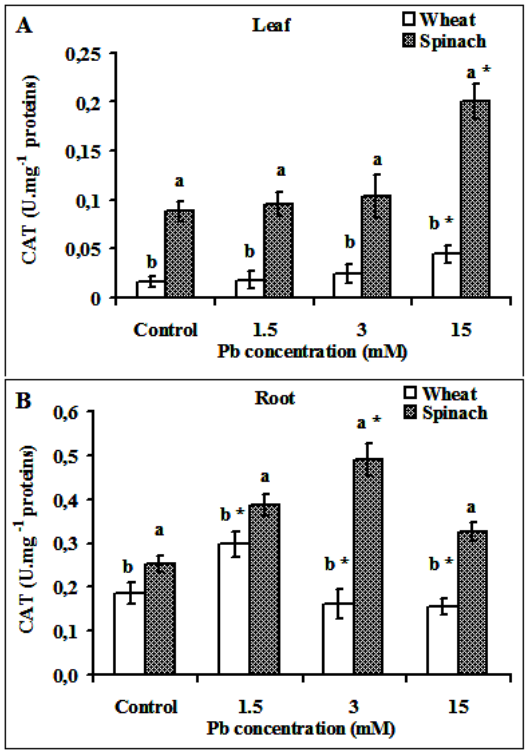

Figure 3. Effect of lead on CAT activities in leaves (A) and roots (B) wheat and spinach. Results are the mean of five replicates ± SD. * indicates significant differences between the treatments and the control of the same plant (*P<0.05). Different letters indicate significant differences between the two species in each treatment.

Ascorbate peroxidase activity

Ascorbate peroxidase activity in leaves and roots of wheat and spinach is given in Figure 5. APX activity in leaves of wheat was nearly the same at all levels stress. In spinach, APX activity of leaves is almost unchanged at 1.5 mM, and increased strongly (256% at 3 mM and 319% at 15 mM compared with the controls), with increasing Pb stress (Figure 5A). In wheat roots, APX activity was the same as in leaves. In spinach, APX activity increased strongly (107% of controls at 3 mM) and then decreased, but still remained higher (120%) than in controls at 15mM (Figure 5B). APX activity in spinach was significantly higher than in wheat, and the difference between both species was more obvious than the other activities.

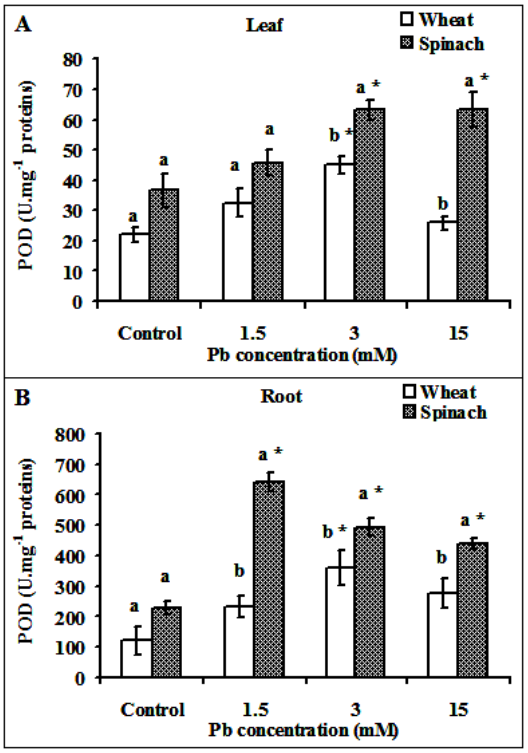

Figure 4. Effect of lead on POD activities in leaves (A) and roots (B) wheat and spinach. Results are the mean of five replicates ± SD. * indicates significant differences between the treatments and the control of the same plant (*P<0.05; **P<0.01). Different letters indicate significant differences between the two species in each treatment.

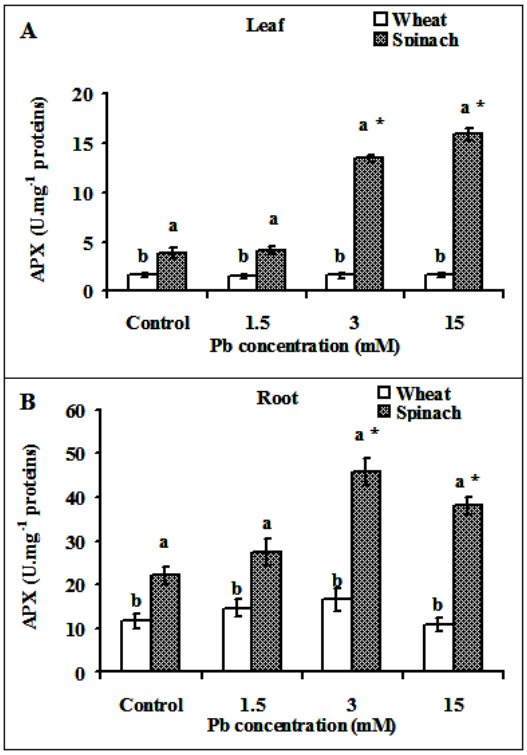

Figure 5. Effect of lead on APX activities in leaves (A) and roots (B) wheat and spinach. Results are the mean of five replicates ± SD. * indicates significant differences between the treatments and the control of the same plant (*P<0.05). Different letters indicate significant differences between the two species in each treatment.

Lipid peroxidation products

As shown in Figure 6, MDA content in leaves of wheat increased with Pb stress, indicating a concentration-dependent free radical generation (194% at 15 mM). In spinach leaves, MDA remains almost unchanged up to 3 mM, but increased strongly (221%) at 15 mM (Figure 6A). In wheat roots, MDA decreases slightly at 1.5mM (28% of controls) and then increased (173% of controls at 15 mM). For spinach, there was an irregular change of MDA content in the roots with Pb stress, footing at 36% at 3 mM, peaking at 106% at 15 mM compared with the control. Lipid peroxidation was significantly lower in spinach than in wheat, in leaves and roots, especially at 3 and 15mM. Thus, spinach seems to have more efficient antioxidant enzyme systems than wheat.

Discussion

Lead is known to inhibit seedling growth on barley (Stiborova *et al.*, 1987), certain legumes (Sudhakar *et al.*, 1992) and wheat (Lamhamdi *et al.*, 2011). Based on the Tolerance Index (T.I.) we observed a concentration-dependent decrease of tolerance of both spinach and wheat. On the both parts T.I. was significantly higher in spinach than in wheat, in all levels of treatment. Spinach which is a vegetable (member of *Amaranthaceae*) likely accumulated lower amounts of lead in its roots, rather than the root system of wheat, which belongs to *Graminae* (Mesmar & Jaber, 1991). This difference may be in part explained because spinach leaves contain antioxidant flavonoids, in particular spinacetin and patuletin, that may help to reduce the levels of reactive oxygen intermediates and thus play an

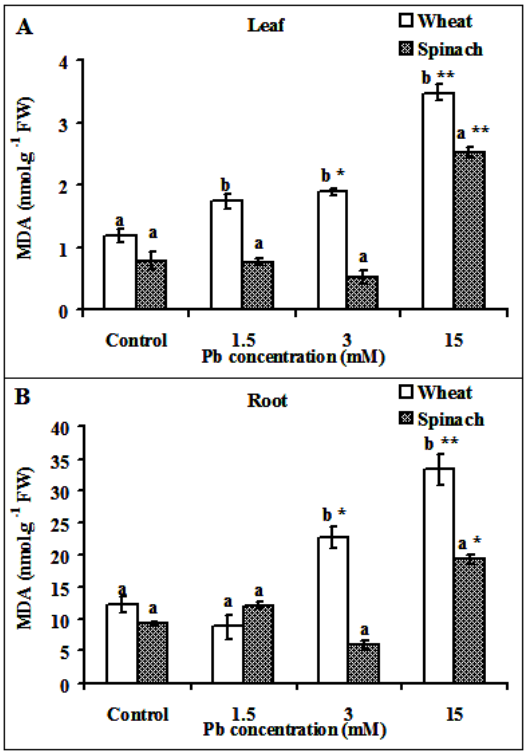

Figure 6. Effect of lead on MDA content in leaves (A) and roots (B) wheat and spinach. Results are the mean of five replicates ± SD. * indicates significant differences between the treatments and the control of the same plant (*P<0.05; **P<0.01). Different letters indicate significant differences between the two species in each treatment.

important role in the defence mechanisms (Herrmann, 1995).

Increase in SOD activity was observed in leaves and roots at 3 mM or 15 mM (Figure 2), but at the highest concentration of lead, the activity of SOD in roots of both samples decreased sharply. The decline in SOD activity at 15 mM indicated that the oxygen scavenging function of SOD was impaired. These data are in agreement with the results from *Alyssum* species (Schickler & Caspi, 1999) and *Allium sativum* (Zhang *et al.*, 2005). SOD activity in spinach leaves is maximal at higher metal concentrations than in wheat, and the response of spinach was significantly stronger in roots and leaves, suggesting that this increase in SOD results in a better protection against oxidant damage (Bowler *et al.*, 1992). In wheat, CAT activity was not affected in the leaves except at 15 mM, but increased in the roots at lower concentration only. In spinach, CAT activity increased in both leaves and roots. The higher SOD and CAT activities in spinach indicates that the H_2O_2 scavenging mechanism is more effective than in wheat, since CAT activity coordinated with SOD activity play a central protective role in the O_2^- and H_2O_2 scavenging process (Badawi *et al.*, 2004). POD and APX are widely distributed in the plant kingdom and are the principal enzymes involved in the elimination of active oxygen species (AOS). Previous studies in other plants have reported increase, decreases and no changes in POD activity in response to heavy metal exposure (Shaw, 1995; Schützendübel *et al.*, 2001, 2002). Figure 4 shows that spinach is able to maintain high levels of POD activity at higher concentrations Pb and there was significant difference in POD activity between spinach and wheat. APX activity in leaves of wheat remains the same at all levels stress. APX activity in spinach was significantly higher than in wheat.

In this study, Pb stress increased free radical generation in spinach and wheat plants, as indicated by the MDA production, which is similar to the effect of heavy metals on higher plants (Verma & Dubey, 2003). This suggests that the toxic effect of heavy metals is probably exerted through free radical generation. MDA content increased significantly in leaves of wheat, but declined in leaves of spinach, except at 15 mM. On the other hand, the MDA content in roots of spinach was unaffected at low metal concentrations, and at 15 mM it was lower than in Wheat. This implies that spinach is better protected from oxidative damage, and can rapidly up-regulate the antioxidative system. We think that the reduction of MDA concentration was due to increased antioxidative enzyme activities, which reduced $O_2^{\cdot-}$ and H_2O_2 levels and membrane damage. Yang *et al.* (2010)

have reported that MDA content remains unchanged during germination of wheat seeds exposed to lead. They also observed a significant enhancement production of extracellular H_2O_2 in germinating seeds cv. Xihan, which might be responsible for lead-inhibitory effect on wheat growth. In spinach, the MDA reduction resulted from the collaboration of antioxidative enzyme activities (SOD, CAT, POD and APX). Our results are in agreement with those of Shalata *et al.* (2001) for roots of *Lycopersicon pennellii* and for roots of salt-tolerant BR5033 maize genotypes (De Azevedo Neto *et al.*, 2006).

Another hypothesis can be proposed, connected with the presence of phytoecdysteroids (PEs) in spinach (Bakrim *et al.*, 2008), and their absence in wheat (Dinan, 1995). PEs are stable and continuously biosynthetized and redistributed in the aerial parts in spinach (Bakrim *et al.*, 2008). It may well be that a parallel process takes place in roots. The latter are also biosynthetic organs in spinach (Schmelz *et al.,* 1998). These molecules could provide a defense against abiotic stress by decreasing oxidative stress, and indeed previous studies have demonstrated that exogenous PEs treatment reduce the toxicity of lead and decrease concentration of lead in cells of *Chlorella vulgaris* (Bajguz & Godlewska-Zylkiewicz, 2004). Chemical similarities between ecdysteroids and brassinosteroids have led to suggestions that ecdysteroids might be active in brassinosteroids responsive systems and *vice versa*, but their structural differences are probably great enough to ensure biochemical specificity of their respective actions (Bajguz & Hayat, 2009).

Our results show that the difference in the antioxidative enzyme activities of leaves and roots, and the level of lipid peroxidation explain the greater tolerance of spinach to Pb stress compared to Wheat. In spinach APX activity was much higher, indicating that it may represent the most important H_2O_2 scavenging enzyme. With regard to spinach ecotoxicology and pollution monitoring, APX activity may be useful as a biomarker of heavy metal exposure. On the other hand, lipid peroxidation level in roots may be useful as a biomarker in wheat. This difference between both species will be used in future experiments aimed to search for molecules able to provide a protection against the deleterious effect of Pb.

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