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MALDI-TOF MS Detection of Endophytic Bacteria Associated with Great Nettle (*Urtica dioica* L.), Grown in Algeria

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Abstract

Any plant with a vascular system has a specific endophytic microflora. The identification of bacteria is essential in plant pathology. Although identification methods are effective, they are costly and time consuming. The purpose of this work is to isolate and to identify the different bacteria from the internal tissues of *Urtica dioica* L. and to study their diversity. This last is based on the different parts of the plant (stems, leaves and roots) and the harvest regions (Dellys and Tlamcen). The identification of bacteria is done by biochemical tests and confirmed by MALDI-TOF MS. Seven genus and eleven species were isolated from the Great Nettle. They belong to the genera *Bacillus*, *Escherichia*, *Pantoea*, *Enterobacter*, *Staphylococcus*, *Enterococcus* and *Paenibacillus*. The majority of these bacteria were isolated from Tlemcen which makes this region the richest in endophytic bacteria compared to that harvested from Dellys. The results show also that the leaves are the most diversified in endophytic bacteria. *Bacillus pumilus*-ME is the common species of the three parts of the plant harvested in both regions. From this work, it emerges that the Great Nettle can be settled by various endophytic bacteria which are differently distributed within the same plant harvested in different regions.

Key words: *Bacillus pumilus*-ME, diversity, endophytic bacteria, MALDI-TOF MS, *Urtica dioica* L.

Introduction

The internal tissues of plants can be a niche for various types of endophytic microorganisms (bacteria and fungi) (Rosenblueth and Martinez-Romero, 2006, Goryluk *et al*., 2009). Endophytic bacteria are very ubiquitous in plants and can be isolated from the stems, leaves, roots, fruits, tubers and nodules of leguminous plants (Kobayashi and Palumbo, 2000).

Some endophytes are very beneficial, even necessary for the growth of their host plants (Dudeja *et al*., 2012, Jasim *et al*., 2014). They prevent some pathogenic organisms from colonizing plants and can also act as biological control agents against insects (Laib, 2014). However, it is probable that beneficial endophytes can become pathogenic under certain stress conditions when the plant no longer controls them (Arnold, 2007).

The traditional methods of bacterial identification are very effective but have the disadvantage of being laborious and time-consuming (Tshikhudo *et al*., 2013). Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is an identification tool that is easy to use, fast, accurate and cost-effective (Gravet and Gessier, 2013, Sauget *et al*., 2017). Due to the lack of robust information tools and effective databases, this technique did not appear in public and private laboratories until 2008 (Tshikhudo *et al*., 2013). It was reserved exclusively for biochemical or research laboratories (Gravet and Gessier, 2013). This technique is based on the generation of mass spectra from whole cells and their comparison with reference spectra after ionization (Sauget *et al*., 2017).

Belonging to the Urticaceae family, *U. dioica* L. is a plant used for food and medicinal purposes. It is also on the list of medicinal plants selected by the French Pharmacopoeia (Draghi, 2005). The aim of this work was to identify the endophytic bacteria isolated from the stems, leaves and roots of the Great Nettle harvested at Dellys and Tlemcen by biochemical tests and by MALDI-TOF MS.

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Experimental

Material and Methods

Description of the study area. The different parts of *U. dioica* L. (stems, leaves and roots) were harvested during the month of February 2016 in two different regions of northern Algeria. The first is Dellys, a coastal town located at 115 km from Algiers. The second is Tlemcen, located in the north-west of Algeria, 520 km west of Algiers.

Isolation of endophytic bacteria. The plant freshly harvested under aseptic conditions and showing no pathological symptoms was sent directly to the laboratory within a period not exceeding 24 h in view of the microbiological studies.

In order to remove the microorganisms present on the cortex, the whole plant (stems, leaves and roots) was washed with tap water, then underwent a series of disinfection with 95% ethanol for 30 s, with sodium hypochlorite 10% and 75% ethanol for 2 min., then rinsed 3 times with sterile distilled water to remove traces of the disinfectant (Evans *et al.*, 2003, Rubini *et al.*, 2005). The superficial tissues were scoured using a scalpel and then crushed using a sterile forceps. A volume of 100 µl was deposited and then spread on the surface of a Petri dish containing nutrient agar (Jasim *et al.*, 2014). At the same time, a Petri dish containing a drop of sterile distilled water from the last washing of the plant served serve as a control. The whole was incubated at 37°C for 24 h. The operation was repeated three times for each of the different parts of *U. dioica* L.

The isolated bacteria were coded by two letters and one number. The first letter derived from the harvest area, and the second from the part of the plant. The number indicates the order in which the bacteria appear.

Macroscopic and biochemical identification. After incubation, the different bacteria associated with the Great Nettle underwent a successive series of transplanting until adequate purification and isolation of the colo-

Table I
Macroscopic and microscopic appearance of isolated bacteria.

Codes	Macroscopic appearance					Microscopic appearance				
	Form	Area	Elevation	Size	Chromo-enesis	Contour	Opacity	Fresh state	Spores	Gram
TS1 TR2 DS2 DL2 DR3	Large round colonies	Rough	Flat	< 4 mm	Yellowish	Irregular	Opaque	Long bacilli in a chain motionless	+	+
TS2 TR1 DS3 DL3 DR3	Medium colonies, rounds	Smooth	Flat	< 2 mm	Beige	Irregular	Opaque	Small bacilli in a chain motionless	+	+
TL6 TS3 TR3 DS1 DL1 DR1	Large colonies	Smooth	Flat	3 mm	Whitish	Irregular	Opaque	Small bacilli in a chain	+	+
TL1 DL4	Small, round and well-insulated colonies	Brilliant	Bomb	> 1 mm	Whitish	Regular	Translucent	Colibacilles, mobile	–	–
TL2	Round	Dried	Slightly bulging	2 mm	Whitish	Regular	Opaque	Cocci very mobile	–	+
TL3	Round colonies	Smooth	Flat	> 1 mm	Whitish	Regular	Opaque	Cocci mobile	–	–
TL4	Small, round colonies Convex	Smooth Brilliant	Bomb	> 1 mm	Whitish	Regular	Opaque	Cocci in cluster	–	+
TL5	Small colonies	Smooth	Flat	> 1 mm	Transparent	Regular	Translucent	Small mobile bacilli	–	–
TR4	Large colonies	Rough	Flat	> 1 mm	Whitish	Irregular	Opaque	Small bacilli	–	+
DR2	Large colonies	Smooth	Flat	< 2 mm	Whitish	Irregular	Opaque	Small, very mobile bacilli	+	+
TR5	Large colonies	Rough	Flat	> 1 mm	Whitish	Irregular	Opaque	Bacillus	–	+

TS = Tlemcen stem, DS = Dellys stem, TR = Tlemcen root, DR = Dellys roots, TL = Tlemcen leaves, DL = Dellys leave

Table II
Biochemical study of isolated bacteria.

Codes	Catalase	Oxidase	Acetoin	Indole	Citrate	Urease	Nitrate	Motility	Mannitol	H_2S	ONPG	TDA	Glucose	Lactose
TS1 TR2 DS2 DL2 DR3	+	–	+	+	–	–	+	–	–	–	–	–	+	–
TS2 TR1 DS3 DL3 DR3	+	+	–	–	+	–	–	+	+	–	+	/	+	–
TL6 TS3 TR3 DS1 DL1 DR1	+	+	+	–	+	–	–	+	+	–	+	–	+	–
DR2	+	+	+	–	–	–	+	+	–	–	–	+	+	–
TL1 DL4	+	–	–	+	–	–	+	+	+	–	+	–	+	+
TR4	+	–	+	–	–	+	–	+	+	–	+	/	+	+
TL3	+	–	+	–	+	–	+	+	+	–	+	–	+	+
TL4	+	–	+	–	+	+	+	–	+	–	–	–	+	+
TL2	–	–	+	–	–	–	+	–	+	–	–	/	+	+
TL5	+	–	+	–	+	–	+	+	+	–	+	+	+	–
TR5	+	+	–	–	+	–	+	+	+	–	+	/	+	+

TS = Tlemcen stem, DS = Dellys stem, TR = Tlemcen root, DR = Dellys roots, TL = Tlemcen leaves, DL = Dellys leaves, + = presence, – = absence.

nies was achieved. The distinction between the different bacteria was based on morphological criteria (form, area, elevation, size, chromogenesis, shape and opacity).

In order to have a first orientation on the identification of the bacterial species detected, we carried out microscopic examinations such as fresh observation and Gram staining. These tests were complemented by the study of some biochemical characteristics (catalase, oxidase, acetoin, indole, citrate, urease, nitrate, motility, mannitol, H_2S, ONPG, TDA, glucose, lactose) that allowed to get closer and closer to the identity of each species.

Identification by MALDI-TOF MS. The matrix was prepared before each series of analysis by diluting a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Sigma H, Lyon, France) in 500 µl of 50% (v/v) acetonitrile, 250 µl of 10% (v/v) trifluoroacetic acid (TFA) and 250 µl of HPLC water. The whole was stirred vigorously, sonicated for 10 min, centrifuged (13 000 × g, 5 min.) and then transferred to a clean polypropylene tube.

Each bacterial colony obtained from a young culture (18 to 24 h) was deposited in duplicate on the MALDI-TOF target plate (Bruker Daltonics TM, Wissembourg, France) and then covered with 1.5 µl of the matrix solution. The whole (target plate and matrix) was dried at room temperature for a few min. and then analyzed (Pfleiderer *et al.*, 2013). A Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) was used for bacterial identification. The spectra of the bacteria obtained were compared with the Bruker computer database using the flexAnalysis v. 3.3 and MALDI-Biotyper v. 3.0 software for data analysis. The isolate was correctly and significantly identified at the species level when the logarithmic score (LSV) was greater than or equal to 1.9 (Seng *et al.*, 2009).

Results

The study of the macroscopic and biochemical aspect gave us a first orientation towards the determination of the bacterial species (Tables I and II).

The efficiency of the disinfection was checked in the control box after 24 h of incubation at 37°C and showed no microbial growth, indicating that the epiphytes were completely removed according to the disinfection protocol.

Based on morphological and biochemical criteria, a total of 57 endophytic bacteria were isolated from

Table III
Identification results by MALDI-TOF/MS.

Codes	Organism best match	Study Area	Numbers			%	Score value (LSV)	Color	Significance of results
			S	L	R				
TS1 TR2 DS2 DL2 DR3	*Bacillus anthracis*	T	2	0	5	20	2.317	G	Correct identification of the genus and species
		D	2	5	0	22.72	2.226	G	
TS2 TR1 DS3 DL3 DR3	*Bacillus megaterium*	T	0	0	5	14.28	2.07	G	
		D	1	2	2	22.72	2.302	G	
TL6 TS3 TR3 DS1 DL1 DR1	*Bacillus pumilus-ME*	T	3	1	1	14.28	2.233	G	
		D	5	2	2	40.90	2.404	G	
DR2	*Bacillus cereus*	D	0	0	1	4.54	2.26	G	
TL1 DL4	*Escherichia coli*	T	0	5	0	14.28	2.354	G	
		D	0	1	0	4.54	2.243	G	
TL5	*Pantoea agglomerans*	T	0	1	0	2.85	2.175	G	
TL3	*Enterobacter amnigenus*	T	0	1	0	2.85	2.293	G	
TL4	*Staphylococcus cohnii*	T	0	1	0	2.85	2.024	G	
TL2	*Enterococcus faecium*	T	0	1	0	2.85	2.405	G	
TR4	*Paenibacillus lautus*	T	0	0	1	2.85	1.875	Y	Correct identification of the genus
TR5	*Paenibacillus glucanolyticus*	T	0	0	1	2.85	1.757	Y	
UN	?	T	2	1	4	20	< 1.7	R	Unidentified
		D	0	0	1	4.54			

TS = Tlemcen stem, DS = Dellys stem, TR = Tlemcen root, DR = Dellys roots, TL = Tlemcen leaves, DL = Dellys leaves, D = Dellys, S = Stem, L = Leaves, R = Root, G = Green, Y = Yellow, R = Red, UN = Unidentified

U. dioica L., among them 35 bacteria from Tlemcen and 22 bacteria from Dellys. These bacteria belong to Bacillaceae, Enterobacteriaceae, Paenibacillaceae, Staphylococcaceae, Enterococcaceae.

The identification of the various isolated bacteria was proved by MALDI-TOF MS. The values of the scores obtained are noted in Table III.

Among the 57 isolates analyzed by MALDI-TOF MS, eight bacteria were not identified. The 11 species identified belong to different families. The results show a dominance of Bacillaceae, represented essentially by four species, namely *Bacillus pumilus-ME*, *Bacillus anthracis*, *Bacillus megaterium* and *Bacillus cereus*. There are followed by Enterobacteriaceae with 3 species (*Escherichia coli*, *Pantoea agglomerans* and *Enterobacter amnigenus*), Paenibacillaceae family with 2 species (*Paenibacillus lautus* and *Paenibacillus glucanolyticus*). The less frequent families are Staphylococcaceae and Enterococcaceae with *S. cohnii* and *E. faecium* respectively.

Analysis of the presence of endophytic bacteria in the two samples of the Great Nettle revealed a heterogeneous distribution of the identified germs.

It appears also that the leaves are richest in endophytic bacteria with 6 species isolated at Tlemcen and 4 species at Dellys. *B. pumilus-ME* is the common species in both regions of the different parts of the Great Nettle (leaves, stems and roots).

As for the effect of the biotope on diversity in endophytic bacteria, it seems that *U. dioica* L. collected in the region of Tlemcen is the richest in bacteria associated with seven genera and eleven species compared to that harvested from Dellys which is represented by 2 genera and 5 species. In addition, 4 bacterial species were isolated from the Great Nettle harvested in both regions. These include *B. anthracis*, *B. megaterium*, *B. pumilus-ME*, and *E. coli*. The 6 endophytic bacteria isolated only from Tlemcen are *P. lautus*, *P. glucanolyticus*, *P. agglomerans*, *E. amnigenus*, *E. faecium* and *S. cohnii*. Finally, *B. cereus* is detected only at Dellys.

Discussion

Endophytic bacteria have already been isolated from medicinal plants by several authors. Indeed, El-deeb *et al*., (2013) working on Shara «*Plectranthus tenuiflorus*», harvested from the Sahara of Saudi Arabia, revealed the presence of a multitude of endophytic bacteria including *Bacillus* sp., *B. megaterium*, *B. pumilus-ME* and *Paenibacillus* sp.

Similarly, Jasim *et al*., (2014) showed the existence of *Bacillus* sp. and *Staphylococcus* sp. in the Ginger rhizome «*Zingiber officinale*». Coêlho *et al*., (2011) isolated *B. cereus* and *B. anthracis* from the seeds and stems of Sumauma «*Ceiba pentandra*» and Mahogany «*Swietenia macrophylla*» from the Amazon.

Furthermore, all species of the genus *Pantoea* can be isolated from fecal matter, soil and plants (Andersson *et al*., 1999), where they may be either pathogenic or commensal (Monier and Lindow, 2005). Among the bacteria of the genus *Pantoea*, *P. agglomerans* is used by plants as a biocontrol agent against phytopathogenic fungi and bacteria (Adriaenssens *et al*., 2011). Although this bacterium is good for plant development, it may also become an opportunistic human pathogen. Cruz *et al*., (2007) have shown that the same species can cause serious infections in children over 6 years of age. According to Kratz *et al*., (2003), Ulloa-Gutierrez *et al*., (2004) *P. agglomerans* is often isolated in humans from soft tissue or bone / joint infections. The transmission of the bacteria to humans is due to trauma caused by plants.

MALDI-TOF mass spectrometry is a technology of microbiology, which makes it possible to identify microorganisms by directly analyzing their proteins. Although MALDI-TOF MS was described by Tshikhudo *et al*., (2013), as the ideal technique for the identification of bacterial cells by the easy determination of peptide fingerprints, De Bruyne *et al*., (2011) report that various factors can influence the quality and reproducibility of bacterial fingerprints, particularly sample preparation, cell lysis method, matrix solutions and organic solvents, which justifies the use of alternative methods to ensure correct identification.

An investigation of the presence of endophytic bacteria from *U. dioica* L. was carried out. The results obtained demonstrate the presence of a diverse endophytic community in the internal tissues of the Great Nettle which are differently distributed within stems, leaves and roots in both regions.

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