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ORIGINAL ARTICLE

Towards a new approach of controlling endophytic bacteria associated with date palm explants using essential oils, aqueous and methanolic extracts from medicinal and aromatic plants

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Abstract

We identified two strains of endophytic bacteria associated with date palm explants by 16S rRNA gene amplification and sequencing, and we explored different approaches to control them. Based on their 16S sequences, the two isolates were identified as *Microbacterium testaceum* and *Serratia marcescens*. Antibacterial activity of essential oils, methanolic and aqueous extracts, from seven plant species against these endophytic bacteria was studied using different methods. The essential oils and the aqueous extracts of *Artemisia herba-alba, Rosmarinus officinalis* and *Thymus satureioides* inhibited the growth of both isolates through the disc diffusion method. The inhibition zones ranged from 18 to 31.5 mm and from 5 to 7 mm for essential oils and aqueous extracts, respectively. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values ranged from 0.025 to 0.033% and 0.033 to 0.05%, respectively. None of the methanolic extracts had any activity against the bacteria. The incorporation of the extracts into the culture medium showed different results depending on culture phase. During the induction phase, none of the extracts was able to inhibit the bacterial growth without causing phytotoxicity. During shoot bud multiplication, only the essential oils of *A. herba-alba* at the concentration of 0.1% inhibited the bacterial growth without causing phytotoxicity. Furthermore, the explants showed normal growth with an average number of 13.1 shoot buds per explant. The use of extract-impregnated plugs showed no inhibitory activity against the bacteria, whereas immersing explants in the antibacterial solutions caused browning and death of plant tissues.

Key message

The endophytic bacteria observed during date palm organogenesis and somatic embryogenesiswere identified for the first time ever using 16S sequencing, and a new biological and efficientapproach to control it was developed.

Keywords 16S sequencing · Endophytic bacteria · Organogenesis · Plant extracts · *Phoenix dactylifera* L.

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Introduction

Date palm is a fruit tree species native to the Middle East, but it is cultivated in many other areas of the world such as North Africa, South Asia, Spain and the United States of America (Krueger 2015). It is grown for its delicious and high-nutrient fruits, and can be used as forage and as a source of fiber and fuel (Krueger 2011). In addition, date palm is highly adaptable to arid areas, where it contributes to preserving their ecosystems threatened by desertification, creates equable microclimate for agriculture and contributes to increasing the income of their inhabitants (Jain 2011; Masmoudi-Allouche et al. 2011; Sedra and Lazrek 2011). These attributes make date palm an important crop on agronomic, economic and ecological levels.

Among date palm cultivars, Mejhoul cv. is the most popular and most sought after cultivar in the world (Sedra 2015). In Morocco, this variety is threatened by bayoud, a severe wilt disease caused by *Fusarium oxysporum* f. sp. *albedinis*. Unfortunately, bayoud has caused a dramatic reduction in the population of cv. Mejhoul (Sedra 2011). To date, the only feasible way to preserve this cultivar is through rapid and large-scale propagation followed by plantation in bayoud-free areas. Along this line, the use of in vitro culture techniques such as somatic embryogenesis and organogenesis might support large-scale multiplication of cv. Mejhoul plants (Mazri and Meziani 2015). The organogenesis technique has the advantage of producing true to type plantlets. The effects of various factors on cv. Mejhoul organogenesis have been evaluated, and protocols for in vitro shoot bud multiplication and plantlet regeneration were reported (Mazri et al. 2016, 2018; Meziani et al. 2015, 2016). However, producing date palm plants through organogenesis is hampered by some physiological disorders such as hyperhydricity, tissue browning, precocious rooting (Mazri and Meziani 2013; Mazri 2014, 2015; Meziani et al. 2016) as well as contaminations with endophytic bacteria of date palm, which can cause drastic losses during the organogenesis process.

Based on cultural and biochemical approaches, previous works identified endophytic bacteria associated with date palm during in vitro propagation as *Bacillus sp*. (Leary et al. 1986). However, molecular-based identification of these bacteria has never been undertaken. Recent advances in new generation sequencing technologies and the growing body of available reference sequence data offer the opportunity for more accurate identification of bacteria that contaminate date palm explants. Generally, contaminations become visible 1 month into the culture process, and can occur even on healthy and thoroughly disinfected explants (Abahmane 2011). To date, reports on the use of chemical or natural additives to inhibit the growth of these bacteria are very limited. Therefore, developing methods to control these bacteria can contribute significantly to improving date palm multiplication using tissue culture techniques.

The antibacterial effects of plant extracts have been previously reported. Specifically, essential oils and other extracts from medicinal and aromatic plants have been shown to have antimicrobial properties. For example, the antibacterial activity of *Artemisia herba-alba* (Yashphe et al. 1979), *Rosmarinus officinalis* (Celiktas et al. 2007), *Thymus satureioides* (Tantaoui-Elaraki et al. 1993), and *Lawsonia inermis* (Babu and Subhasree 2009) has been scientifically proven. However, the effects of such plants against endophytic bacteria of date palm have never been investigated. Therefore, the use of plant extracts can offer new opportunities to develop effective management strategies against contaminations of palm tissues with endophytic bacteria.

The purposes of the present study were to (1) isolate and identify endophytic bacteria associated with date palm during in vitro organogenesis; (2) determine the antibacterial activity of the methanolic and aqueous extracts as well as the essential oils of *Artemisia herba-alba, Lawsonia inermis, Rosmarinus officinalis, Thymus satureioides, Acacia tortilis* subsp. *raddiana, Ormenis africana* and *Zygophyllum gaetulum* against these bacteria. Though these medicinal and aromatic plant species are abundant in Errachidia and Zagora regions (Morocco), their potential antimicrobial properties have not been fully studied; and (3) evaluate the effects of these plant extracts on date palm organogenesis.

Materials and methods

Plant material and extract preparation

Plant material

A. herba-alba, A. tortilis subsp. *raddiana, O. africana, R. officinalis, T. satureioides, Z. gaetulum* and *L. inermis* were collected in May 2016 from the regions of Errachidia (32°20′34.4″N 4°08′27.2″W) and Zagora (30°22′18.1″N 5°47′44.8″W), Morocco. Either the leaves or seeds of these plant species were used (Table 1). The leaves and seeds were thoroughly washed with sterile distilled water, dried in the darkness at room temperature for 15 days then ground to powder.

Methanolic and aqueous extract preparation

The dried material was macerated in methanol or in sterile distilled water (10 g dried powder:100 mL methanol or sterile distilled water, w/v) for 48 h at room temperature then filtered through Whatman no. 1 filter paper. The solvents

Table 1 Plant species used against the endophytic bacteria of date palm

Plant species	Location of collection	Date of collection	Plant part used for extraction	Extract used	Yield % (w/w)
Acacia tortilis subsp. *raddiana*	Zagora	May 5th, 2016	Seed	Methanolic extract	7.19
				Aqueous extract	6.20
Artemisia herba-alba	Errachidia	May 30th, 2016	Leaf	Methanolic extract	5.15
				Aqueous extract	4.98
				Essential oils	1.05
Lawsonia inermis	Zagora	May 5th, 2016	Leaf	Methanolic extract	5.47
				Aqueous extract	4.02
Ormenis Africana	Zagora	May 5th, 2016	Leaf	Methanolic extract	6.76
				Aqueous extract	6.93
Rosmarinus officinalis	Errachidia	May 30th, 2016	Leaf	Methanolic extract	5.60
				Aqueous extract	4.90
				Essential oils	1.27
Thymus satureioides	Errachidia	May 30th, 2016	Leaf	Methanolic extract	5.40
				Aqueous extract	4.12
				Essential oils	1.35
Zygophyllum gaetulum	Zagora	May 5th, 2016	Leaf	Methanolic extract	5.87
				Aqueous extract	7.89

were evaporated by rotary evaporator and the residue was recovered and stored at 4 °C for later use.

Essential oil extraction

The plant material was dried in shade for 15 days then ground to powder. The dried ground powder (100 g) was Clevenger-hydrodistilled as described by Roohinejad et al. (2018). Briefly, the ground powder was mixed with water in a flask of 2 l capacity then boiled for 3 h. Steam and essential oil vapors passed through a 40 cm length refrigerant and cooled by condensation. The essential oils and water were then separated by decantation. The recovered essential oils were stored in opaque containers at 4 °C for later use.

Antibacterial activity determination

Isolation and identification of bacterial strains

Two morphologically distinct bacterial strains, which appear in the culture medium as transparent white and white–pink zones, were used in this study. These two bacterial strains grow in the culture medium around explants and adventitious roots (Fig. 1a) and may cause drastic losses during in vitro propagation of date palm. Both strains were isolated from the culture medium around explant roots and streak-purified on LPGA medium (7 g L^{-1} yeast extract, 7 g L^{-1} peptone, 7 g L^{-1} glucose and 18 g L^{-1} agar). Genomic DNA of a single colony derived strain was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer instructions with minor modifications, and 16S rRNA genes were amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCCGCA-3′) in a 50 µL reaction volume using PCR Supermix High Fidelity master mix (Invitrogen, Carlsbad, CA) with 10 pM each primer and 100 ng template DNA following the thermocycling protocol of Takeuchi et al. (1996). Amplicons were pair end Illumina sequenced at the National Center for Scientific and Technical Research (CNRST, Rabat, Morocco). For each strain, forward and reverse sequences were trimmed to 800 bp of good quality sequence and aligned using MEGA (v5.2; Tamura et al. 2011) to construct consensus sequences. The two strains were BLAST-identified by comparing their 16S rRNA gene sequences to existing sequence databases.

Determination of extract antibacterial activity by the paper disc diffusion method

The two strains were characterized for their sensitivity to the three types of plant extracts used in this study. For each type of extract, 6 mm diameter sterile filter paper discs were soaked with 5 µL of that extract and placed on LPGA plates immediately after 100 µL of bacterial suspension was spread-plated. Sterile distilled water-soaked filter was used as control test. After 2 days of growth at 27 °C, growth inhibition zone sizes were measured as the radius from the edge of the disc to the edge of any clear inhibition zone. Each strain-extract interaction was replicated three

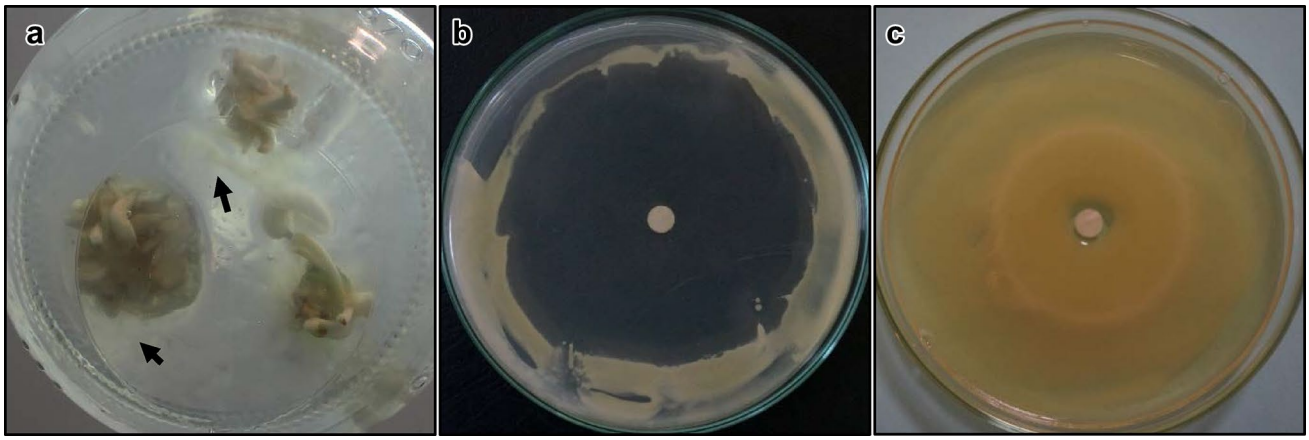

Fig. 1 Antibacterial activity of plant extracts against *Microbacterium testaceum* by the paper disc diffusion method. The bacterial strains were isolated from the culture medium used for date palm organogenesis then cultured on LPGA medium. **a** *Microbacterium testaceum* strains in the culture medium around explants (highlighted with arrows). **b** Antibacterial effectiveness of the essential oils of *Thymus satureioides*. **c** Antibacterial effectiveness of the aqueous extracts of *Thymus satureioides*

times, and mean inhibition zone sizes (Is) were determined. Interactions were considered not inhibitory (−) for Is < 8 mm, inhibitory (+) for 9 mm < Is < 14 mm, highly inhibitory (++) for 15 mm < Is < 19 mm, and extremely inhibitory (+++) for Is > 20 mm (Ponce et al. 2003).

Determination of extract antibacterial activity by volatile phases

Sterile filter paper discs (6 mm diameter) were impregnated with 5, 10 or 15 µL of the extracts then placed on the inner surface of the inverted lid of Petri dishes. The Petri dishes were closed and incubated at 27 °C. The inhibitory effect of the volatile extracts was determined by the measurement of colony diameter (mm) after 2 days incubation. As a control, filter paper discs impregnated with sterile distilled water were used. All analyses were applied in triplicate.

Determination of the minimum inhibitory concentration (MIC) and the minimum bacteriocidal concentration (MBC) of extracts

In order to determine the MIC and the MBC of extracts, solutions were prepared by mixing 100 µL of each extract and 900 µL of 0.2% (w/v) agar agar. Afterwards, different concentrations (0.0125–0.2%) of each solution were added to the bacterial suspensions. The MIC was defined as the lowest concentration that inhibited bacterial growth after incubation at 27 °C for 24 h, whereas the MBC was defined as the lowest concentration of plant extracts that killed ≥ 99.9% of the original inoculum.

Effects of plant extracts on date palm organogenesis

Plant material and culture procedure

Plantlets were produced according to the organogenesis protocol previously described by Meziani et al. (2016). Briefly, offshoots of date palm cv. Mejhoul (3-year-old) were collected from Erfoud, Morocco. The shoot tip was immediately extracted and disinfected by immersion in a solution of 0.03% (w/v) potassium permanganate in commercial liquid chlorine bleach for 20 min, followed by three rinses in sterile distilled water for 10 min. Shoot tip explants were excised and cultured on half-strength Murashige and Skoog medium (1/2MS; Murashige and Skoog 1962) supplemented with 14.2 µM indole-3-acetic acid (IAA), 13.4 µM 1-naphthaleneacetic acid and 0.5 µM 6-(dimethylallylamino)purine (2iP) (induction medium) for 9 months (darkness; 25 °C). Afterwards, established shoot buds were cultured on 1/2MS supplemented with 0.9 µM 2-naphthoxyacetic acid, 1.1 µM IAA, 1.8 µM kinetin and 1.9 µM 2iP (multiplication medium) for 3 months (16 h photoperiod; 13.5 µmol m^{-2} s^{-1} light intensity; 25 °C). Shoots were singled out and transferred to hormone-free 1/2MS medium for 3 months (16 h photoperiod; 40 µmol m^{-2} s^{-1} light intensity; 25 °C). The rooted plantlets were acclimatized as described by Mazri et al. (2017). All media were supplemented with 1.5 g L^{-1} polyvinylpyrrolidone, 30 g L^{-1} sucrose and gelled with 6 g L^{-1} agar. The medium pH was adjusted to 5.8 prior to autoclaving at 121 °C for 25 min.

Bacterial elimination by supplementing the culture media with plant extracts

1-month-old-contaminated explants grown on either induction or multiplication medium were subcultured onto media containing various concentrations of plant extracts. The extracts were incorporated into autoclaved culture media (121 °C for 25 min) using Millipore filters at concentrations ranging from 0% (control) to 0.5% (with increment by 0.1%) and 1%. Each contaminated explant was placed into a 55-mM Pyrex brand test tube, containing 13 mL culture medium. All treatments were replicated ten times.

Bacterial elimination by immersing explants in plant extracts

One-month-old-contaminated explants grown on either induction or multiplication medium were immersed for 5 s in water-diluted plant extracts using the same concentrations as explained above (0–0.5% and 1%). The dilutions were prepared using sterile distilled water. Each explant was subcultured into a test tube containing 13 mL culture medium. All treatments were replicated ten times.

Bacterial elimination by soaking sterile cotton plugs with plant extracts

One-month-old-contaminated explants grown on either induction or multiplication medium were placed in test tubes containing 13 mL culture medium (one explant per tube). The tubes were plugged with sterile cotton previously soaked with plant extracts for 5 s then used to plug test tubes at the same concentrations as explained earlier (0–0.5% and 1%). Sterile distilled water was used for dilution preparation. The experiment was conducted in ten replicates per treatment.

Data collection and statistical analysis

After 9 months of culture on the induction medium and 3 months of culture on the multiplication medium, the rates of bacteria elimination and explant phytotoxicity were calculated. At the end of the multiplication phase, the average number of shoot buds per explant was calculated. Phytotoxicity of plant extracts was determined visually by checking for tissue browning.

All experiments were conducted using a completely randomized design. Data were subjected to analysis of variance using SPSS v. 21 (IBM SPSS Inc., Chicago, IL, USA). The Student–Newman–Keuls test was used for mean separation at 5% probability. Percentage data values were arcsin-transformed before running analyses.

Results

Yield of extracts

The yields of methanolic and aqueous extracts and those of essential oils differed between plant species (Table 1). The yields of methanolic extracts ranged from 5.15% for *A. herba-alba* leaves to 7.19% for the seeds of *A. tortilis* subsp. *raddiana*. In regards to aqueous extracts, the yields varied between 4.02% in the leaves of *L. inermis* and 7.89% in those of *Z. gaetulum*. The yields of essential oils were 1.05, 1.27 and 1.35% for *A. herba-alba, R. officinalis* and *T. satureioides*, respectively.

Bacteria identification

Based on alignment of their 16S sequences with reference sequences from the NCBI Genebank database, the two bacterial strains associated with date palm tissue during in vitro propagation showed highest identities of 97% and 96% to *Microbacterium testaceum* and *Serratia marcescens*, respectively.

Antibacterial activity determination

Determination of extract antibacterial activity by the paper disc diffusion method

The essential oils of *T. satureioides* showed the maximum antibacterial activity with an inhibition zone of 31.5 mm (Fig. 1b). This was followed by the essential oils of *A. herba-alba* (18.5 mm) and *R. officinalis* (18 mm). The aqueous extracts of *A. herba-alba, R. officinalis* and *T. satureioides* exhibited a moderate activity with an inhibition zone ranging from 5 to 7 mm (Fig. 1c). The methanolic extracts of all plants did not show any antibacterial activity (Table 2).

Determination of extract antibacterial activity by volatile phases

In volatile phase, neither the essential oils nor the methanolic and aqueous extracts inhibited the growth of the bacteria (Table 2). According to these results, the volatile phase cannot be used to eliminate the endophytic bacteria of date palm.

Table 2 Antibacterial activity of plant extracts: inhibition zone size, minimum inhibitory concentration and minimum bacteriocidal concentration against the endophytic bacteria of date palm

Antibacterial assay	Plant extract*	*Microbacterium testaceum*			*Serratia marcescens*		
		Is*	MIC* (%)	MBC* (%)	Is	MIC (%)	MBC (%)
Paper disc diffusion	*A. tortilis* subsp. *raddiana* ME	NI	–	–	NI	–	–
	A. tortilis subsp. *raddiana* AE	NI	–	–	NI	–	–
	A. herba-alba ME	NI	–	–	NI	–	–
	A. herba-alba AE	–	–	–	NI	–	–
	A. herba-alba EO	++	0.033	0.05	++	0.033	0.05
	L. inermis ME	NI	–	–	NI	–	–
	L. inermis AE	NI	–	–	NI	–	–
	O. Africana ME	NI	–	–	NI	–	–
	O. Africana AE	NI	–	–	NI	–	–
	R. officinalis ME	NI	–	–	NI	–	–
	R. officinalis AE	–	–	–	NI	–	–
	R. officinalis EO	++	0.025	0.05	++	0.033	0.05
	T. satureioides ME	NI	–	–	NI	–	–
	T. satureioides AE	–	–	–	NI	–	–
	T. satureioides EO	+++	0.025	0.033	++	0.033	0.05
	Z. gaetulum ME	NI	–	–	NI	–	–
	Z. gaetulum AE	NI	–	–	NI	–	–
	Sterile distilled water (control)	NI	–	–	NI	–	–
Volatile phase	*A. tortilis* subsp. *raddiana* ME	NI	–	–	NI	–	–
	A. tortilis subsp. *raddiana* AE	NI	–	–	NI	–	–
	A. herba-alba ME	NI	–	–	NI	–	–
	A. herba-alba AE	NI	–	–	NI	–	–
	A. herba-alba EO	NI	–	–	NI	–	–
	L. inermis ME	NI	–	–	NI	–	–
	L. inermis AE	NI	–	–	NI	–	–
	O. Africana ME	NI	–	–	NI	–	–
	O. Africana AE	NI	–	–	NI	–	–
	R. officinalis ME	NI	–	–	NI	–	–
	R. officinalis AE	NI	–	–	NI	–	–
	R. officinalis EO	NI	–	–	NI	–	–
	T. satureioides ME	NI	–	–	NI	–	–
	T. satureioides AE	NI	–	–	NI	–	–
	T. satureioides EO	NI	–	–	NI	–	–
	Z. gaetulum ME	NI	–	–	NI	–	–
	Z. gaetulum AE	NI	–	–	NI	–	–
	Sterile distilled water (control)	NI	–	–	NI	–	–

**AE* aqueous extract, *EO* essential oils, *ME* methanolic extract, *Is* inhibition zone size, *MBC* minimum bacteriocidal concentration, *MIC* minimum inhibitory concentration, *NI* no inhibition

– for a diameter less than 8 mm

++ for a diameter ranging from 15 to 19 mm

+++ for a diameter larger than 20 mm

Determination of the MIC and MBC values of different extracts

Table 2 shows the MIC and the MBC results of the antibacterial assays against the endophytic bacteria of date palm. The MIC values ranged from 0.025 to 0.033% while the MBC values ranged from 0.033 to 0.05%. The essential oils of *T. satureioides* appeared to be the most active compound, with a MIC value of 0.025% and a MBC value of 0.033%.

Effects of plant extracts on date palm organogenesis

Bacterial elimination by supplementing the culture media with plant extracts

None of the extracts at any concentration inhibited bacterial growth during explant induction without causing phytotoxicity (Table 3; Fig. 2). This suggests that these extracts are not appropriate for controlling bacterial contamination during the induction phase. During shoot bud multiplication, all the studied concentrations (0.1–1%) were able to inhibit the growth of the endophytic bacteria. However, high concentrations of essential oils were toxic to explants. For example, the concentration of 1% caused browning and death after only 6 days of culture. Interestingly, we found that the essential oils of *A. herba-alba* showed no phytotoxicity at the concentration of 0.1%. This was followed by the essential oils of *R. officinalis* (10% mortality at 0.1%; Table 4). With regard to shoot bud multiplication, the highest average number of shoot buds per explant was 13.1 and was observed when the concentration of 0.1% of *A. herba-alba* essential oils was used (Table 4). The shoots showed normal growth during the elongation and rooting phase and a survival rate of 86% was observed after acclimatization.

Fig. 2 Explant phytotoxicity during the induction phase. The culture medium contained 0.5% of the essential oils of *Artemisia herba-alba*

Bacterial elimination by immersing explants in plant extracts

Immersing explants in the antibacterial solutions appeared to be phytotoxic. In fact, regardless of the extract used, all the concentrations (0.1–1%) used in this study caused explants to turn brown and die during the first week of culture.

Table 3 Effect of different plant extracts on explant contamination and phytotoxicity during the induction phase

Plant extract	Extract concentration in the culture medium (%)	Explant contamination after 9 months of culture (%)	Phytotoxicity* (%)
Control	0	100 a	0 a
A. herba-alba EO	0.1	100 a	0 a
	0.2	100 a	0 a
	0.3	100 a	0 a
	0.4	0 b	100 c
	0.5	0 b	100 c
	1	0 b	100 c
R. officinalis EO	0.1	100 a	0 a
	0.2	100 a	0 a
	0.3	100 a	0 a
	0.4	0 b	100 c
	0.5	0 b	100 c
	1	0 b	100 c
T. satureioides EO	0.1	100 a	0 a
	0.2	100 a	10 ± 31.6 b
	0.3	100 a	20 ± 42.1 b
	0.4	0 b	100 c
	0.5	0 b	100 c
	1	0 b	100 c

*The values are means ± SD (n = 10). Different letters indicate significant differences among the studied parameters (Student–Newman–Keuls $P \leq 0.05$)

Table 4 Effect of different plant extracts on explant contamination, phytotoxicity and shoot bud proliferation during the multiplication phase

Plant extract	Extract concentration in the culture medium (%)	Explant contamination after 3 months of culture (%)	Phytotoxicity (%)*	Average number of shoot buds per explant after 3 months of culture*
Control	0	100 a	0 a	–
A. herba-alba EO	0.1	0 b	0 a	13.1 ± 1.9 a
	0.2	0 b	40.0 ± 51.6 ab	11.5 ± 1.0 ab
	0.3	0 b	60.0 ± 51.6 bc	11.3 ± 1.5 ab
	0.4	0 b	70.0 ± 48.3 bc	10.5 ± 1.2 ab
	0.5	0 b	70.0 ± 48.3 bc	10.3 ± 1.5 ab
	1	0 b	100 c	–
R. officinalis EO	0.1	0 b	10 ± 31.6 a	12.3 ± 0.8 ab
	0.2	0 b	60 ± 51.6 bc	10.2 ± 2.6 ab
	0.3	0 b	70.0 ± 48.3 bc	9.3 ± 1.5 b
	0.4	0 b	70.0 ± 48.3 bc	9.3 ± 0.5 b
	0.5	0 b	100 c	–
	1	0 b	100 c	–
T. satureioides EO	0.1	0 b	60 ± 51.6 bc	9.5 ± 1.7 b
	0.2	0 b	100 c	–
	0.3	0 b	100 c	–
	0.4	0 b	100 c	–
	0.5	0 b	100 c	–
	1	0 b	100 c	–

*The values are means ± SD (n = 10). Different letters indicate significant differences among the studied parameters (Student–Newman–Keuls $P \leq 0.05$)

Bacterial elimination by soaking sterile cotton plugs with plant extracts

Regardless of the extract used, this method was not effective in eliminating the bacteria. In fact, the bacteria reappeared in the culture medium after each subculture. This shows that the direct contact method is the only efficient way to eliminate the endophytic bacteria of date palm.

Discussion

Medicinal and aromatic plants have attracted much attention over the past decades because of their various properties. This has led many researchers to investigate the antimicrobial effectiveness of their products such as organic acids, essentials oils, methanolic and aqueous extracts, among others (El Asbahani et al. 2015; Inoue and Craker 2014; Viuda-Martos et al. 2011). In fact, many studies have been conducted to identify natural antimicrobials able to replace the synthetic ones (Abdallah 2011). Herein, the in vitro antibacterial activity of various plant extracts against the endophytic bacteria of date palm as well as the approach used to treat contaminated explants were investigated.

Based on biochemical approaches as well as colony morphology and cultural characteristics, the endophytic bacteria associated with date palm during micropropagation have been previously reported to belong to the genus *Bacillus* (Leary et al. 1986; Charkaoui 1997). To the best of our knowledge, identification of these strains using 16S rRNA gene amplification and sequencing has never been undertaken. Our results revealed that these bacterial strains belong to *Microbacterium testaceum* and *Serratia marcescens*. These endophytic bacteria were also isolated from other plants such as potato and rice, respectively (Gyaneshwar et al. 2001; Morohoshi et al. 2011).

As far as we know, the antibacterial activity of extracts from *A. herba-alba, L. inermis, R. officinalis, T. satureioides, A. tortilis* subsp. *raddiana, O. africana* and *Z. gaetulum* has never been evaluated against the endophytic bacteria of date palm, even though the antibacterial activity of some of these plant species was already demonstrated against other bacteria (Babu and Subhasree 2009; Celiktas et al. 2007; Tantaoui-Elaraki et al. 1993; Yashphe et al. 1979), confirming the antibacterial activities found in this study.

In previous works, it was found that different types of extracts from the same plant might have different effects on bacterial growth (Najjaa et al. 2007). This is in agreement with the results of our work. In fact, the antibacterial activity of essential oils was more potent than that of aqueous extracts. Essential oils, which are a group of secondary metabolites, possess potent bioactive properties, including

a natural protection against pathogens (Laborda et al. 2013). They are mainly composed of terpenoids and phenylpropanoids, and the most abundant terpenoids in essential oils are monoterpenes (De Sousa 2011). Surprisingly, all of the methanolic extracts displayed no activity against the endophytic bacteria of date palm. This may be due to the extraction procedure and the solvent used, and their effects on the final chemical composition of the extracts. Indeed, it was reported that extraction conditions have a strong effect on the chemical composition of the extracts (Bittencourt et al. 2015).

In the present study, it was found that the volatile phase is not effective against the endophytic bacteria of date palm. Indeed, the antibacterial activity of extracts was relevant only in the case of disc diffusion method (the contact phase). It appears that the effectiveness of these methods depends on the plant extract and the bacteria/fungi type. For example, Shao et al. (2013) found that the volatile phase of tea tree oil is more toxic than its contact phase to *Botrytis cinerea*. Soylu et al. (2010) also reported that the volatile phase of the essential oils of various plant species is more effective than the contact phase against *Botrytis cinerea*. Along this line, it was reported that the volatile phase of the essential oils of plant species possesses more antimicrobial activity against plant pathogenic fungi and bacteria (Edris and Farrag 2003; Soylu et al. 2005). On the other hand, Ojaghian et al. (2016) reported that both the volatile and contact phases of E-cinnamaldehyde were able to significantly reduce the growth of *Sclerotinia sclerotiorum* after 6 days. Our results suggested that the contact phase is more effective than the volatile phase against the endophytic bacteria of date palm. We also found that the MIC and MBC values varied with the plant species and the extract used. This might be due to the different chemical composition of each plant extract, which influences the antibacterial activity effectiveness.

It is well known that the endophytic bacteria of date palm hamper the propagation of this species through either organogenesis (Abahmane 2011) or somatic embryogenesis (Abd-El Kareim 2009). Studies related to controlling the growth of these bacteria are very scarce. Abd-El Kareim (2009) used actinomycetes (*Streptomyces bobilii* and *S. chloramphenicol*) at different concentrations during date palm somatic embryogenesis to control these bacteria. Al-Mussawii (2010) and Al-Dosary et al. (2011) reported the efficiency of four different antibiotics (amoxicillin, chloramphenicol, gentamycin and streptomycin) against these bacteria while Benjama and Charkaoui (1997) used a combination of 20 mg L^{-1} novobiocin and 10 mg L^{-1} gentamycin to inhibit their growth. These authors reported that increasing the concentration of antibiotics resulted in severe phytotoxicity towards explants. This is in good agreement with our results. In fact, we found that using natural compounds at high concentrations also causes phytotoxicity.

In the present study, various plant extracts were used and very interesting results were obtained with the essential oils of *A. herba-alba*. However, the approach used to treat contaminated explants showed different effects on bacterial growth and explant reactivity. Indeed, the incorporation of plant extracts into the culture medium was the only efficient approach to inhibit bacterial growth. The use of extract-impregnated plugs was not efficient in inhibiting the growth of bacteria while immersing explants in plant extracts caused them to turn brown and die.

Regarding shoot bud induction and multiplication, Benjama and Charkaoui (1997) reported that the use of antibiotics does not have a negative effect on the multiplication rate. In our case, this depends on the plant species, the extract concentration and the culture phase. For example, during the induction phase, none of the extracts was able to inhibit the bacterial growth without causing phytotoxicity. During the multiplication phase, the use of *A. herba-alba* essential oils at 0.1% did not show any negative effect on shoot bud multiplication. This shows that explants in induction are more sensitive to phytotoxicity than shoot buds in multiplication. Comparing our results (13.1 shoot buds per explant) with previous findings on date palm organogenesis, in which uncontaminated explants were used (13 shoot buds per explant; Meziani et al. 2016), it can be noted that the multiplication rates are almost identical. In addition, the shoots showed normal growth during the elongation and rooting phase, and a survival rate of 86% was observed after acclimatization. This is consistent with previous works on date palm cv. Mejhoul organogenesis, when the survival rates reported after acclimatization ranged from 70 to 97.5% (Mazri et al. 2016; Meziani et al. 2015, 2016). All these findings indicate that the use of *A. herba-alba* essential oils at the concentration of 0.1% inhibit the growth of the endophytic bacteria of date palm during the multiplication phase without affecting the regeneration capacity of explants.

Conclusions

In conclusion, we identified the endophytic bacteria associated with date palm explants using 16S sequencing. Our results suggest the possibility of using the essential oils of *A. herba-alba* against *Microbacterium testaceum* and *Serratia marcescens* during the multiplication phase, which inhibited the bacterial growth at the concentration of 0.1%, with no apparent phytotoxicity in explants. Further studies are currently underway to determine the chemical composition of the essential oils used in the present study and to evaluate their activity against other microbial contaminants that hamper date palm micropropagation.

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Author contributions RM, MAM, CA and FJ conceived and designed research. RM, HEI and ML prepared plant extracts and essential oils. RM, AE, HEI and ML performed antibacterial activity experiments. RM, MAM, HEI and ML performed organogenesis experiments. GD and FG performed DNA sequencing and bacteria identification. MAM and AE wrote the manuscript. MAM conducted statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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