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RESEARCH PAPERS

Bacterial endophytes of weeds are effective biocontrol agents of *Agrobacterium* spp., *Pectobacterium* spp., and promote growth of tomato plants

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Summary. Bacterial endophytes were isolated from native plants growing in a fallow field. Taxonomy of these bacteria, and their beneficial effects to plants, were determined. Seventeen strains were selected from a group of 73 isolates on the basis of origin, colony morphology and antagonistic properties and were characterized by 16S rRNA gene sequence and phylogenetic analyses. These strains were assayed *in vivo* against pathogenic strains of *Agrobacterium* and *Pectobacterium* spp. Their ability to improve plant growth was also evaluated. The Gram positive *Bacillus amyloliquefaciens*, *B. cereus*, *B. methylotrophicus*, *B. pumilus and Curtobacterium flaccumfaciens* and the Gram negative *Pseudomonas brassicacearum* were identified. The *Bacillus* and *Pseudomonas* were shared among five plant species while C. *flaccumfaciens* was isolated only from *Euphorbia* spp. Biocontrol activity of endophytic strains was evaluated on potato disks inoculated with *Pectobacterium* spp. and on tomato plants grown in sterile soil, root-bacterized with endophytes and stem inoculated with *Agrobacterium* spp. A reduction of soft rot caused by *Pectobacterium* spp. on three potato varieties treated with *Bacillus* pp. strains was observed. *Bacillus methylotrophicus* strain OS4 strongly reduced gall development induced by *Agrobacterium* spp. and gave 100% germination of tomato seeds compared with 75.5% for the non-treated seeds. *Pseudomonas brassicacearum* strain PS1 enhanced tomato seed germination and increased plant growth parameters. These results indicate that native plants harbour various endophytic bacterial species that possess potentially valuable biocontrol and growth promotion activities.

Keywords: *Bacillus* spp., *Pseudomonas brassicacearu m*, PGP, native plants.

Abbreviations: PGP, Plant Growth Promotion; SDW, Sterile Distilled Water; LB, Luria Broth; YPGA, Yeast Peptone Glucose Agar.

Introduction

Bacterial endophytes live inside host plants without causing symptoms and may exert several beneficial activities such as growth promotion, biocontrol of pathogens, induction of systemic host resistance and bioremediation (Bacon and Hinton 2006; Ryan *et al.*, 2008; Forchetti *et al.*, 2010). Endophytes commonly originate from the soil in which host plants are grown. Plant genotype, growth stage and physiological status, type of plant tissues, soil conditions and agricultural practices determine colonization of plants by different bacteria, and the composition of bacterial endophytic communities (Hardoim *et al.*, 2008). Genetic factors are also presumed have a role enabling a specific bacterium to become endophytic (Reinhold-Hurek and Hurek 1998; Rosenblueth and Martinez-Romero 2006). Microbes profit from plants because of the enhanced availability of nutrients,

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whereas plants can receive benefits from bacterial associates by growth enhancement or stress reduction. These mutualistic interactions could have emerged as the result of selection exerted on these associations (Hardoim *et al.,* 2008).

Much research regarding bacterial endophytes isolated from cultivated plant species has been published, while only a few studies have focused on characterization of endophytic microflora of weeds (Chaintreuil *et al.,* 2000; Zinniel *et al.,* 2002). These plants are native and pioneering, they are very abundant in nature, grow in dense populations and do not undergo selection or breeding. Weeds are usually considered for their negative effect on germination and growth of surrounding cultivated plants, measured as reductions of crop yields. However, these plants exert a positive influence on soil microbial diversity and subsequent soil health and quality (Sturz *et al.,* 2001).

Endophytes have a wide spectrum of effects on host plants, which is related to the production of secondary metabolites that alter host growth and phenotype (Bacon and Hinton, 2006), and may increase host resistance to biotic and abiotic stress (D'Alessandro *et al.*, 2014; Yaish *et al.*, 2015). The ability of weeds to grow prolifically, germinate quickly, produce many seeds and keep photosynthetic leaves active throughout the winter, may be partly due to molecules produced by endophytic microrganisms (Strobel *et al.,* 2004).

The purpose of the present study was four-fold:

i) to survey native plants growing in a fallow field for the presence of bacterial endophytes; ii) to determine the taxonomic positions of these bacteria; iii) to determine their antagonistic activity against bacterial pathogens; and iv) to determine if the bacteria have beneficial effects on crop plants.

Material and methods

Plant material

Spontaneous growing weeds were collected from a fallow field of the Experimental Station of the Agronomy Department (University of Blida, Algeria), and identified according to the flora key of Quezel and Santa (1962). The field was previously inspected in order to determine the dominant plant species. For each species, three plants were randomly chosen and analyzed for presence of endophytic bacteria. Plants were carefully uprooted, put in plastic bags and transported to the lab.

Isolation of bacteria

Roots were separated from aerial parts of the plants and separately processed for bacteria isolation. After washing under running tap water, 1 g samples were randomly cut from each part, and these were disinfected by immersion for 5 min in a solution containing 2% sodium hypochlorite and 1% Tween 20. Samples were then treated with a solution containing 70% ethanol for 2 min and then washed twice with SDW. Three aliquots of 100 µL of the second wash were spread on YPGA plates to check the sterility of the plant surfaces. Following disinfection, samples were each placed between two sterile filter papers, then ground in a sterile mortar with 1 mL of SDW and left for 15–20 min to permit the release of endophytes. Suspensions were serially diluted and a volume of 150 µL of each dilution was spread on a YPGA plate (Schaad *et al.,* 2001). The plates were incubated at 28°C for one week. The most representative colony types were selected and streaked twice on YPGA for purification.

Different colony profiles based on morphological criteria (shape, colour, elevation, diameter and margin) were described, originating from individual plant species.

In vitro antagonistic activity

Seventy-three colonies of different morphologies and origins were selected for antagonistic activity assays. Seven pathogenic bacterial species (*Agrobacterium tumefaciens*, *Agrobacterium vitis*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia amylovora*, *Pectobacterium* spp., *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *citri*) were chosen from the plant pathogenic bacteria collection of the Department of Agronomy, University of Blida, Algeria. Putative antagonistic bacteria were grown on YPGA plates for 48 h at 28°C. Each bacterial strain was scraped from the plates and suspended in SDW to a known concentration (10^8cfu mL^{-1}). Concentration of suspensions was determined spectrophotometrically by comparing the OD of suspensions to the reference curve of each strain. The reference curves were established by serial dilutions and plate counts and the correspondence between OD at λ 600 nm and cfumL^{-1} concentration

was determined. Three 50 µL droplets of each bacterial suspension were placed on the surface of a YPG agar plate, and the inoculated plates were and incubated for 48 h at 28°C. At the same time, pathogenic bacteria were streaked on YPGA medium and grown under the conditions described for endophytes. After incubation, the suspensions of pathogenic bacteria (10^7cfu mL^{-1}) were prepared as described above and sprayed onto the surface of the plates containing the droplet-inoculated endophytic bacteria. Plates were incubated again for 24 to 48 h at 28°C. Development of inhibition zones around the bacterial inoculation points was considered as positive antagonistic activity. The diameter of each colony plus the inhibition zone was measured to assess the relative inhibition efficacy of the different isolates.

Characterisation of bacteria

Seventeen isolates selected on the basis of their origins, colony morphology and *in vitro* antagonistic activity, were tested for Gram reaction (Schaad *et al.*, 2001) and characterised by the partial 16S rRNA gene sequence analysis. Bacterial strains were grown in 5 mL of LB overnight at 27°C. One mL of each culture was used for DNA extraction using a DNeasy kit (Qiagen), following the protocols of the kit manufacturer for Gram positive or Gram negative bacteria.

The amplifications of the 1500 bp sequences were performed in 25 µL volumes using 200 ng DNA, 20 pmol of each primer fD1 (5′ – AGAGTTTGATCCTGGCTCAG – 3′) and rP1/rP2 (5′ – GGYTACCTTGTTACGACTT – 3′; Y=C/T)) (Pious and Thyvalappil, 2009), 50 µM dNTPs and 0.5 units of Taq DNA polymerase (Invitrogen). The amplification cycle was the same as reported by Pious *et al.* (2008). PCR products were sequenced by Primm s.r.l. (Milano, Italy) using the primer set involved in the PCR reactions. Similarities of partial 16S rDNA nucleotide sequences with known sequences in the NCBI GenBanK database were determined by BLASTn (http://blast.ncbi.nlm.nih.gov/). Partial sequence data for the 16S rRNA genes have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under the following accession numbers:KP851946, for PS1; KP851947 for CR1; KP851948 for CR2; KP851949 for EHA2; KP851950 for EHF3; KP851951 for EHF5; KP851952 for EHR1; KP851953 for EPR3; KP851954 for OR1; KP851955 for OR2; KP851956 for OS1; KP851957 for OS2; KP851958 for OS4; KP851959 for PA2; KP851960 for PF1; KP851961 for PF3; and KP851962 for PR1. Nucleotide sequences coding for rRNA in phylogenetically closely related bacterial species were retrieved from NCBI GenBanK in order to carry out a phylogenetic analysis. Sequences were aligned with Clustal X (Thompson *et al.*, 1997) and the alignment profiles were then used to establish the evolutionary distances by applying Kimura's two parameter model (Kimura, 1983) implemented in the MEGA5 program (Kumar *et al.*, 2004; Tamura *et al.*, 2011). The best phylogenetic tree was created using the neighbour-joining method (Saitou and Nei 1987) using the same program. Bootstrap analysis with 1000 replicates was performed to assess confidence levels for the branches (Felsenstein, 1985).

In vivo antagonistic activity against *Agrobacterium* spp.

Seeds of tomato cv. St. Pierre were surface-sterilized with 2% sodium hypochlorite solution for 5 min, rinsed thoroughly with SDW and then placed on sterile discs of Whatman filter paper inside Petri dishes. A mixture composed of 2/3 parts of field soil and 1/3 part peat was prepared and autoclaved twice for 60 min at 120°C, with 24 h interval between each autoclaving. Sterile substrate was distributed in plastic pots (10 × 6.5 cm) where the sterilized seeds were sown. Three different *Bacillus* species and two *P. brassicacearum* strains were chosen among the endophytes isolated from weeds and tested against *A. tumefaciens* strain E14 (Krimi *et al.*, 2006), and *A.vitis* strains AL9/95, BU20/95 andAV25/95. All en dophytic bacteria were grown on YPGA at 28°C for 48 h. The concentration of each bacterial culture was determined by a spectrophotometer at 600 nm, and was adjusted to 10^7 cfu mL^{-1} for antagonists and 10^6 cfu mL^{-1} for pathogens.

Tomato plants were explanted at the two-leaf stage, rinsed to remove soil particles from the roots and then soaked for 24 h in the suspensions of the antagonistic bacteria or in SDW for negative controls. Bacterized plants were then transplanted again into the sterile potted soil. Twenty-four hours after root bacterization, plant stems were each wounded in three places with a sterile scalpel and 20 µL of the 10^6 cfu mL^{-1}suspensions of *A. tumefaciens* and *A. vitis* strains were injected into the wounds. The same volume of SDW was used to treat control plants. Inoculated stems were wrapped with aluminum foil to prevent desiccation of the inoculum.

The experiment was carried out in a greenhouse with a daily photoperiod of 16 h light and 8 h of darkness at a temperature of 25–30°C. Treatments were arranged in a completely randomized design with nine replicates for each combination of pathogen/antagonist. Tumour weights were recorded 9 weeks after inoculation. The experiment was repeated once.

In vivo antagonistic activity against *Pectobacterium* spp.

Five endophytic bacteria belonging to the *Bacillus* genus were chosen, on the basis of the results of *in vitro* antagonistic tests, to perform biocontrol assays against *Pectobacterium* spp. The efficacy of endophytes was determined against six pathogenic strains on tubers of *Solanum tuberosum* L. cv. Bartina, Desiree or Spunta. Bacterial suspensions were prepared from cultures grown on YPGA at 28°C for 48 h. The concentration of suspensions was 10^7cfu mL^{-1} for antagonists and 10^6 cfu mL^{-1} for *Pectobacterium* spp. Healthy potato tubers were thoroughly rinsed under running tap water and then were disinfected with a 4% solution of calcium hypochlorite for 1 h. Tubers were then rinsedthree times with SDW. Twelve disks of 25 mm diameter and 15 mm thickness were aseptically cut from tubers and dipped for 2 min in the suspensions of the endophytic isolates. A volume of 50 µL of each *P. carotovorum* strain suspension was placed into the wells of a microplate (Costar, cell culture cluster dish), then the previously bacterized potato disks were placed into the wells. For each combination pathogen/endophyte six replications were made.

Four treatments were compared: i) potato disks treated withSDW; ii) potato disks with endophytic bacteria and inoculated with *P. carotovorum* strains; iii) potato disks with endophytic bacteria only; iiii) potato disks inoculated with *Pectobacterium* spp. strains. Four days after inoculation, the size of the rotten area of each potato slice was measured using a caliper. The experiment was repeated once.

Plant growth promotion (PGP) activity

In vitro *experiments*

Eight bacterial strains were assayed for their PGP activity *in vitro* and *in vivo*. Bacteria were grown for 48 h at 28°C on LB broth. Cultures were then filtered through sterile Millipore filters (0.2 µm pore size). A volume of 0.25 mL of each sterile filtrate was added to a sterile glass tube containing 15 mL of sterile MS medium (Murashige and Skoog 1962). Tomato seeds (*Lycopersicon esculentum* cv. Saint-Pierre) were washed under running tap water and disinfected with a solution of sodium hypochloride (2%) for 5 min. Seeds were then dipped in 70% ethanol for 5 min and washed thoroughly three times with SDW. Seeds were then placed into the glass tubes containing MS medium supplemented with bacterial filtrates. Controls differed by having SDW instead of bacterial filtrates. The tubes were kept at room temperature (23 ± 2°C) under a 3000 lux light and a 12 h daily photoperiod. The test was performed in a randomized complete block experimental design with 12 repetitions for each strain. Seed germination and plant growth parameters were recorded daily.

Greenhouse experiments

Seeds of tomato were surface-sterilized with 2% sodium hypochlorite for 2 min, rinsed thoroughly in SDW and let dry on sterile discs of Whatman filter paper placed inside Petri dishes. Tomato seeds were covered with the bacterial suspensions (10^8 cfu mL^{-1}) of endophytic strains and incubated at room temperature at 28°C for 72 h before sowing. In parallel, a mixture composed of equal parts of peat, sand and field soil was prepared and autoclaved twice for 20 min at 120°C with 24 h between autoclavings. Sterile substrate was distributed in plastic pots (6 × 6 × 5.5 cm) where the bacterized seeds were transferred. Seeds were again each bacterized with 1 mL of bacterial suspensions and then covered with soil. As a negative control, seeds were treated with SDW. Pots were placed in the greenhouse according to a completely randomized block experimental design. For each bacterial strain 15 pots each containing three seeds were used. Treatments were arranged in a completely randomized experimental design with nine treatments and 15 repetitions. Plant growth parameters were recorded 45 d after sowing, using six parameters: percentage of seed germination; plant height; fresh and dry weight of roots; fresh and dry weight of shoots. The experiment was repeated once.

Statistical analyses

All data were analyzed by ANOVA. Efficacy of endophytic strains against *Agrobacterium* spp. and *Pectobacterium* spp. was assessed, respectively, by evaluating the reductions of tumour weight (mg)

from tomato stems and the dimensions of rotted areas (mm) on potato slices. PGP activity of endophytic bacteria was determined by calculating the percentage increases in seed germination and plant growth parameters. The significance of the results was determined by Duncan's tests.

Results

Characterisation of endophytic isolates associated to tissues of weeds

The most representative plants that were collected from the fallow field were *Calendula arvensis*, *Euphorbia helioscopia*, *Euphorbia peplus*, *Plantago lanceolata* and *Urtica dioica*.

Eight different bacterial profiles (P1 to P8), based on morphological criteria, were found among 73 isolates that developed different colonies on agar plates. Profile P8 included all bacterial colonies that were less than 1mm in diameter and did not show clear morphology. Most of the identified endophytic species were Gram positive and included six different morphological profiles. Only one profile was recorded within the Gram negative group (Table 1).

Blast analysis of the 16S rDNA sequences in the Gene bank database revealed that among the Gram positive bacteria, 13 strains were affiliated to the *Bacillus* genus and one strain was identified as *Curtobacterium flaccumfaciens*. The three Gram negative bacteria were identified as *Pseudomonas brassicacearum* (Table 1). Phylogenetic analysis indicated that the *Bacillus* group included strains that shared 99% similarity with *B. amyloliquefaciens*, *B. cereus*, *B. methylotrophicus* and *B. pumilus*. Strain OR1 shared 99% similarity with both *B. toyonensis* and *B. thuringiensis*, while four *Bacillus* strains were not identified at species level since they shared only 97% similarity identity with reference strains (Table 1 and Figure 1). The different bacterial species were shared among the five plant species, except that *C. flaccumfaciens* that was only isolated from *Euphorbia* spp.

Table 1. Origin and characterisationof the most representative bacterial endophytes isolated from weeds.

Strain	Host origin	Gram reaction	Species	Colony profile
CR1	*Calendula arvensis*	+	*Bacillus amyloliquefaciens*	P1
CR2	*C. arvensis*	-	*Pseudomonas brassicacearum*	P2
EHA2	*Euphorbia helioscopia*	+	*Bacillus* spp.	P3
EHR1	*E. helioscopia*	+	*Bacillus cereus*	P3
EHF3	*E. helioscopia*	+	*Curtobacterium flaccumfaciens*	P4
EHF5	*E. helioscopia*	+	*B. amyloliquefaciens*	P5
EPR3	*E. peplus*	-	*P.brassicacearum*	P2
OR1	*Urtica dioica*	+	*B. toyonensis* /*B. thuringiensis*	P3
OR2	*U. dioica*	+	*B. amyloliquefaciens*	P6
OS1	*U. dioica*	+	*B.cereus*	P3
OS2	*U. dioica*	+	*B. pumilus*	P7
OS4	*U. dioica*	+	*B. methylotrophicus*	P6
PA2	*Plantago lanceolata*	+	*B.*spp.	P6
PF1	*P. lanceolata*	+	*B.* spp.	P7
PF3	*P. lanceolata*	+	*B. methylotrophicus*	P1
PR1	*P. lanceolata*	+	*B.* spp.	P3
PS1	*P. lanceolata*	-	*P. brassicacearum*	P2

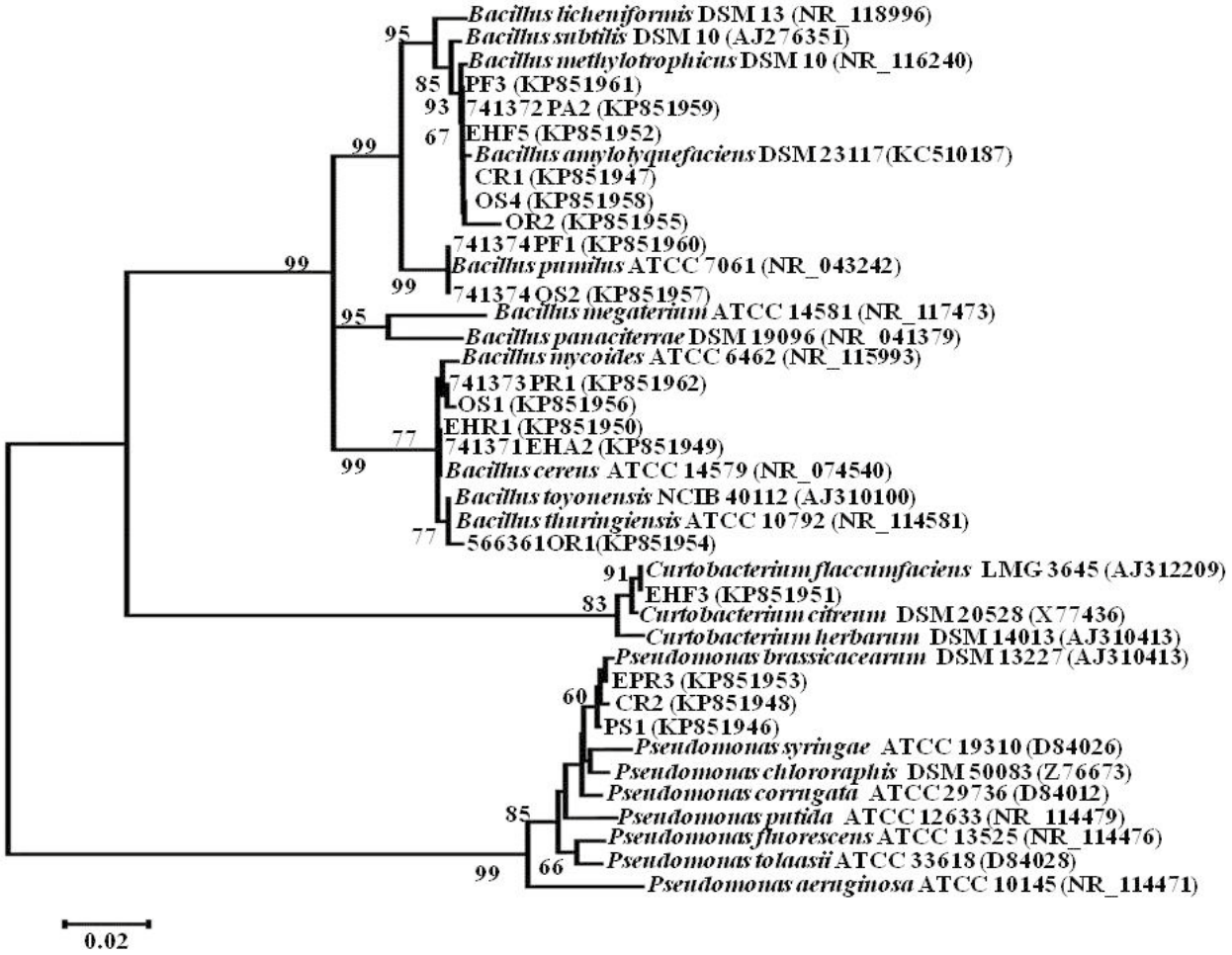

Figure 1. Phylogenetic tree showing evolutionary relationships of endophytic strains of *Bacillus* spp., *Curtobacterium flaccumfaciens* and *Pseudomonas* spp., as indicated by using neighbour-joining analysis. The tree was constructed using partial sequence (1500 bp) of the 16S rDNA gene.

Antagonistic activity of endophytes against plant pathogenic bacteria

The seventy-three bacteria were active against at least two of the seven pathogenic bacteria tested. The mean diameter of the clear zones around colonies of the antagonists was between 11.3 to 41.3 mm. All strains identified by 16S rDNA analysis, except for *C. flaccumfaciens* EHF3, exhibited strong antagonistic activity against the seven phytopathogenic bacteria. Strain EHF3 produced a hypersensitive reaction on tobacco leaves and was pathogenic on three bean varieties (data not shown). The antagonistic activity of nine strains representative of the bacteria isolated from the weeds is reported in Table 2.

Biocontrol activity of endophytic strains against *Agrobacterium* and *Pectobacterium* spp.

The five endophytic bacteria used for bacterization of tomato roots produced marked reductions in weights of tumours induced on tomato plants stem-inoculated with pathogenic agrobacteria. The endophytes showed biocontrol efficacy mainly related to the specific pathogen strains. For instance, reductions of tumour size of 84 to 96%, respectively, were obtained against *A. vitis* strains AL9/95 and Bu20/95. Less efficacy of endophytic strains in protecting tomato plants was observed against *A. tumefaciens* E14 and *A. vitis* AV25 (Table 3). Endophytic strains *Bacillus pumilus* OS2 and *Ba-*

Table 2. *In vitro* antagonistic activity of representative endophytic bacterial strains of different species against seven plant pathogenic bacteria.

Profile	Endophytic strains	Average size of inhibition zone (mm)[a]						
		At	Av	Cmm	Ea	P	Rs	Xc
P6	*Bacillus methylotrophicus* OS4	40	34.6	40.3	26	33	41.3	23
P7	*Bacillus* spp. PF1	18	19.6	20	20	15.3	12	21.6
P1	*B. methylotrophicus* PF3	36.6	18.6	29.6	23.3	23	20.3	24.6
P5	*B. amyloliquefaciens* EHF5	33	30.3	16.3	13.6	29.3	22	23
P2	*Pseudomonas brassicacearum* CR2	30	15.6	19.3	35.6	20.6	13.3	24.3
P6	*B. amyloliquefaciens* OR2	20	18	24	18.3	16	11.3	20.6
P7	*B. pumilus* OS2	15	30.3	26.3	15	20.6	20	31
P2	*P. brassicacearum* PS1	27	20	33	28.3	21	14.6	28.6
P3	*B.cereus* EHR1	21	17.3	21.3	25.6	13.6	15.3	17.3

[a] At: *Agrobacterium tumefaciens*, Av: *A.vitis*, Cmm: *Clavibacter michiganensis* subsp. *michiganensis*, Ea: *Erwinia amylovora*, P: *Pectobacterium* spp., Rs: *Ralstonia solanacearum*, Xc: *Xanthomonas axonopodis* pv *citri*.

Table 3. Mean weights of tumours (mg) induced by *Agrobacterium vitis* and *A. tumefaciens* on stem-inoculated tomato plants root-treated with different endophytic bacteria.

Endophytic bacteria[a]	Pathogenic bacterial strains			
	AL9/95 (%)	AV25/95 (%)	Bu20/95 (%)	E14 (%)
PS1	6.2 b[b] (87)	63.4 ab (23)	2.8 c (96)	60.2 b (30)
OS2	9.2 b (90)	49.1 bc (42)	6.3 bc (95)	51.8 bc (39)
EPR3	13.6 b (84)	78.3 a (7)	12.3 b (86)	37.2 c (57)
EHR1	9.6 b (88)	73.2 a (13)	4.2 bc (91)	45.5 bc (46)
OS4	7.6 b (91)	36.3 c (57)	4.1 bc (91)	56.7 b (33)
SDW	91.8 a	82.7 a	99.1 a	85.7 a

[a] PS1=*Pseudomonas brassicacearum*; OS2=*Bacillus pumilus*; EPR3=*Pseudomonas brassicacearum*; EHR1=*Bacillus cereus*; OS1=*Bacillus cereus*; OS4=*Bacillus methylotrophycus*; SDW=sterile distilled water.
[b] Data followed by the same letters are not statistically different according to the Duncan test ($P< 0.01$). The values reported in brackets are percentage reductions of tumour weights due to the plant bacterization with endophytic strains.

cillus methylotrophicus OS4 were the most effective against all of the pathogenic agrobacteria strains tested (Table 3).

Most of the endophytic strains reduced the size of the rotted areas of potato disks that were inoculated with *Pectobacterium* spp. Statistical analyses showed that the effect of pathogenic and antagonistic strains and their interactions were significant ($P<0.001$). The effectiveness of endophytes against the six pathogenic strains was similar on tuber discs of the cv. Bartina, Desiree and Spunta. In Figure 2, only data for cv. Spunta variety are presented. *Bacillus* spp. strains EHA2 and PA2 were the most efficient, since rotted tissues did not exceed 4 mm in diameter ($P< 0.001$). *Bacillus methylotrophicus* OR2 was almost ineffective against all the pathogenic strains.

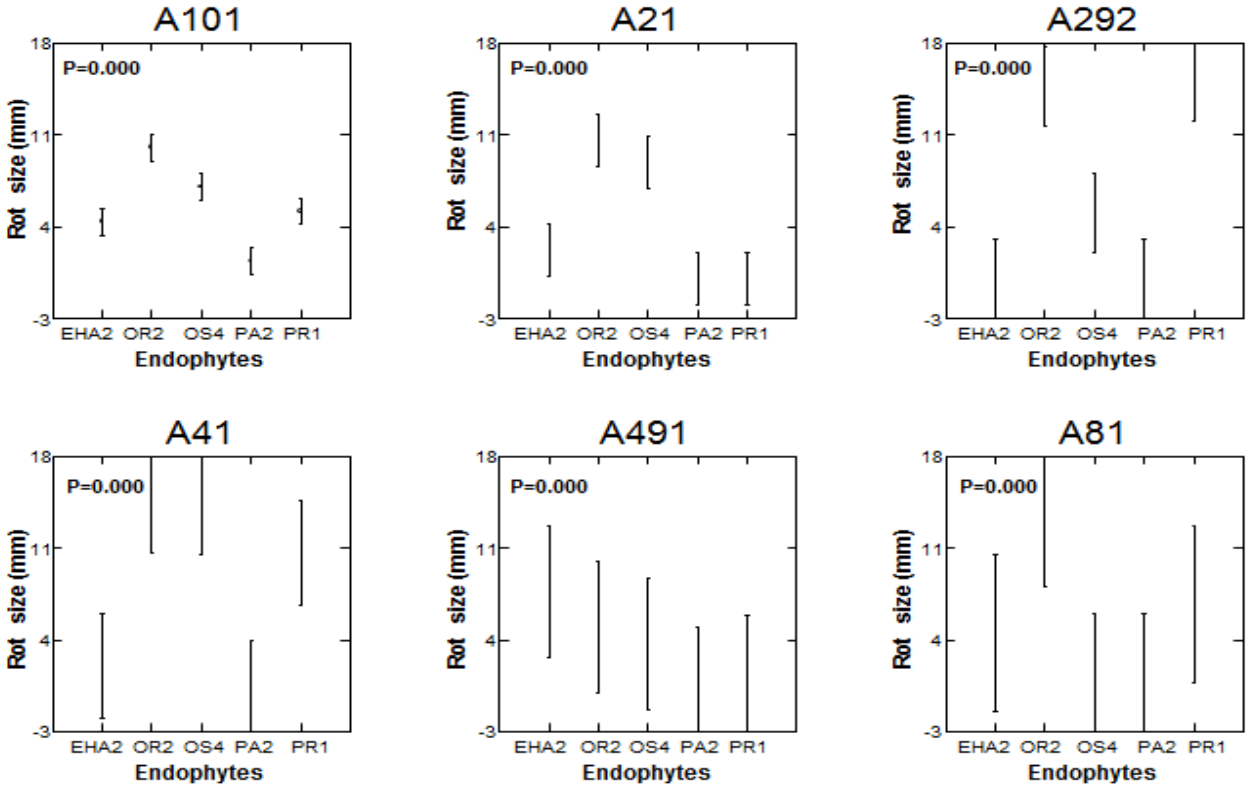

Figure 2. Mean dimensions of rots caused by *Pectobacterium* spp. inoculated *in vitro* on disks of potato tubers of the variety Spunta, treated with different strains of endophytic bacteria. EHA2=*Bacillus* spp.; OR2= *B. methylotrophicus*; OS4= *B. methylotrophicus*; PA2=*Bacillus* spp.; PR1=*Bacillus* spp. A101, A21, A292, A41, A491 and A81 are *Pectobacterium* spp. strains identified by biochemical, physiological and pathogenicity tests following the procedure described by Schaad *et al.*, 2001.

Evaluation of plant growth promotion activity of bacterial endophytes

All strains had positive effects on germination of tomato seeds. Bacterized seeds started to germinate early. The percentage of germinated seeds was greater than in the controls both *in vitro* and *in vivo* (Table 4). *Bacillus amyloliquefaciens* strain OS4 and *B. methylotrophicus* strain PF3 were the most active : at 10 d after bacterization more than 88% of the seeds were germinated as opposed to 53% of the controls. *Bacillus amyloliquefaciens* OS4 and *P. brassicacearum* PS1 strains were the most effective *in vivo* (Table 4).

Bacterized tomato plants had increased growth parameters both *in vitro* and in greenhouse trials (Table 5). *In vitro*, bacteria particularly enhanced root mass of plants since mean fresh and dry weights increased. *Bacillus amyloliquefaciens* CR1, *B.cereus* EHR1, *B. methylotrophicus* PF3 and *P. brassicacearum* PS1 were the most effective strains. Their activity is illustrated in Figure 3 where results are presented as proportional (%) increases percentage of plant growth parameters. These four bacterial strains produced marked increases in whole plant dry weights *in vivo*: *P. brassicacearum* PS1 increased root dry weight of 72% while *B. cereus* strain EHR1 was the most effective for increases in plant shoot weights (57%).

Discussion

This research aimed to characterize and study beneficial and antagonistic properties of endophytic bacteria living in weed hosts belonging to different botanical families, collected from a fallow field. Native plants are naturally adapted to environmental

Table 4. Mean percentage of germinated seeds at 5, 10 and 15 d after bacterization with endophytic strains *in vitro* and *in vivo*.

Endophytic bacteria [a]	*In vitro* (cultural filtrate)			*In vivo* (bacterial suspension)		
	5 d	10 d	15 d	5 d	10 d	15 d
CR1	2.7 a [b]	77.7 c	80.5 ab	4.4 bc	55.5 a	77.7 a
EHF5	13.9 cd	72.2 bc	77.7 a	2.2 b	64.4 b	84.4 b
EHR1	11.1 bc	66.6 b	80.5 ab	4.4 bc	75.5 cd	88.8 c
EPR3	8.3 b	80.5 cd	86.1 bc	2.2 b	71.1 c	88.8 c
OS2	8.3 b	69.4 b	86.1 bc	2.2 b	77.7 d	86.6 bc
OS4	11.1 bc	88.8 d	91.6 c	2.2 b	82.2 e	100 d
PF3	16.6 d	88.9 d	88.9 c	11.1 d	68.8 bc	84.4 b
PS1	8.3 b	80.5 cd	83.3 b	6.6 c	77.7 d	91.1 cd
Control	2.7 a	52.7 a	77.7 a	0 a	53.3 a	75.5 a

[a] CR1=*Bacillus amyloliquefaciens*; EHF5=*Bacillus amyloliquefaciens*; EHR1=*Bacillus cereus*; EPR3=*Pseudomonas brassicacearum*.; OS2=*Bacillus pumilus*; OS4=*Bacillus methylotrophicus*; PF3=*Bacillus methylotrophicus*.; PS1=*Pseudomonas brassicacearum*.; SDW=sterile distilled water.
[b] Data followed by the same letters are not statistically different according to Duncan test ($P< 0.01$).

Table 5. Mean parameters of tomato plants treated with different endophytic bacterial strains.

Endophytic strains	Stem length		Shoot fresh weight		Shoot dry weight		Root fresh weight		Root dry weight	
	In vitro (mm)**	*In vivo* (cm)**	*In vitro* (mg) **	*In vivo* (g) *	*In vitro* (mg)**	*In vivo* (g)**	*In vitro* (mg)**	*In vivo* (g) **	*In vitro* (mg)**	*In vivo* (mg)**
CR1	58.6 ab	23.2bc	90.6 bc	17.2ab	5.5 ab	0.31bc	8.0 bc	2.3 b	0.88c	30.8c
EHF5	58.5 b	21.8 d	86.5 c	17.2ab	5.1 bc	0.29bc	6.5 d	2.2bc	0.69 e	29.5cd
EHR1	60.9 ab	27.7 a	99.33ab	18 a	5.4 ab	0.42 a	6.4 d	2.2bc	0.80d	44 b
EPR3	61.4 ab	22.4cd	90.4 bc	15 bc	5. 4 ab	0.27 c	4.8 e	1.9cd	0.78 d	22 ef
OS2	55.5 bc	24.8bc	73.4 d	17 ab	4.4 c	0.27 c	6.0 d	2.1bc	0.62 f	25.4de
OS4	60.1 ab	22.5cd	87. 4 c	16.6ab	5.0 bc	0.26 c	7.1 cd	2.0 c	0.92bc	23.9 e
PF3	65.9 a	23.2 c	106.2 a	17.4ab	6.0 a	0.32 b	12.6 a	2.3 b	1.08 a	33.6 c
PS1	60.5 ab	25.7ab	92.3 bc	17.6 b	4.9 bc	0.33 b	9.1 b	2.6 a	0.99 b	68.9 a
SDW	51.0 c	19 e	66.6 d	13.9 c	3.5 d	0.18 d	4.2 e	1.6 d	0.5 g	19.3 f

CR1=*Bacillus amyloliquefaciens*; EHF5=*Bacillus amyloliquefacienss*; EHR1=*Bacillus cereus*; EPR3=*Pseudomonas brassicacearum*.; OS2=*Bacillus pumilus*; OS4=*Bacillus methylotrophicus*; PF3=*Bacillus methylotrophicus*.; PS1=*Pseudomonas brassicacearun*.; SDW=sterile distilled water
Data followed by the same letters are not statistically different according to Duncan test (** $P< 0,01$; * $0,01< p< 0.05$).

stress conditions which can often be extremely harsh in natural habitats. Their competitiveness, resistance to abiotic and biotic factors and abundant seed production abilities could be partly related to their endophytic microflora. Endophytes are often capable of eliciting drastic physiological changes that modulate the growth and development of host plants. Beneficial effects of endophytes may be exacerbated

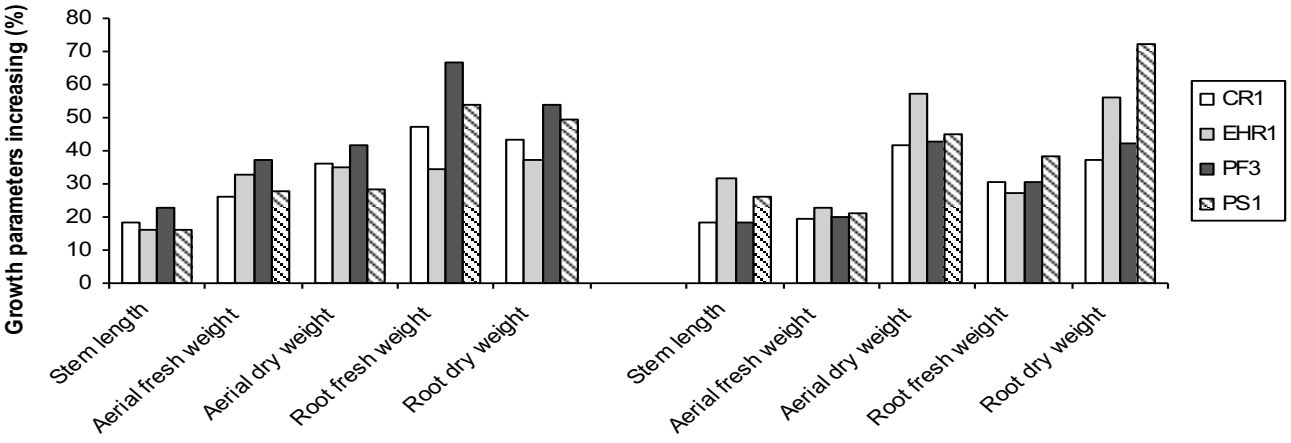

Figure 3. Mean Percentage increases of plant growth parameters for the most effective endophytic bacterial strains assessed in this study (left: *in vitro*; right: *in vivo*). CR1=*Bacillus amyloliquefaciens*; EHR1=*Bacillus cereus*; PF3=*Bacillus methylotrophicus*; PS1=*Pseudomonas brassicacearum*

when hosts are growing under stressful conditions (Hardoim *et al*., 2008).

Endophytic isolates belonging to *Bacillus* were identified as *B. amyloliquefaciens*, *B. cereus*, *B. methylotrophicus*, and *B. pumilus*. For some *Bacillus* isolates, the sequence similarity was less than 97% so that they could not be affiliated to any species. Strain OR1 shared 99% similarity with both *B. toyonensis* and *B. thuringiensis*. Sequence analysis of the 16S gene is not always an appropriate method to identify *Bacillus* species, given the complexity and variability of this group of bacteria. This was also evidenced by the presence of different colony morphologies within the individual species identified in this study. A multiphasic approach is often needed to precisely identify *Bacillus* strains (Ash *et al.*, 1991; Derekova *et al.*, 2008). Most of the endophytic species were common to the different plants analysed, except for *C. flaccumfaciens* which was only isolated from the two *Euphorbia* species. Dong *et al.* (2003) suggested that hosts can participate actively in endophytic colonization, and that this process is not passive; it requires the active participation of the bacterium to enter the plant host. Several reports have indicated that *C. flaccumfaciens* can function as a biological control agent against many pathogens, and may either induce systemic resistance (Raupach and Kloepper, 1998) or produce antibiotics (Sturz and Matheson, 1996). The *C. flaccumfaciens* strain isolated in the present study did not show any antagonistic effects or PGP activity, and it was not able to produce active enzymes or hormones *in vitro* (data not presented). *Pseudomonas* strains were isolated from three different host species (*C. arvensis*, *E. peplus* and *P. lanceolata*) and all were affiliated to *P. brassicacearum*. This bacterium was reported as a novel species in 2000 by Achouak*et al.* (2000), and was associated with roots of *Brassica napus* and *Arabidopsis thaliana*. It has the ability to suppress plant pathogens by producing active compounds such as 2,4-diacetyl-phloroglucinol, HCN, siderophores and proteases (Zhou *et al.*, 2012). Two endophytic strains induced a marked reduction of tumour size against two out of four *Agrobacterium* spp. strains. Inhibition of gall development was affected by the virulence of *Agrobacterium* strains; the antagonistic efficiency of endophytes decreased when the virulence of pathogens increased. Reduction of tumour size could be due to the ability of endophytic strains to induce host resistance (SAR/ISR), since antagonist and pathogen were inoculated at different sites on tomato seedlings. Endophytes were applied for root bacterization while pathogenic strains were inoculated by stem wounding.

The plant hormone ethylene plays a critical role during crown gall development and morphogenesis, as demonstrated by the high concentrations of this compound in transformed plant tissues (Wachter *et al.*, 1999). Toklikishvili *et al.*, (2010) showed that many bacteria produce ACC deaminase, which can cleave the immediate precursor of ethylene resulting in a reduction of ethylene and suppression of tumour growth. Our results suggests that effective

bacteria may synthesize the enzyme ACC deaminase (ACCD) which in turn prevents the overproduction of ethylene in the tumour tissues and alters the balance of hormones essential for tumorigenesis. This hypothesis needs to be confirmed by testing the ability of endophytic strains active against *Agrobacterium* spp. to produce ACCD.

Two *Bacillus* spp. endophytic strains reduced soft rot on potato disks inoculated with different *Pectobacterium* spp. strains. Finding effective strains for control of soft rot is important because this disease is not efficiently controlled by chemical and cultural measures, and these bacterial pathogens are soilborne and have wide host-ranges. Pathogenicity of *Pectobacterium* spp. is related to the activity of pectolytic enzymes and regulated by quorum sensing, which is a population density-dependent modulation of the bacterial phenotypes (Barnard *et al.*, 2007). Reduction of soft rot symptoms observed here could therefore be due to the ability of endophytic bacteria to maintain pathogen populations below the density required to induce disease (Baz*et al.*, 2012). The endophytic strains tested exhibited *in vitro* and *in vivo* antibiosis against pathogenic bacteria which could be explained by the production of different active metabolites. In particular, *P. brassicacearum* isolates produced detectable amounts of hydrogen cyanide (HCN) *in vitro* (data not presented).

The PGP assay showed that all tested strains increased tomato seed germination and plant growth. In comparison to the controls, all endophytic bacteria stimulated germination, both for *in vitro* and *in vivo* trials, and bacterized seeds germinated more rapidly than untreated seeds. However, *B. methylotrophicus* OS4 and *P. brassicacearum* PS1 strains were the most effective, producing large increases in seed germination *in vivo*. Most strains also promoted growth of tomato seedlings in assays conducted *in vitro* and *in vivo*, as shown by increased of fresh and dry biomass of the plants. The bacterial filtrates, as well as whole bacterial cells, induced significant increases of root growth that may have positively influenced the development of plant shoots. This vegetative bio-stimulation could be due to secondary metabolites secreted by the bacteria that were present in the filtrates. It is known that stimulation of germination by beneficial bacteria is due to the secretion of hormones such as IAA and ethylene, that are implicated in the breaking of seed dormancy (Glick, 2005). Some strains stimulated seed germination but did not greatly affect root biomass. This could be explained by their ability to produce high levels of ethylene which breaks seed dormancy and inhibits root elongation (Glick, 2005). Beneficial effects of endophytes are often greater than those of many rhizosphere-colonizing bacteria because they interact specifically with the hostplants they specifically associate with (Hardoim *et al.*, 2008). Bacteria isolated from native plants might possess relevant and diverse biological activities beneficial to the hosts, since environmental stresses and plant genotypes are selective factors for the most competitive strains. This may support the hypothesis that endophytic microflora can be the results of co-evolution strategies (Forchetti *et al.*, 2010).

To our knowledge, this is the first report of *B. methylotrophycus* as an endophyte. Moreover, our results of *in vivo* trials showed that *B. methylotrophicus* OS4 and *P. brassicacearum* PS1 were the most interesting beneficial strains that we isolated. *Bacillus methylotrophicus* strain OS4 strongly reduced gall development induced by *Agrobacterium* spp., and gave 100% germination of tomato seeds compared with 75.5% for the controls. *Pseudomonas brassicacearum* strain PS1 also actively enhanced tomato seed germination and markedly increased all plant growth parameters.

These strains are potential beneficial agents for use in biopesticide or biofertilizer formulations. However, further studies are needed to determine their synergistic interactions, their ability to colonize plant tissues of different hosts, and to evaluate their competitiveness under field conditions.

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