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ORIGINAL PAPER

Characterization of endophytic *Bacillus* strains from tomato plants (*Lycopersicon esculentum*) displaying antifungal activity against *Botrytis cinerea* Pers

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Abstract Eighty endophytic bacteria were isolated from healthy tissues of roots, stems, leaves and fruits of tomato plants (*Lycopersicon esculentum*). Four strains, named BL1, BT5, BR8 and BF11 were selected for their antagonism against *Botrytis cinerea*, a phytopathogenic fungus responsible of gray mold in several important crops, with growth inhibitory activity ranging from 27 to 53 %. Morphological, biochemical, and molecular parameters as 16S rDNA sequencing demonstrated that the selected bacterial strains were related to *Bacillus* species which are known to produce and secrete a lot of lipopeptides with strong inhibitory effect against pathogen mycelial growth. Electrospray mass spectrometry analysis showed that these strains produced heterogeneous mixture of antibiotics belonging to fengycin and surfactin for BL1 and BT5, to iturin and surfactin for BR8, to bacillomycin D, fengycin and surfactin for BF11. Furthermore, these bacteria exhibited biocontrol potential by reducing the disease severity when tested on detached leaflets. Based on their antifungal activity against *Botrytis cinerea*, these strains could be used for biological control of plant diseases.

Keywords *Bacillus* sp. · *Botrytis cinerea* · Endophytic bacteria · Antifungal activity · Lipopeptides · Tomato

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Introduction

Botrytis cinerea is a necrotrophic pathogen that infects more than 200 plant species resulting in significant yield losses (Elad et al. 2004). It is the causal agent of gray mold diseases in many economically important fruits, vegetables, and flowers. Control of plant diseases still relies on the use of synthetic fungicides. However, the emergence of fungicide resistance that conducts to the loss of effectiveness of these compounds (Rosslenbroich and Stuebler 2000), the potential harmful effects of fungicides on the environment as well as on human health and the increasing public demands for reduction in pesticide use (Ippolito and Nigro 2000), emphasize the need for alternative disease control strategies. Among explored alternatives, the use of microbial biocontrol agents has shown significant potential.

Endophytic bacteria colonize healthy plant tissues intercellularly and/or intracellularly without causing any obvious symptoms of infections or diseases (Fisher and Petrini 1987). Plants constitute vast and diverse niches that can be exploited by an extensive variety of microorganisms including endophytes (Azevedo et al. 2000). Both Gram-positive and Gram-negative bacteria endophytes have been isolated from a large diversity of plants, e.g., citrus (Kalai-Grami et al. 2014), grapevines (Bell et al. 1995), maize (Araújo et al. 2000), potato (Sessitsch and Berg 2004), rice (Stolzfus et al. 1997), tomato (Pillay and Nowak 1997) and wheat (Coombs and Franco 2003). Such bacteria are well adapted to live inside the plant and therefore could behave as efficient biological control agents (Lin et al. 2013). Many endophytic bacteria are able to produce bioactive compounds that exert antifungal activity against plant pathogenic fungi such as *Fusarium*, *Botrytis cinerea, Phoma tracheiphila* and bacterial genuses such as *Lactobacillus*, *Xanthomonas*, *Pseudomonas* and *Bacillus*.

Bacillus species are ubiquitous and involved in the degradation of organic polymers in soil (Emmert and Handelsman 1999). Several strains belonging to this genus have shown great promises in the control of a wide range of phytopathogenic fungi owing to their production of lipopeptides as iturin, surfactin and fengycin, or proteases and chitinase that degrade fungal structural polymers, and antifungal volatiles (Katz and Demain 1977). Moreover, this genus forms endospores that are resistant to desiccation, heat, UV irradiation, and organic solvents (Sadoff 1972) and constitutes therefore an ideal candidate for use in biocontrol.

In the present study, endophytic bacteria that inhibit the growth of *Botrytis cinerea* were isolated from healthy tomato plants and characterized by biochemical, physiological and molecular tools. The lipopeptide antibiotics were extracted from the culture filtrate of these strains and characterized by electrospray ionization–mass spectrometry (ESI–MS). The effect of endophytic bacteria on disease severity reduction was tested on detached tomato leaflets.

Materials and methods

Fungal pathogen culture

A strain of *Botrytis cinerea*, provided by Dr. Hajlaoui (National Institute of Agronomic Research, Tunisia), was used in these experiments. It was routinely grown on potato dextrose agar (PDA, FLUKA) for 10 days at 25 °C under light. Conidial suspension was obtained by flooding the fungal culture with sterile distilled water containing 0.01 % tween 80, gentle rubbing of the mycelium and filtering through four layer cheesecloth. Conidia concentration was determined with a haemacytometer and adjusted to 10^6 conidia ml^{-1} with sterile distilled water.

Isolation of endophytic bacteria

Endophytic bacterial strains were isolated from various organs of healthy tomato plants. Leaves, roots, stems and fruits were sterilized for 5 min with 1 % sodium hypochlorite solution and 70 % ethanol for 1 min and washed 3 times with sterile distilled water. Each sample was aseptically cut into small pieces and deposited on Petri dishes containing Luria–Bertani medium (LB). After plates' incubation for 2 days at 30 °C, representative colonies were streaked onto new LB plates. Bacteria were stored at −20 °C in LB broth containing 25 % glycerol.

Dual culture assay

Each isolate was streaked at the middle of a Petri plate containing PDA medium and a mycelial disk (4 mm) from the peripheral region of 7 days old *Botrytis cinerea*'s culture was placed at approximately 2 cm from the bacteria. Plates were incubated at 25 °C for 7 days and bioactivity evaluated by measuring the percentage of growth inhibition (Korsten et al. 1995) as indicated below:

$$GI\% = 100\frac{R1 - R2}{R1}$$

R1 is the distance of fungal growth from the point of inoculation to the colony on control plates, and R2, the distance of fungal growth from the point of inoculation to the direction of the antagonist. Selected strains were identified using biochemical and molecular tools. Control plates were not inoculated with bacteria.

Antifungal activity of cell-free supernatant

To evaluate the antifungal activity of cell-free supernatant, the well diffusion method was used (Kalai-Grami et al. 2013). Disks of 4 mm diameter of 7 days old *Botrytis cinerea* culture was cut and placed in the center of PDA plates. Four wells of 4 mm diameter were punctured around the fungus. One hundred µl of twofold serial dilutions (1, 1/2, 1/4, 1/8) of cell-free supernatant or LB broth (control) were deposited in each well. Plates were incubated at 25 °C for 7 days. The antifungal activity titer (AU ml^{-1}) was defined as the reciprocal of the highest dilution that produced a detectable inhibition zone (Ben Slimene et al. 2012).

Morphological and biochemical identification of bacterial strains

Morphological characteristics and motility were assessed using light microscopy, 50 µl of bacterial culture were deposited on glass slide and then observed with an optical microscope (Olympia) 100× magnification.

The 4 selected bacteria were tested for catalase and oxidase activities and Gram stained. Strains were then identified with the analytical profile index API (BioMérieux, Marcy l'Etoile, France). The fermentation of 49 carbohydrates was tested with API 50 CHB/E medium for *Bacillus* and related genera. Acids produced, decreased pH, and changes in colours either to yellow, blue or green were observed after 24 and 48 h. Enzymes activities (β-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylase, urease, tryptophane desaminase and gelatinase),

citrate utilisation, glucose fermentation and H_2S, indole and acetoin production were carried out with API 20E for Gram negative bacteria. Colour reactions were scored against a chart provided by the manufacturer and results analyzed with API WEB (BioMérieux).

DNA extraction, 16S rDNA amplification and sequencing

Each strain was inoculated into 5 ml of LB broth medium and incubated at 30 °C for 18 h on a rotary shaker at 150 rpm min^{-1}. DNA extraction was done using DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's standard protocol. 16S rDNA was amplified by standard PCR using universal primers FD1 (5′-AGAGTTT GATCATGGCTCAG-3′) and RD1 (5′-AAGGAGGTGAT CCAGCCGCA-3′) (Edwards et al. 1989). The PCR product was purified using MinElute Gel Extraction kit (QIAGEN) and used directly for sequencing. Sequences similar to the 16S rDNA were carried out using the NCBI BLAST. Sequences from the selected bacteria and those identified by BLAST were aligned with ClustalW program (Thompson et al. 1994).

Extraction and identification of lipopeptides

Extraction of lipopeptides was carried out using the method of Kim et al. (2004) with minor modifications. After cell free medium centrifugation at 10,000*g* for 15 min and 4 °C, the collected supernatant was acidified with 3 N HCl to pH 2 and stored overnight at 4 °C, for lipopeptide precipitation. The precipitates were then collected by centrifugation at 10,000*g* for 20 min at 4 °C and dissolved in chloroform/methanol (2:1, v:v). After evaporation of solvent at 50 °C using a rotary vacuum evaporator RE 200 (BIBBY, Sterilin Ltd., UK), the precipitate was weighed and solubilized in methanol at a concentration of 50 mg ml^{-1}.

Each lipopeptide sample was tuned by direct injection into QTRAP mass spectrometer (Applied Biosystem) equipped with an electrospray ionization interface, at a flow rate of 10 µl min^{-1}. MS analysis was performed in positive ion mode. The ion source parameters were as follows: ion spray voltage: 5500 V; nebulizer gas (gas 1): 20 psi, curtain gas (nitrogen 99.9 %):10 psi.

Paper disk-agar assay method

For paper disk-agar diffusion assay, the method described by Raahave (1974) was used. Conidial suspension was spread on PDA plates and twofold serial dilutions of 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg ml^{-1} of the lipopeptide crude extracts. Twenty microliters of each solution were deposited on 5 mm sterile Whatman paper disk. Control disk amended with 20 µl methanol only was tested. Disks were allowed to dry then applied on the agar surface. Results were recorded after 72 h at 25 °C by measuring the diameter of the clear zone around the paper disks.

Detached leaflets assay

Detached leaflets free from wounds and lesions, from 4-week-old tomato plants were surface-sterilized by soaking in 2 % aqueous sodium hypochlorite for 3 min. Leaflets were then thoroughly rinsed with sterile distilled water, dried and placed into Petri dishes containing water-soaked filter paper. Bacterial suspension was deposited at a final concentration of 10^8 CFU ml^{-1} and sprayed onto leaflets. Three needle-prick wound were applied to each leaflet. These wounds were then inoculated with 20 µl of *Botrytis cinerea* conidial suspension (10^6 conidia ml^{-1}). Inoculated leaflets were maintained at 25 °C in the dark for 5 days under 95 % relative humidity. Experiments were performed in triplicate, each consisting in nine leaflets excised from three plants. At the end of the incubation period, percent lesion area was assessed visually according to the method of Rajkumar et al. (2005), based on a 0-4 scale: 0; no symptoms, 1; 1–12 %, 2; 13–25 %, 3; 26–50 %, 4; 51–100 % of leaflet area covered with brown lesions. The disease severity was calculated based on the following formula:

$$Disease\ severity = \frac{\sum(Scale \times number\ of\ leaflet\ of\ each\ scale)}{(Total\ number\ of\ leaflet \times highest\ scale)} \times 100$$

Statistical analysis

All experiments were carried out in triplicate and data expressed as mean ± SD. The experimental data were established using Duncan's Multiple Range tests ($p = 0.05$) following the one-way analysis of variance (ANOVA). All statistical analysis was performed using "Statistica v 5.1" software (Statsoft 1998).

Results

Antagonistic activity

Eighty bacterial strains were screened for their antagonistic activity against *Botrytis cinerea* that was evaluated using the diameter of the inhibition zone (Fig. 1). Four antagonistic strains called BL1, BT5, BR8 and BF11 were the

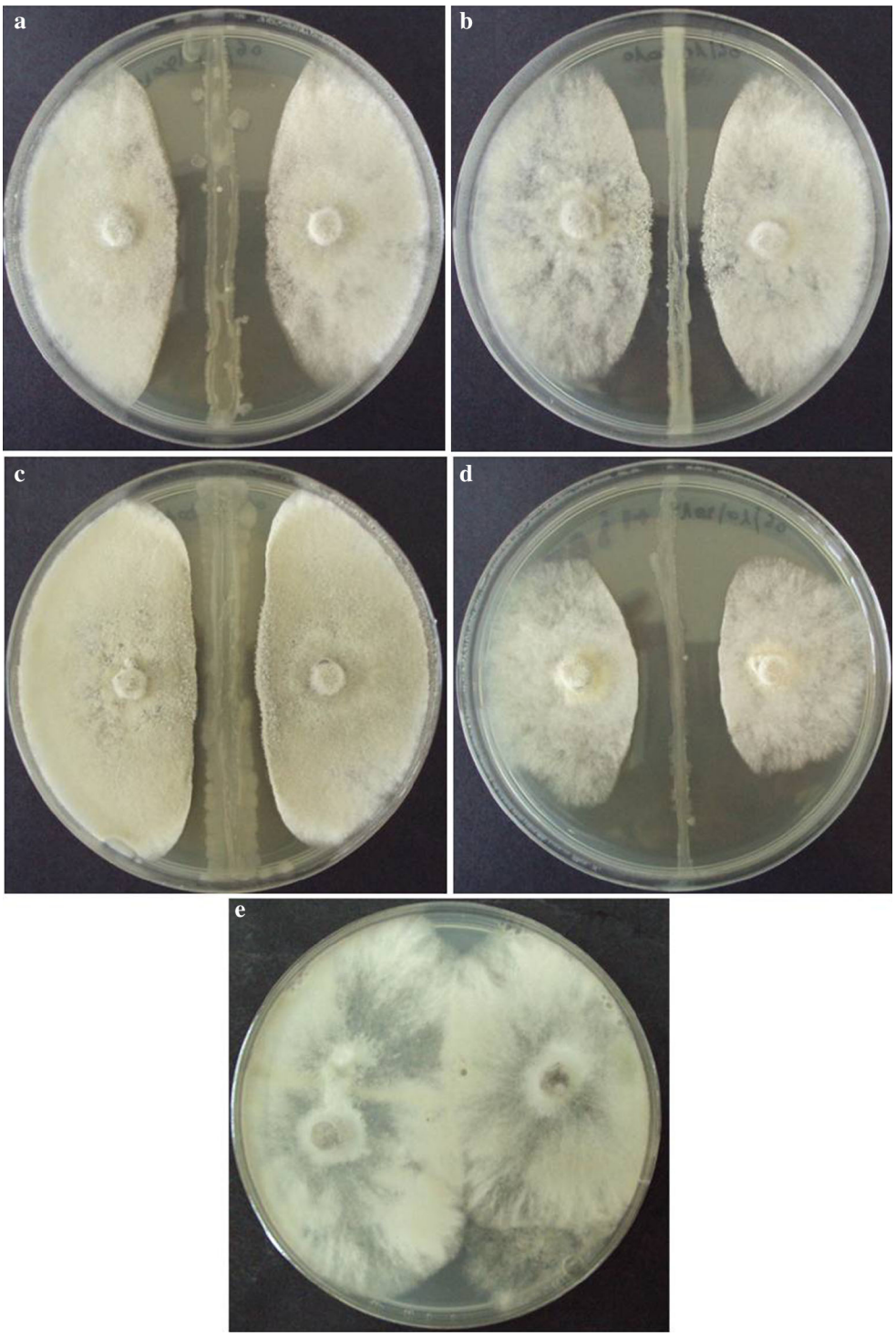

Fig. 1 In vitro antagonistic activity of bacterial strains BL1 (**a**), BT5 (**b**), BR8 (**c**) and BF11 (**d**) against *Botrytis cinera* used as control (**e**)

most active, inhibiting fungal mycelia growth by 46, 42, 27 and 53 % over control, respectively (Table 1). All subsequent experiments were conducted using these strains. Cell-free supernatant from BL1, BT5 and BF11 strains exhibiting an antifungal activity of 40 (AU ml^{-1}) inhibited the growth of the fungal pathogen as well (Table 1).

Table 1 Antifungal activity of selected bacterial strains and their cell free supernatants against *Botrytis cinerea*

Bacterial strains	Origin	Growth inhibition (%)	Antifungal activity of CFS (AU ml^{-1})	Volume of CFS (µl)[a]
BL1	Tomato leaves	45.88 ± 4.61b	40	25
BT5	Tomato stems	42.35 ± 4.42b	40	25
BR8	Tomato roots	27.06 ± 2.66c	10	100
BF11	Tomato leaves	52.94 ± 3.77a	40	25

(±) Standard deviation. Values with different small letters are significantly different at $p < 0.05$

CFS cell free supernatant

[a] Volume of CFS tested

Table 2 Morphological and biochemical characteristics of the four selected bacteria

Characteristics	BL1	BT5	BR8	BF11
Colour	Creamy	Creamy	Creamy	Creamy
Shape	Rod	Rod	Rod	Rod
Motility	+	+	+	+
Endospore	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Gram	+	+	+	+
D-Lactose fermentation	−	−	−	+
D-Xylose fermentation	+	+	−	−
Glycogen fermentation	+	−	+	+
Arginine dihydrolase	−	−	−	−
β-Galactosidase	+	+	+	−
Gelatinase	+	+	+	+
Lysine decarboxylase	+	−	−	−
Ornithine decarboxylase	−	−	−	−
Tryptophane desaminase	−	−	−	−
Urease	−	+	−	−
Acetoin production	+	+	+	+
H_2S production	−	−	−	−
Indole production	−	−	−	−
Citrate utilisation	+	+	+	+
Glucose fermentation	+	+	+	+

Identification of the endophyte strains

The four endophyte strains belonged to the genus *Bacillus* (Table 2) according to their morphological and biochemical properties and to the standardized API strip system. *Bacillus* species are mostly rhizospheric and endophytic bacteria, Gram's reaction, catalase and oxidase positive, exhibit motility and endospore formation in liquid culture, and UV fluorescence negative. DNA homology studies carried by comparison with GenBank database of the 16S rDNA gene sequence, indicated that BL1 (accession number KR071854) shared 99.71 % homology with *Bacillus mojavensis* NBRC 15718^T strain, that BT5 (accession number KR071855) exhibited 99.93 % homology with *Brevibacterium halotolerans* DSM 8802^T strain, that BR8 (accession number KR071856) showed up to 99 % homology with *Bacillus subtilis*, and that BF11 (accession number KR071857) shared 99 % homology with *Bacillus amyloliquefaciens* BCRC 11601^T strain.

Antifungal activity of lipopeptide crude extract

As *Bacillus* species produced lipopeptides responsible of their antifungal activity, we focused on their inhibitory effect against phytopathogenic fungi using the paper disk-agar diffusion method. The in vitro antifungal activity of lipopeptides is presented in Table 3. The cell-free supernatant containing lipopeptide from the four strains significantly inhibit the growth of *B. cinerea* versus control and BF11 strain exhibited the strongest activity at a crude extract concentration of 7.81 µg ml^{-1}. The three other strains namely BL1, BT5 and BR8 elicited the same level of inhibition at 62.50 µg ml^{-1} an eightfold higher concentration.

Lipopeptide identification

Lipopeptides from cell-free supernatant of endophyte strains were identified by ESI–MS. Comparative analysis of mass spectra obtained from BL1, BT5, BR8 and BF11 strains revealed very clear peak clusters at m/z values between 1008–1092, 1031–1097 and 1435–1505 which could be attributed to surfactins (C13–C16 and C19), bacillomycin D (C14–C16) and fengycins (C14–C19) respectively. This analysis revealed also the presence of compounds at m/z values 1085–1141 which were assigned as iturins C17–C21 (Table 4). All strains produced surfactins C13 to C15. Iturins were solely produced by BR8. BL1, BT5 and BF11 strains produced fengycins and bacillomycin D C14, C15 and C16 were produced by BF11. The high antifungal activity of BF11 is likely due to

Table 3 Diameter of the inhibition zones for lipopeptide extracts from endophytic strains against *Botrytis cinerea*

Bacterial strains	Inhibition zone (mm)							
	Lipopeptide concentration (μg ml^{-1})							
	1000	500	250	125	62.5	31.25	15.62	7.81
BL1	19.7 ± 0.18aB	17.5 ± 0.16bB	16.3 ± 0.09cB	12.3 ± 0.18dA	7.5 ± 0.28eB	nd	nd	nd
BT5	10.3 ± 0.53aD	9 ± 0.92bD	9 ± 0.92bD	7.8 ± 0.27cB	6.2 ± 1.16dC	nd	nd	nd
BR8	12.2 ± 0.71aC	10 ± 0.92bC	8.2 ± 0.71cC	7 ± 0.46dC	5.3 ± 0.53eD	nd	nd	nd
BF11	23.5 ± 0.80aA	21.8 ± 0.71bA	18.2 ± 0.96cA	12.3 ± 0.96dA	10 ± 0.92eA	9 ± 0.92fA	6.2 ± 0.71gA	5.5 ± 0.46hA

Values followed by different letters are significantly different ($p < 0.05$). The small letters indicate significance between the various lipopeptide concentrations for the same strain. The great letters indicate significance between the various strains for each lipopeptide concentration

the presence of both fengycin and bacillomycin D antibiotics whereas the lower activity of BL1 and BT5 is probably linked to the sole fengycin and that of BR8 to iturin production.

Tomato leaflets assay

When applied on detached leaflets, all four endophyte strains were able to inhibit the lesions induced by fungal infection (Fig. 2). BF11 was the most protective strain as it reduced disease severity till 11 % when compared to positive control of *B. cinerea* on its own which induced 94 % of disease severity. The three other bacteria provided a moderate protection that reached 50 % of disease severity (Fig. 3).

Discussion

Endophytes are microorganisms isolated from surface sterilized plant organs, which colonize the same environment than various pathogens and as such could constitute convenient biocontrol agents in the fight against multiple plant diseases caused by soil borne pathogens (Manso and Nunes 2011). Numerous studies have demonstrated the potential of endophytic bacteria in the control of pathogens in cultured plants. Paul et al. (2013) demonstrated that endophytic bacteria, isolated from healthy tissues of chili pepper plants (*Capsicum annum* L.) belonged to three bacteria genus as *Pseudomonas*, *Bacillus* and *Burkholderia* and exhibited antifungal activity against *Alternaria panax*, *Botrytis cinerea*, *Colletrotichum acutatum*, *Fusarium oxysporum* and *Phytophtora capsici*.

In the present study, several bacterial strains were isolated from various organs of healthy tomato plants cultivated in an area regularly infected with *B. cinerea*. Four isolates exhibiting high antifungal activity against *B. cinerea* were selected and analyzed. Sequencing of the 16S rDNA indicated that the four selected isolates were phylogenetically related to the *Bacillus* genus i.e., BL1 to *B. mojavensis*, BT5 to *B. halotolerans,* BR8 to *B. subtilis* and BF11 to *B. amyloliquefaciens*. Several bacterial strains belonging to the *Bacillus* sp. displaying biocontrol activities against fungal pathogens have been isolated from various plants such as *B. subtilis* strain Lu144 from mulberry leaves (Xianling et al. 2008), *Bacillus endoradicis* sp. nov. from soybean root (Zhang et al. 2012) or *Bacillus amyloliquefaciens* BZ6-1 from peanut (Wang and Liang 2014).

As *Bacillus* species secrete a large variety of secondary metabolites, cell-free supernatants were tested for their antifungal activity against *B. cinerea.* The antifungal compounds purified from the culture broth strongly inhibited the growth of *B. cinerea*. *Bacillus* species are well known producers of biologically active compounds as lipopeptides with potent antifungal activities (Yun-Feng et al. 2012). Our data are in agreement with those of Yoshida et al. (2001), who demonstrated the inhibition of mulberry anthracnose by secreted antifungal compounds from *B. amyloliquefaciens* RC-2 strain. Other studies reported about the antifungal activity of *Bacillus pumilus* 3PPE and *B. amyloliquefaciens* 2TOE strain against *B. cinerea* (Mari et al. 1996) or on the antifungal activity of *B. amyloliquefaciens* against *Phoma tracheiphila* (Kalai-Grami et al. 2013).

ESI–MS analysis of the secreted lipopeptides allowed the identification of peptides belonging to surfactin, fengycin and iturin families. Iturin exhibited strong antifungal activity, fengycin inhibited the growth of filamentous fungi (Touré et al. 2004) and surfactin enhanced the antifungal activity of iturin A but is not fungitoxic on its own (Maget-Dana et al. 1992). The present strains produce various lipopeptide isoforms. Thus, BL1 (*B. mojavensis*) and BT5 (*B. halotolerans*) expressed fengycin and surfactin whereas BR8 (*B. subtilis*) produced iturin and surfactin. BF11 (*B. amyloliquefaciens*) secreted bacillomycin D, fengycin and

Table 4 Mass spectrometry analysis of lipopeptide antibiotics produced by selected bacterial strains

Bacterial strains	Experimental peak mass (m/z)	Theoretical peak mass (m/z)	Assignment
BL1	1449.8371	1449.8	Fengycin C13, $[M + H]^+$
	1463.7162	1463.6	Fengycin C14, $[M + H]^+$
	1477.7395	1477.6	Fengycin C15, $[M + H]^+$
	1491.7332	1491.6	Fengycin C16, $[M + H]^+$
	1505.7946	1505.6	Fengycin C17, $[M + H]^+$
	1008.8013, 1030.7496, 1046.7412	1008.6, 1030.6, 1046.7	Surfactin C13, $[M + H, Na, K]^+$
	1022.8034	1022.6	Surfactin C14, $[M + H]^+$
	1036.8069, 1058.7852, 1074.7599	1036.6, 1058.5, 1074.5	Surfactin C15, $[M + H, Na, K]^+$
BT5	1463.8000	1463.6	Fengycin C14, $[M + H]^+$
	1477.7027	1477.6	Fengycin C15, $[M + H]^+$
	1491.8804	1491.6	Fengycin C16, $[M + H]^+$
	1505.7628	1505.6	Fengycin C17, $[M + H]^+$
	1008.8368, 1030.7888, 1046.7283	1008.6, 1030.6, 1046.7	Surfactin C13, $[M + H, Na, K]^+$
	1022.7816, 1044.7932, 1060.7307	1022.6, 1044.5, 1060.7	Surfactin C14, $[M + H, Na, K]^+$
	1036.8069, 1058.7641, 1074.7596	1036.6, 1058.5, 1074.5	Surfactin C15, $[M + H, Na, K]^+$
BR8	1085.7346	1085.8	Iturin C17, $[M + H]^+$
	1141.6219	1141.7	Iturin C21, $[M + H]^+$
	1008.8308	1008.6	Surfactin C13, $[M + H]^+$
	1022.8589	1022.6	Surfactin C14, $[M + H]^+$
	1036.8560, 1058.7959, 1074.7815	1036.6, 1058.9, 1074.5	Surfactin C15, $[M + H, Na, K]^+$
	1050.8617	1050.6	Surfactin C16, $[M + H]^+$
	1092.7661	1092.8	Surfactin C19, $[M + H]^+$
BF11	1031.6558, 1053.6534, 1069.6018	1031.6, 1053.7, 1069.6	Bacillomycin D C14, $[M + H, Na, K]^+$
	1045.7111, 1067.6555, 1083.6006	1045.6, 1067.7, 1083.6	Bacillomycin D C15, $[M + H, Na, K]^+$
	1097.6055	1097.7	Bacillomycin D C16, $[M + K]^+$
	1435.6965	1435.8	Fengycin C12, $[M + H]^+$
	1449.7485	1449.8	Fengycin C13, $[M + H]^+$
	1463.7367	1463.6	Fengycin C14, $[M + H]^+$
	1477.7400	1477.6	Fengycin C15, $[M + H]^+$
	1491.7902	1491.6	Fengycin C16, $[M + H]^+$
	1505.7971	1505.6	Fengycin C17, $[M + H]^+$
	1008.8489, 1030.7903, 1046.6592	1008.6, 1030.6, 1046.7	Surfactin C13, $[M + H, Na, K]^+$
	1022.7663, 1060.7307	1022.6, 1060.7	Surfactin C14, $[M + H, K]^+$
	1036.8167, 1058.7959, 1074.7604	1036.6, 1058.5, 1074.5	Surfactin C15, $[M + H, Na, K]^+$

surfactin. The potential of the selected bacteria to produce multiple antimicrobial peptides is obviously at the basis of their effectiveness in the inhibition of *B. cinerea* growth. Moreover they afford the opportunity to investigate the mechanism of action of the lipopeptides in particular the synergism that could occur between the various isoforms.

The production of surfactin and iturin by *B. subtilis* strain has been reported previously by Ben Slimene et al. (2012). Asaka and Shoda (1996) showed that *B. subtilis* BR14 strain which produced iturin A and surfactin was effective in the control of damping-off caused by *Rhizoctonia solani* in tomato plants. The high protection offered by this strain is likely due to the synergism between surfactin and iturin (Maget-Dana et al. 1992) which are able to exert significant pressure on pathogens (Phae et al. 1990).

B. mojavensis and *B. halotolerans* strains, which belong to the *B. subtilis* group, are surfactin and fengycin producers. However the antifungal activity is mostly linked to fengycins which are able to create pores that drastically affects the cell membrane permeability (Deleu et al. 2005) emphasizing the relevance of fengycins in the biocontrol of phytopathogenic fungi (Chan et al. 2009). Concerning surfactin they rather acted synergistically with iturin A (Akpa et al. 2001). *B. mojavensis* strain isolated from

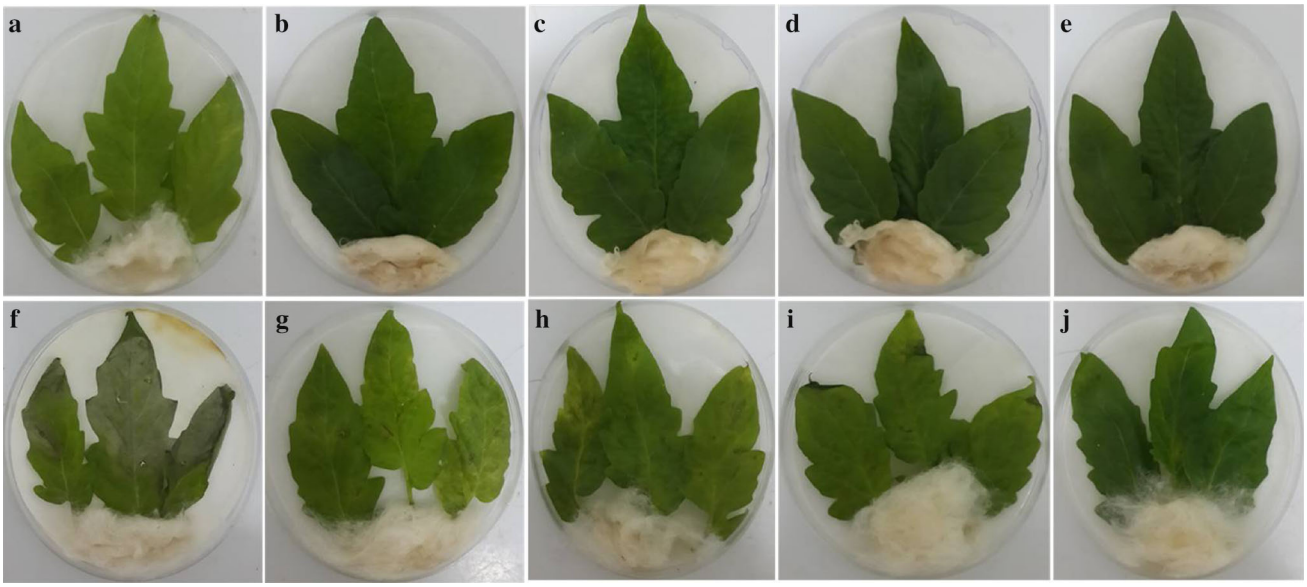

Fig. 2 Protective effect of endophytic strains against necrotic lesions induced by *Botrytis cinerea*. Negative control untreated leaflets (**a**), leaflets treated with endophytic bacteria BL1 (**b**), BT5 (**c**), BR8 (**d**) and BF11 (**e**). Positive control leaflets infected with *Botrytis cinerea* (**f**), leaflets pre-treated with BL1 (**g**), BT5 (**h**), BR8 (**i**) and BF11 (**j**) and infected with pathogen

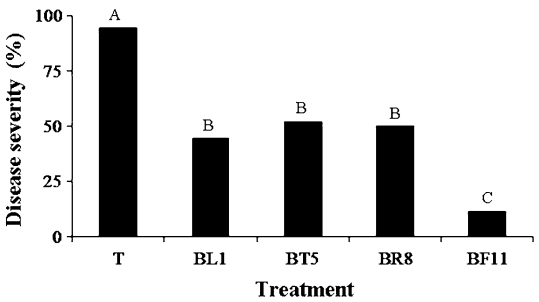

Fig. 3 Reduction in disease severity of detached leaflets treated with bacterial strains. *Different letters* indicate significance between treatments

maize secreted inhibitory compounds into the medium that exhibited antifungal activity against *Fusarium monilifrome* (Bacon and Hinton 2002) and *B. mojavensis* strain AB1 isolated from coffee twigs produced thermostable compounds with strong antifungal activity against a wide range of phytopathogenic fungi (Nair et al. 2002). However little is known about *B. halotolerans* as a biocontrol agent. To our knowledge, the present study describes for the first time the isolation of an endophytic *B. halotolerans* strain from healthy tomato plants. Further studies to assess the antifungal activities of purified lipopeptide compounds and their mode of action are in progress.

B. amyloliquefaciens specie which is closely related to *B. subtilis*, has been reported as an antagonist in various plant diseases (Yoshida et al. 2001). Yin et al. (2011), evaluating the antifungal activity of endophytic *B. amyloliquefaciens* from poplar against *B. dothidea*, suggested that this effect was due to cyclic lipopeptides. The ability in producing bioactive lipopeptides against fungal pathogen has also been reported by Arrebola et al. (2010), who further showed that the control of postharvest fungal pathogens by *B. amyloliquefaciens* PPCB004 mainly result from the synergism between iturin A, surfactin and fengycin. Similarly the highly efficient antagonistic activity of BF11 could likely result from the synergism between bacillomycin D, surfactin and fengycin.

Our selected endophytic bacteria were also effective in reducing the disease severity of detached leaflets inoculated with the pathogen in a similar fashion as described for tomato leaves and cucumber seedling cotyledons (Wang et al. 2009). This effect may be due to the ability of the antagonistic isolates to inhibit hyphal growth of the pathogen; it is indeed established that abolition of the pathogen development contributes to the reduction disease incidence. In this respect, Daayf et al. (2003) reported a higher protection on whole plant than on detached leaves. This latter experimental assay is a model of choice for the determination of the potential biocontrol of antagonistic strains before *in planta* assays particularly when testing the BF11 strain, which is the most efficient one exerting an almost complete reduction of the disease.

The production of a large array of lipopeptides by endophytic bacteria is an interesting feature with potential practical applications. In this context, *B. amyloliquefaciens* BF11 strain is a promising candidate for the biological control of gray mold of tomatoes caused by *B. cinerea*. The

current research is so a substantial contribution to the preservation of the environment by means of an alternative to chemical fungicides by bioactive molecules as lipopeptides-producing endophytic bacteria.

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