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REGULAR ARTICLE

# Antagonistic bacteria of composted agro-industrial residues exhibit antibiosis against soil-borne fungal plant pathogens and protection of tomato plants from *Fusarium oxysporum* f.sp. *radicis-lycopersici*

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**Abstract** Rhizospheric and root-associated/endophytic (RAE) bacteria were isolated from tomato plants grown in three suppressive compost-based plant growth media derived from the olive mill, winery and *Agaricus bisporus* production agro-industries. Forty-four (35 rhizospheric and 9 RAE) out of 329 bacterial strains showed in vitro antagonistic activity against at least one of the soil-borne fungal pathogens, *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), *F. oxysporum* f.sp. *raphani*, *Phytophthora cinnamomi*, *P. nicotianae* and *Rhizoctonia solani*. The high percentage of total isolates showing antagonistic properties

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(13%) and their common chitinase and β-glucanase activities indicate that the cell wall constituents of yeasts and macrofungi that proliferate in these compost media may have become a substrate that favours the establishment of antagonistic bacteria to soil-borne fungal pathogens. The selected bacterial strains were further evaluated for their suppressiveness to tomato crown and root rot disease caused by FORL. A total of six rhizospheric isolates, related to known members of the genera *Bacillus*, *Lysinibacillus*, *Enterobacter* and *Serratia* and one RAE associated with *Alcaligenes faecalis* subsp. were selected, showing statistically significant decrease of plant disease incidence. Inhibitory effects of extracellular products of the most effective rhizospheric biocontrol agent, *Enterobacter* sp. AR1.22, but not of the RAE *Alcaligenes* sp. AE1.16 were observed on the growth pattern of FORL. Furthermore, application of cell-free culture extracts, produced by *Enterobacter* sp. AR1.22, to tomato roots led to plant protection against FORL, indicating a mode of biological control action through antibiosis.

**Keywords** Rhizosphere · Suppressive compost · Endophytic · Chitinase · Fungal antagonist · *Enterobacter* · Antibiosis

## Introduction

Several studies have demonstrated the suppression of soil-borne pathogens by composts derived from agro-industrial wastes including olive processing by-products, spent mushroom substrates, and grape/winery wastes (Noble and Coventry 2005; Termorshuizen et al. 2007; Ntougias et al. 2008).

Suppressiveness of composts to plant diseases has been attributed to complex interactions between abiotic characteristics and/or microbial populations and plants, involving microbial competition for nutrient substrates and ecological niches, antibiosis, microbial production of lytic enzymes, fatty acid degradation, parasitism, changes in nutrient availability and host-mediated induction of resistance (Lorito et al. 1993; Erhart et al. 1999; Hoitink and Boehm 1999; McKellar and Nelson 2003; Kavroulakis et al. 2005; Borrero et al. 2006; Perez-Piqueres et al. 2006). Synthesis and excretion of simple bioactive compounds (amino acids, organic acids, vitamins), plant growth hormones and chelators alter physiological conditions related to plant growth and may also affect the enhanced plant tolerance to diseases conveyed by certain composts (Van Loon 2007).

As a consequence, the capacity of composts to suppress plant diseases is highly variable, even when using similar composted materials and application rates (Erhart et al. 1999; Bonanomi et al. 2007; Noble and Coventry 2005; Termorshuizen et al. 2007). Inoculation of mature composts with efficient biocontrol agents could potentially improve the efficiency and the spectrum of disease suppression (Zhang et al. 1998; Trillas et al. 2006; Siddiqui et al. 2008) but this route has not been systematically explored.

In contrast to the general suppression of Oomycetes pathogens, which appears to be related to the proliferation and activity of broad microbial consortia (Hoitink et al. 1996), specific microbial agents appear to be responsible for the efficiency of compost amendments on the control of Eumycetes, such as *Fusarium* and *Rhizoctonia* pathogens (Hoitink and Boehm 1999; Weller et al. 2002).

Plant chitinases and β-glucanases inhibit fungal growth (Mauch et al. 1988; Arlorio et al. 1992) and have also been shown to inhibit plant colonization and to reduce the plant disease incidence caused by pathogenic fungi (Broglie et al. 1991; Vierheilig et al. 1993). Microorganisms expressing these activities have also been shown to effectively inhibit fungal growth and to lower disease incidence caused by soil-borne pathogens (Chet et al. 1990; Jung et al. 2003; Nagarajkumar et al. 2004). Microbial hydrogen cyanide (HCN) production is another characteristic related to suppression of plant pathogens (Voisard et al. 1989), and efficient producers of both HCN and lytic enzymes appear to be competent biological control agents (Nagarajkumar et al. 2004)

In this study we made use of three compost media derived from olive mill, winery and *Agaricus bisporus* agro-industrial waste materials, which were shown to be highly suppressive to the tomato pathogens *Phytophthora nicotianae* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) (Ntougias et al. 2008). All three composts are rich in chitin rather than cellulose and we hypothesized that this common property could lead to the proliferation of chitinase and β-glucanase bacterial producers. The objectives of this work were i) to isolate from these media novel rhizospheric and root-associated/endophytic (RAE) bacterial strains as potential biocontrol agents; ii) to

test the hypothesis of whether these compost media could be selective for biocontrol agents showing lytic properties against chitin and $\beta$-glucans; iii) to identify effective biocontrol agents by evaluating their in vitro antagonistic activity against major soil-borne pathogens and their suppressiveness to tomato crown and root rot disease caused by FORL, and iv) to investigate whether cell-free culture extracts from the most effective biocontrol agents can suppress plant disease.

## Materials and methods

### Plant pathogens

Plant root pathogen FORL Jarvis & Shoemaker (strain CBS 101587) was obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; *Fusarium oxysporum* f.sp. *raphani* Kendr. & Snyder was kindly donated by CMJ Pieterse (University of Utrecht, The Netherlands), while *Phytophthora nicotianae* Breda de Haan (strain IK-23), *Phytophthora cinnamomi* Rands and *Rhizoctonia solani* were provided by the Benaki Phytopathological Institute, Athens, Greece.

### Compost material used

Three composts, derived from agro-industrial residues and by-products, i.e. grape-marc wastes (GM), extracted olive press cake (EPC, press cake remaining after the extraction of residual kernel and pulp oil from olive press cake), olive tree leaves (OL), olive mill wastewaters (OMW) and spent mushroom compost from the cultivation of *Agaricus* species (SMC) were prepared on site at the Institute of Kalamata and have been previously described (Ntougias et al. 2008). The composts were labelled GM+EPC, OL+OMW and SMC, in reference to the raw materials used to make the respective composting blends. All three composts had demonstrated high levels of suppressiveness to disease caused by *P. nicotianae* and FORL in tomato plants, as previously reported (Ntougias et al. 2008).

### Isolation of antagonistic bacteria

Sphagnum peat, (Sunu Kura, Seda joint-Stock company), treated with 20 g $l^{-1}$ $CaCO_3$, was mixed with each of the composts at a ratio of 1:1 w $w^{-1}$, in order to obtain RAE and rhizospheric bacteria. Tomato plants (*Solanum lycopersicon* cv. ACE55) were grown on the peat–compost mixtures in 300 $cm^3$ volume pots. Seeds were surface-sterilized in 2.5% NaOCl and three seeds were directly sown in each pot. The pots were placed in a growth chamber at 65% relative humidity (RH) (light: 16 h, 25°C; dark: 8 h, 20°C) and irrigated daily. Plants were grown for 1 month.

To isolate rhizospheric bacteria, plants were removed carefully from the pots and 1 g (wet weight) of compost-based potting medium, loosely attached to plant roots, was put in 25-ml glass test tubes containing 9.5 ml of sterile 8.5 g $l^{-1}$ NaCl solution (Ntougias et al. 2004). The mixture was shaken thoroughly, tenfold dilution series were prepared and 0.1 ml of the suspensions was sequentially aliquoted onto isolation media. For the isolation of RAE candidates, tomato plant roots were surface-sterilized with 2.5% NaOCl for 10 min and extensively washed with sterilized tap water. Roots were partitioned and placed on solid isolation media, i.e. Luria-Bertani (LB) and King's B (KB) supplemented with the antifungal compound cycloheximide (100 µg $ml^{-1}$). All plates were incubated at 25°C and bacterial isolates were obtained for up to 7 days. Since we have not performed further microscopic analysis to verify the ingression of these bacteria inside the root tissues, these isolates represent either true endophytes or bacteria tightly attached to the root surface.

### In vitro evaluation of the antagonistic activity of isolated bacteria against soil-borne fungi

The RAE and the rhizospheric bacteria isolated were evaluated for their in vitro antagonistic activity against FORL, *F. oxysporum* f.sp. *raphani*, *P. cinnamomi*, *P. nicotianae* and *R. solani*. Bacterial isolates were incubated in broth medium (LB or KB based on the medium from which they were isolated) at 25°C. Fungal pathogens were grown on potato dextrose agar (PDA). A 6-mm plug from the leading edge of a 5-days-old culture of each fungal pathogen tested was placed at the centre of a 9-cm diameter Petri dish containing PDA. Aliquots of 0.1 ml of each growing bacterial culture were placed at approximately 3 mm on the edges at three sides of the plate (five replicate plates per each biocontrol agent–pathogen combination). *Escherichia coli* strain DH5α (no antagonistic activity) was used as negative control. Isolates that formed zones of inhibition (haloes without

mycelial growth or distorted hyphae) over 2 mm were selected.

### In vitro detection of secreted potential antifungal metabolites

For the detection of secreted bacterial $\beta$-glucanase and chitinase, 1.7% agar plates containing 0.1% lichenan (Walsh et al. 1995) and 0.1% colloidal chitin (Garbeva et al. 2004) were used, respectively. The plates were inspected for enzymatic degradation indicated by a clearing zone following 5 days of growth at 25°C. HCN was detected by cyanide indicator paper (Castric 1975). The *E.coli* strain DH5$\alpha$ was used as negative control.

### *In planta* evaluation of the antagonistic activity of RAE and rhizospheric isolates against FORL

To evaluate the bacterial antagonistic activity *in planta*, conidial suspensions of FORL ($10^5$ conidia $cm^{-3}$ of potting medium) and cell suspensions ($10^6$ $ml^{-1}$) of each bacterial isolate were prepared as follows: FORL was grown on potato dextrose broth (PDB) at 25°C in the dark, for 5 days. Following removal of mycelial fragments by sieving, conidia were recovered by centrifugation at 5,000 g, counted and resuspended in the appropriate volume of 0.85% w/v NaCl. The pre-selected bacterial strains were incubated in broth medium (LB or KB) at 25°C. Cells were harvested by centrifugation at 5,000 g for 10 min, counted on the basis of standard growth curves and resuspended in the appropriate volume of 0.85% w/v NaCl.

Seeds of tomato plants (cv. ACE55) were surface-sterilized in 2.5% NaOCl, pre-germinated for two days on moist paper and then sown into 15-cm diameter pots (five seeds per pot), each containing 300 $cm^3$ of peat, blended with NPK + micronutrients fertilizer (5.8% $NO_3^-$-N, 3.9% $NH_4^+$-N, 10.6% urea-N, 20% $P_2O_5$, 20% $K_2O$, 0.5% $MgSO_4$, 0.03% Fe ethylene diamine tetraacetic acid (EDTA), 0.02% Zn-EDTA, 0.02% Mn-EDTA, 0.01% Cu-EDTA and 0.02% B) to a concentration of 0.8 g $l^{-1}$. The pots were placed in a growth chamber at 65% RH (light: 16 h, 25°C; dark: 8 h, 20°C) and watered to the initial weight on alternate days. Once a week the plants were fertilized with a balanced nutrient solution including micronutrients.

Potting media were inoculated with the bacterial strains 1 week after the sowing of tomato seeds. One ml of bacterial cell suspension ($10^6$ $ml^{-1}$) was applied for each growing plant. Pots planted with tomato plants in the absence of bacterial inoculum were used as control. The plant pathogen FORL was applied at $10^5$ conidia per $cm^3$ of potting mixture at the stage of the first true leaf emergence (approximately 2 weeks after sowing). Plants were scored daily either as healthy (no or scarce disease symptoms) or as diseased (severe wilting or dead plants). Five replications (pots) per treatment were included for the *in planta* evaluation of the RAE and rhizospheric bacteria tested. The experiment was performed twice for all isolates.

### Molecular characterization of the bacterial antagonists: 16S rRNA gene sequencing and phylogenetic analysis

DNA from pelleted cells of the selected bacterial isolates was extracted as described by Wilson (1992). The nearly full-length 16S rRNA gene was amplified using primers Afor (5′-GGAGAGTTAGATCTTGGC TCAG-3′) (positions 6–27 by *E. coli* numbering) and Crev (5′-AGAAAGGAGGTGATCCAGCC-3′) (positions 1542–1525 by *E. coli* numbering) (Ntougias et al. 2006). PCR reactions were performed in a PTC-200 thermocycler (MJ Research Inc., USA), with a denaturation step of 2 min at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s primer annealing at 55°C and 1 min polymerization at 72°C. The PCR was completed by 5 min DNA chain extension at 72°C. The amplified fragments were cloned into the pGEM-T Easy Vector (Promega, WI, USA) and transformed into *E. coli* JM-109 competent cells. For each isolate, three independent clones were grown in LB and plasmid DNA was extracted using the "NucleoSpin plasmid Quick Pure" kit (Macherey-Nagel, Germany). PCR sequencing was performed at the Institute of Molecular Biology and Biotechnology (IMBB), Heraklion, Greece, as previously described (Ntougias et al. 2006).

The 16S rRNA gene sequences were assembled using the CAP3 programme (http://pbil.univ-lyon1.fr/cap3.php). Similarity searches were performed using "BLASTN" (http://www.ncbi.nlm.nih.gov/BLAST/) and "Seqmatch" (Ribosomal Database Project II—http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp),

and the sequences of their closest relatives identified in both searches were used for further phylogenetic analysis. Alignment was carried out using the "ClustalW Submission Form" (http://www.ebi.ac.uk/clustalw/), evolutionary distances were calculated using the method of Jukes and Cantor (1969) and topology was inferred using the "neighbour-joining" method (Saitou and Nei 1987) based on bootstrap analysis of 1,000 replicates. A phylogenetic tree was constructed using TREECON for Windows (Version 1.3b) (Van de Peer and De Wachter 1993).

### In vitro inhibitory effect of culture extracts of *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16 on the growth of FORL

Cell-free culture extracts of biocontrol agents *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16 were obtained through centrifugation of 48-h bacterial cultures in LB, followed by filter-sterilization (0.2 μm filter, Nalgene, Rochester, NY). Inhibitory effects of culture extracts on the growth of plant pathogens were examined using 9-cm PDA plates, containing cell-free extract and deionized water in a ratio of 1:1 v/v. PDA plates (including half strength), in the absence of culture extracts, were used as controls. A 6-mm plug from the leading edge of a 5-days-old culture of FORL was placed in the centre of both extract-containing and control PDA plates. Cultures were incubated at 25°C and the radial growth was recorded daily. The experiment was performed twice.

### Evaluation of *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16 culture extracts to suppress tomato crown and root rot disease caused by FORL

Cell-free extracts of *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16 were obtained, as described above. Potting media and tomato plants were prepared and grown, respectively, as previously described. Six experimental sets (each consisting of five replicate pots per treatment) were carried out to assess the effect of increasing concentrations of putative antifungal compounds, secreted by the bacterial isolates in the culture medium. Twenty ml of culture extract was applied to each pot once a week, with the first application being performed 1 week after sowing. The first experimental set received culture extract for 1 week only (i.e. week 2) and was irrigated with tap water thereafter. The second set received culture extract for two consecutive weeks (i.e. weeks 2 and 3) and tap water thereafter, whereas the third set received culture extract for three consecutive weeks (i.e. weeks 2, 3 and 4) and then tap water, et cetera. Controls were prepared by replacing culture extracts either with tap water or LB medium. The plant pathogen FORL was applied at $10^5$ conidia per $cm^3$ of potting mixture at the stage of the first true leaf emergence (2 weeks after sowing). Plants were scored daily either as healthy or as diseased.

### Statistical analysis

Experimental data were analyzed using standard analysis of variance (ANOVA) followed by Duncan multiple comparison tests ($a<0.05$). Standard errors were calculated for all mean values. Arcsine transformation was applied to all data expressed as percentages, before analysis.

## Results

### Isolation and in vitro evaluation of the antagonistic activity of bacterial strains against soil-borne fungal pathogens

Forty-four (35 rhizospheric and 9 RAE) out of the 329 bacterial isolates showed antagonistic activity against at least one of the fungal plant pathogens tested (Table 1). In vitro inhibition of all fungal pathogens tested was observed for 25 isolates (Table 1), whereas seven strains (AR1.15, BR1.1, BR1.3, BR1.6, BR1.10, AR3.4 and AR3.9) inhibited growth of FORL and *F. oxysporum* f.sp. *raphani* only; three strains (AR1.17, BR1.4 and BR1.11) inhibited growth of FORL, *F. oxysporum* f.sp. *raphani* and *R. solani* only; five strains (AR1.7, AR1.8, BR1.8, BR1.14 and BR2.4) inhibited growth of *P. nicotianae, P. cinnamomi* and *R. solani* only; and two strains (AR1.16 and AE1.16) inhibited growth of *P. nicotianae* and *P. cinnamomi* only. For the majority of the selected bacterial strains (26 out of 44), the inhibition of fungal pathogens was influenced, in a complex manner, by the growth medium used (LB or KB), depending on the biocontrol agent and the plant pathogen tested. However, pathogen inhibition was clearly favoured

**Table 1** Screen for selection of potential bacterial biocontrol agents

| Bacterial isolates | Biochemical reactions tested[a] HCN production | In vitro inhibition of soil-borne fungi growing on LB or KB media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) | | *Fusarium oxysporum* f.sp. *raphani* | | *Phytophthora nicotianae* | | *Phytophthora cinnamomi* | | *Rhizoctonia solani* | |
| | | LB | KB | LB | KB | LB | KB | LB | KB | LB | KB |
| GM-EPC | | | | | | | | | | | |
| AR1.1 | − | + | + | + | + | + | + | + | + | + | + |
| AR1.2 | − | + | + | + | + | + | + | + | + | + | + |
| AR1.3 | − | + | + | + | + | + | + | + | + | + | − |
| AR1.4 | + | + | + | + | + | + | + | + | + | + | + |
| AR1.5 | − | + | + | + | + | + | + | + | + | + | + |
| AR1.7 | − | − | − | − | − | + | + | + | + | + | + |
| AR1.8 | − | − | − | − | − | + | + | + | + | + | + |
| AR1.9 | + | + | + | + | + | + | + | + | + | + | + |
| AR1.10 | − | − | − | − | − | − | + | − | + | − | + |
| AR1.12 | − | + | − | + | + | + | + | + | + | − | + |
| AR1.15 | − | + | − | + | + | − | − | − | − | − | − |
| AR1.16 | − | − | − | − | − | + | − | + | − | − | − |
| AR1.17 | + | + | − | + | + | − | − | − | − | − | + |
| AR1.22 | + | + | + | + | + | + | + | + | + | + | + |
| AE1.16 | + | − | − | − | − | + | + | + | + | − | − |
| BR1.1 | − | + | − | + | + | − | − | − | − | − | − |
| BR1.2 | + | + | + | + | + | + | + | + | + | + | + |
| BR1.3 | − | + | − | + | + | − | − | − | − | − | − |
| BR1.4 | + | + | − | + | + | − | − | − | − | − | + |
| BR1.6 | − | + | − | + | + | − | − | − | − | − | − |
| BR1.8 | − | − | − | − | − | − | + | − | + | − | + |
| BR1.9 | + | + | + | + | + | + | + | + | + | + | + |
| BR1.10 | − | + | − | + | + | − | − | − | − | − | − |
| BR1.11 | + | + | − | + | + | − | − | − | − | − | + |
| BR1.13 | + | − | − | − | − | + | ND | + | ND | ND | ND |
| BR1.14 | − | − | − | − | − | − | + | − | + | − | + |
| OL-OMW | | | | | | | | | | | |
| AR2.1 | + | + | + | + | + | + | + | + | + | + | + |
| AR2.4a | − | + | + | + | + | + | + | + | + | + | + |
| AR2.4bii | − | + | + | + | + | + | + | + | + | + | + |
| AR2.6 | + | + | + | + | + | + | + | + | + | + | + |
| AE2.3 | − | + | − | + | − | + | − | + | − | + | − |
| AE2.4 | + | + | − | + | − | + | − | + | − | + | − |
| BR2.1 | − | − | + | − | + | − | + | − | + | − | + |
| BR2.3 | − | − | − | − | − | + | ND | + | ND | ND | ND |
| BR2.4 | − | − | − | − | − | − | + | − | + | − | + |
| BR2.5 | − | + | − | + | + | + | + | + | + | − | + |
| SMC | | | | | | | | | | | |
| AR3.4 | − | + | − | + | − | − | − | − | − | − | − |

**Table 1** (continued)

| Bacterial isolates | Biochemical reactions tested[a] HCN production | In vitro inhibition of soil-borne fungi growing on LB or KB media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) | | *Fusarium oxysporum* f.sp. *raphani* | | *Phytophthora nicotianae* | | *Phytophthora cinnamomi* | | *Rhizoctonia solani* | |
| | | LB | KB | LB | KB | LB | KB | LB | KB | LB | KB |
| AR3.9 | − | + | − | + | − | − | − | − | − | − | − |
| AE3.1 | − | + | − | + | − | + | − | + | − | + | − |
| AE3.3 | − | + | − | + | − | + | − | + | − | + | − |
| AE3.4 | + | + | − | + | − | + | − | + | − | + | − |
| AE3.5 | − | + | − | + | − | + | − | + | − | + | − |
| AE3.6 | − | + | − | + | − | + | − | + | − | + | − |
| AE3.8 | − | + | − | + | − | + | − | + | − | + | − |

[a] All isolates were tested positive for chitinase and $\beta$-glucanase (lichenanase) activity

*LB* Luria-Bertani medium, *KB* King's B medium, *GM+EPC* grape marc (GM) and extracted press cake (EPC)-based compost medium, *OL+OMW* olive tree leaves (OL) and olive mill wastewaters (OMW)-based compost medium, +/−: presence/ absence of inhibitory action of fungal growth, *ND* not determined

by the use of LB medium in the case of bacteria isolated from the SMC-derived compost and the effect was not observed when KB was used (Table 1). The majority of biocontrol agents selected were isolated from GM+EPC-based potting medium (26 strains in total, including one RAE), whereas many RAE (six strains) were isolated from plants growing on SMC-based amendments (Table 1).

In vitro detection of secreted potential antifungal metabolites

Chitinase and $\beta$-glucanase (lichenanase) activity was detected in all bacterial strains exhibiting in vitro antagonistic activity against soil-borne plant pathogens, whereas only 14 strains were positive for HCN production (Table 1).

Evaluation of RAE and rhizospheric bacteria isolated for suppressiveness to tomato crown and root rot disease caused by FORL

*In planta* experiments showed that inoculation of tomato plants with the rhizospheric isolates AR1.2, AR1.5, AR1.22, BR1.4, AR2.4bii and BR2.1 resulted in a statistically significant reduction in plant disease incidence (20–60%), depending on the strain tested (Fig. 1a). The greatest *in planta* antagonistic activity against FORL was observed in potting media inoculated with the strain AR1.22 (40–54% decrease in disease severity) (Fig. 1a). Inoculation of potting media with the RAE strains revealed that strain AE1.16 reduced disease incidence by approximately 40–50%, while the remaining RAE did not cause statistically significant reduction in disease severity compared to the control potting medium with tomato plants in the absence of bacterial biocontrol agent (Fig. 1b).

Molecular characterization of the bacterial antagonists selected from *in planta* experiments

Seven bacterial strains that were identified as the most effective biocontrol agents were characterized at the species level. Their phylogenetic allocation and 16S rRNA gene sequence identities are presented in Fig. 2 and their description is presented in Table 2. Four rhizospheric strains were isolated from the GM+EPC-based compost medium. Three strains, namely AR1.2, AR1.5 and BR1.45 were highly related to *Bacillus* spp., whereas the closest relative of strain AR1.22 was *Enterobacter ludwigii* (99.7% identity in 16S rRNA gene) and the next closest species was *E.*

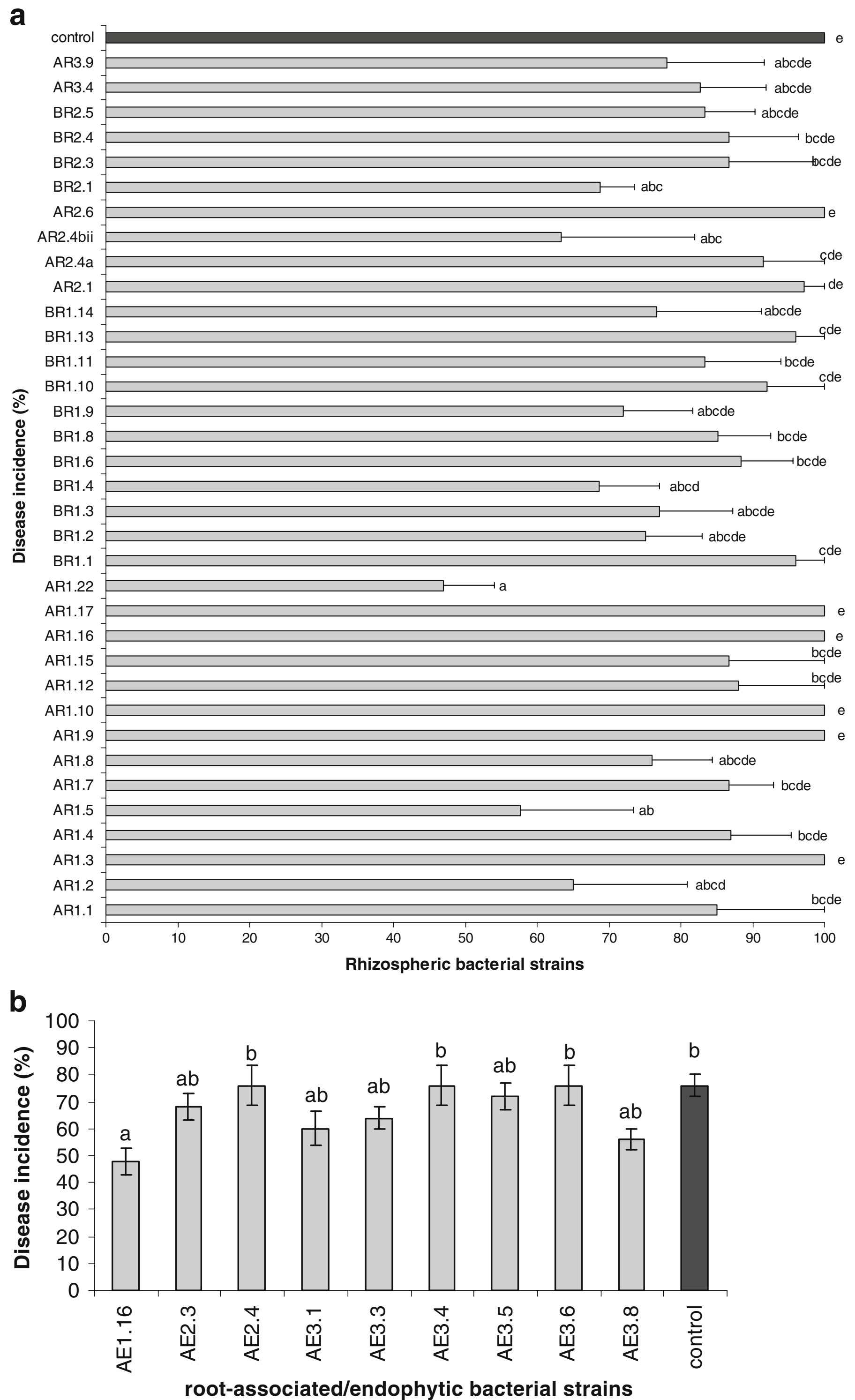


**Fig. 1** **a**. *In planta* evaluation of rhizospheric bacteria selected in the present study against *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). Disease incidence is recorded as percentage of dead plants. *Letters at the top of the bars* indicate statistically significant differences (Duncan's test, $a<0.05$; $n=5$). **b.** *In planta* evaluation of root-associated/ endophytic (RAE) bacteria selected in the present study against FORL. Disease incidence is recorded as percentage of dead plants. *Letters at the top of the bars* indicate statistically significant differences (Duncan's test, $a<0.05$; $n=5$). Results from a representative experiment are shown

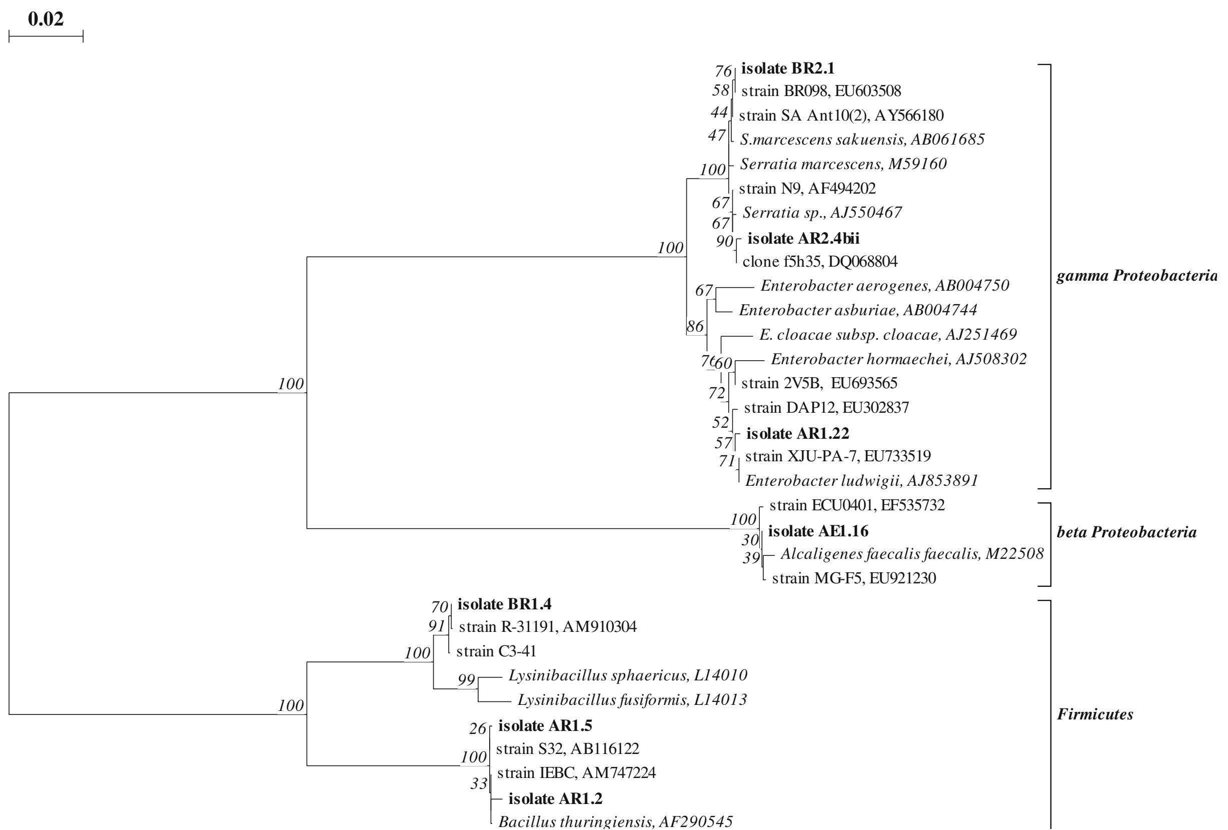


**Fig. 2** Phylogenetic tree based on the 16S rRNA gene sequence of effective biocontrol agents (depicted in bold typeface), isolated from compost-based amendments (or their seeded plants) made by agro-industrial by-products abundant in the Mediterranean region. Evolutionary distances were calculated using the method of Jukes and Cantor (1969) and the topology was inferred using the "neighbor-joining" algorithm (Saitou and Nei 1987), based on a bootstrap analysis of 1,000 replicates (values on branches denote % of bootstrap support). *Scale bar* represents 0.02 inferred substitutions per nucleotide position

*cloacae*. Bacterial strains AR2.4bii and BR2.1, isolated from OL+OMW-based compost medium, were affiliated with *Serratia marcescens* strains, one of which has been isolated from soybean rhizosphere and has been reported as antagonistic to *Ralstonia solanacearum* (GenBank direct submission AJ550467) However, isolates AR2.4bii and BR2.1 differed phylogenetically at subspecies/strain level (Table 2). The only RAE selected (strain AE1.16) was associated with *Alcaligenes faecalis* subsp. *faecalis* (99.7% identity in 16S rRNA gene).

## In vitro and *in planta* inhibitory effects of culture extracts produced by *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16

Culture extracts produced by the rhizospheric *Enterobacter* sp. AR1.22 isolate inhibited by 20% the radial growth of FORL, whereas no effect on the growth of the pathogen was observed with the culture extracts of the RAE *Alcaligenes* sp. AE1.16 isolate (Fig. 3).

In accordance, no inhibitory effect was observed in the disease severity caused by FORL in tomato plants, following successive weekly applications of *Alcaligenes* sp. AE1.16 culture extract, (data not shown). On the contrary, the cummulative application of *Enterobacter* sp. AR1.22 culture extracts to tomato plants had a significant effect on the reduction in disease severity caused by FORL. The reduction was positively related to the number of successive applications (Fig. 4). Thus, no difference in disease incidence was recorded in plants treated once or twice with culture extracts compared to control (water- or LB- treated) plants. Interestingly, extract applications carried out one and 2 weeks prior to plant inoculation with the pathogen (experimental sets 1 and 2; Fig. 4)

**Table 2** Closest phylogenetic relatives of most effective bacterial biocontrol agents identified in this study

| Bacterial isolate | Isolation medium/type-location/compost | Accession number | Phylogenetic relative | Identity (%) | References |
|---|---|---|---|---|---|
| AE1.16 | LB/RAE/GM+EPC | GQ284565 (1,492 bp) | strain MG-F5 (1,360 bp aligned) | 99.9 | EU921230/ |
| | | | *Alcaligenes faecalis* subsp. *faecalis* strain ATCC 8750 (1,476 bp aligned) | 99.7 | Kersters and De Ley 1984 |
| AR1.2 | LB/rhizospheric/ GM+EPC | GQ284563 (1,504 bp) | *Bacillus thuringiensis* strain ATCC 10792 (1,472 bp aligned) | 99.7 | Schnepf et al. 1998 |
| AR1.5 | LB/rhizospheric/ GM+EPC | GQ284562 (1,505 bp) | *Bacillus thuringiensis* strain ATCC 10792 (1,472 bp aligned) | 99.9 | Schnepf et al. 1998 |
| AR1.22 | LB/rhizospheric/ GM+EPC | GQ284566 (1,493 bp) | *Enterobacter ludwigii* strain DSMZ 16688 (1,495 bp aligned) | 99.7 | Hoffmann et al. 2005 |
| AR2.4bii | LB/rhizospheric/ OL+OMW | GQ284560 (1,491 bp) | clone f5h35 (1,486 bp aligned) | 99.9 | Dunn and Stabb 2005 |
| | | | *Serratia marcescens* subsp. *marcescens* strain ATCC 13880 (1,494 bp aligned) | 99.5 | Grimont and Grimont 1984 |
| BR1.4 | KB/rhizospheric/ GM+EPC | GQ284564 (1,503 bp) | strain R-31191 (1,507 bp aligned)/ | 99.9 | Coorevits et al. 2008 |
| | | | *Lysinibacillus sphaericus* strain ATCC 14577 (1,488 bp aligned)/ | 99.8 | Priest et al. 1994 |
| BR2.1 | KB/rhizospheric/ OL+OMW | GQ284561 (1,491 bp) | strain BR098 (1,464 bp aligned) | 100 | EU603508/ |
| | | | *Serratia marcescens* subsp. *marcescens* strain ATCC 13880 (1,493 bp aligned)/ | 99.8 | Grimont and Grimont 1984 |
| | | | *Serratia marcescens* subsp. *sakuensis* strain DSM 17174 (1,493 bp aligned) | 99.8 | Ajithkumar et al. 2003 |

*LB* Luria-Bertani medium, *GM+EPC* grape marc (GM) and extracted press cake (EPC)-based compost medium, *OL+OMW* olive tree leaves (OL) and olive mill wastewaters (OMW)-based compost medium

conferred no protective effect on the plants; on the contrary, the application of extracellular compounds in the presence of the pathogen (experimental sets 4, 5 and 6) led to a 40% decrease in disease severity compared to control plants.

## Discussion

In vitro testing of isolates from plant growth media is a common screen strategy for the selection of indigenous microbiota with biocontrol properties

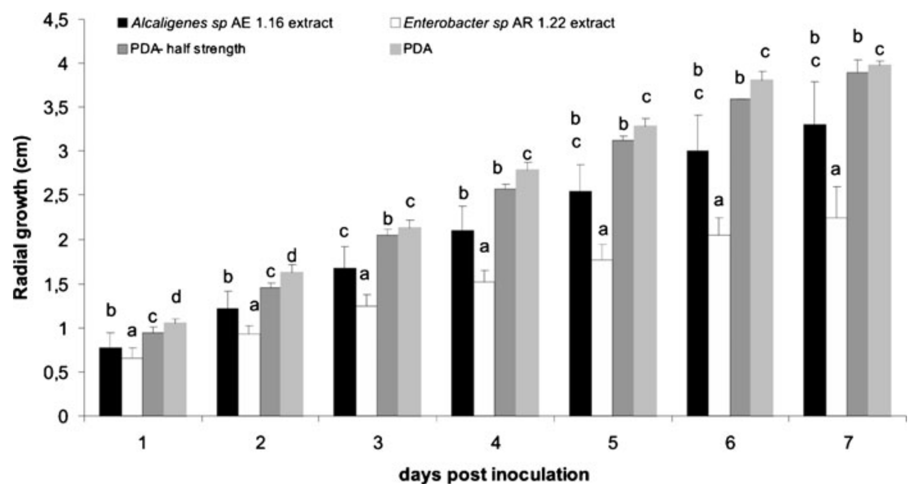


**Fig. 3** In vitro inhibitory effects of culture extracts produced by *Enterobacter* sp. AR1.22 and *Alcaligenes* sp. AE1.16 on the growth of the root pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). *Letters at the top of the bars* indicate statistically significant differences (Duncan's test, $a<0.05$; $n=4$). Results from a representative experiment are shown

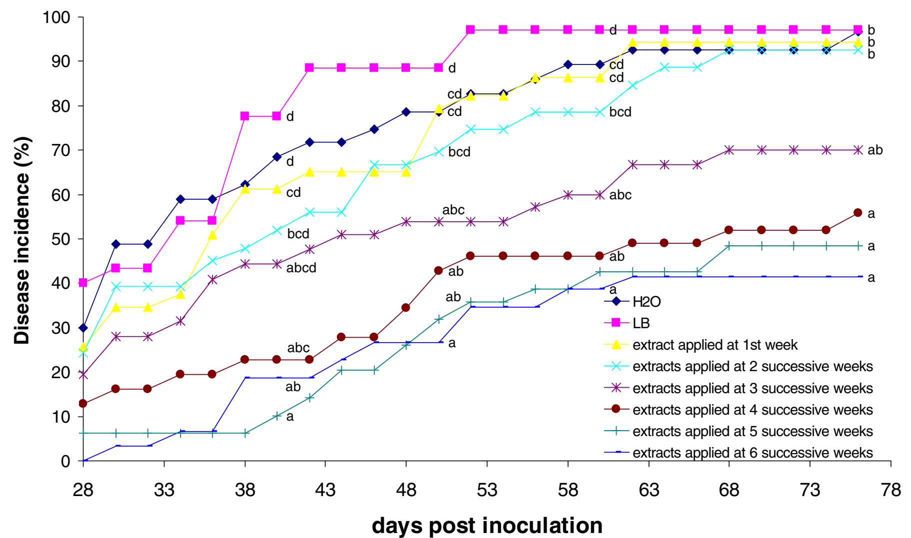


**Fig. 4** *In planta* inhibitory effects of *Enterobacter* sp. AR1.22 culture extracts, applied successively on a weekly basis for 1–6 weeks on the disease severity of the root pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). Disease incidence is recorded as a percentage of dead plants. Different letters shown for representative time interval points, indicate statistically significant differences (Duncan's test, $a<0.05$; $n=5$). Results from a representative experiment are shown

(Ntougias et al. 2004; Termorshuizen et al. 2007). The compost media used in the present study have been shown to drastically reduce disease incidence caused by FORL and *P. nicotianae* in tomato plants, and also leaf infection by *Septoria lycopersici*, which is an indication of the capability of the compost to induce systemic resistance in plants (Ntougias et al. 2008). The high percentage of isolates from the three compost media showing in vitro biocontrol capability depicts the plant protection properties that were reported for these composts.

In the present study, all bacterial strains, showing in vitro inhibition against plant pathogens, exhibited both chitinase and $\beta$-glucanase activity, clearly indicating a lytic action against fungal pathogens. In accordance, inhibition of plant pathogens has been reported to be more effective when glucanases and chitinases acted synergistically (Lim et al. 1991; Lorito et al. 1993). The three compost media used in the present study are rich in chitin rather than cellulose. It could be envisaged that this common property of the media favors the proliferation of chitinase and $\beta$-glucanase bacterial producers. The spent mushroom substrate is well-colonized and rich in fungal cell-wall material of *A. bisporus*, which has been successfully used as substrate for the production of chitinolytic enzymes (Dahiya et al. 2005); grape marc is particularly rich in yeasts (Streichsbier et al. 1982) and in grape skins that directly produce anti-fungal chitinases (Robinson et al. 1997; Fernandez-Caballero et al. 2009); olive mill wastewaters are particularly rich in yeast cell walls (Ben Sassi et al. 2006; Amaral et al. 2008) and favour the proliferation of basidiomycetous yeasts, when applied to environmental media (Karpouzas et al. 2009). Chitinase production has been shown to increase in the presence of chitin-rich cell walls of basidiomycetes (Bruce et al. 1995). Fungal and bacterial antagonists may not efficiently produce chitinases in the presence of simple sugars, such as glucose (Morrissey et al. 1976; de la Cruz et al. 1993; Gupta et al. 1995), which are produced by the degradation of cellulose-rich substrates. On the other hand, their activity is therefore expected to be favored in the mature composts used in this study. Important plant pathogens, such as *Fusarium* and *Rhizoctonia* species, are efficient cellulose degraders and may thrive on cellulose-rich substrates. Indeed, suppression of *Rhizoctonia* species has been related to compost media with low availability in cellulose and simple sugars (Chung et al. 1988; Hoitink et al. 1996).

HCN production, detected in 14 out of the 44 isolates showing in vitro suppressiveness, did not appear to be specifically related to the protection against one or more of the pathogens tested. In line with previous reports (Senthilkumar et al. 2009), no relation between HCN and the production of lytic enzymes was observed in this study.

Only seven out of the 44 potential biocontrol agents identified in vitro were also effective *in planta* and only one RAE biocontrol agent was positive to both in vitro and *in planta* assays. This is commonly observed in the complex rhizosphere environment and

underlines the necessity for *in planta* experiments. Interestingly, 4 out of 7 of the *in planta* biocontrol agents identified in the present study belong to the genera *Bacillus* and *Serratia*. Gram-positive bacteria mainly of the genus *Bacillus* have been found to proliferate in media containing high chitinase-producing bacterial populations (Strohl 1997; Chae et al. 2006). Indeed, Chae et al. (2006) reported the dominance of *Bacillus*, *Paenibacillus* and *Serratia* spp. in chitin-containing rice compost. The role of *S. marcescens* strains in the biological control of several plant pathogens has been repeatedly reported: they are efficient producers of several chitinases (Brurberg et al. 1996) and their antagonistic properties are attributed to the excretion of lytic enzymes that degrade the cell walls of the pathogenic microorganisms (Ordentlich et al. 1988; Someya et al. 2005). The closest relatives of the biocontrol strain BR1.4 were *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) strains, which are antagonistic to plant pathogens (Choudhary and Johri 2009). Besides, two biocontrol strains were related to *B. thuringiensis*, an organism known for its protective properties against plant pathogens beyond insect control (Raddadi et al. 2007). The isolated RAE *Alkaligenes* sp. AE1.16 also produces extracellular chitinases and glucanases. Previous studies, in which biocontrol strains related to the genus *Alcaligenes* had been isolated, report these agents as chitinase producers with a lytic mode of action (Vaidya et al. 2001). However, this strain exhibited biocontrol activity only when plants were inoculated with living bacterial cells, while culture extracts failed to inhibit fungal growth in vitro. This implies that the mechanisms employed by this strain to confer plant resistance may extend beyond antibiosis and could be related to the fact that AE1.16 resides most probably in the plant tissues rather than the rhizosphere. This, however, and the verification of the true endophytic nature of the isolate require further clarification.

Isolate AR1.22, the most effective rhizospheric biocontrol agent in the present study, was phylogenetically related to *E. ludwigii* and *E. cloacae* strains. *E. ludwigii* strain BNM 0357, isolated from the *Lolium perenne* rhizosphere, has recently been identified as an effective biocontrol agent inhibiting *F. solani* vegetative growth, through competition for nutrients and excretion of diffusible antifungal compounds (Shoebitz et al. 2009). Furthermore, the antagonistic properties of *E. cloacae* strains have been extensively reported (Roberts et al. 1994; van Dijk and Nelson 2000). The colonization of plant roots (Roberts et al. 1994) and the competition for fatty acids (van Dijk and Nelson 2000) have previously been proposed as the protective mechanism of *E. cloacae* strains. Our experiments on the *in planta* and in vitro effects of the *Enterobacter* sp. AR1.22 culture extract revealed a significant pathogen inhibition, the mechanism of which most probably relies on antibiosis. However, we cannot exclude the existence of a protection mechanism associated with root colonization. This antagonistic mode of action was dependent on the dose or the persistence of the inhibitory substances in the rhizosphere. Thus, when culture extract was applied for a long enough period of time, growth of the pathogen and its ingression into the plant tissues were restricted, leading to significant reduction in disease incidence (Fig. 4). On the contrary, the application of culture filtrates to the plants before the inoculation with the pathogen could not confer any level of resistance, indicating that the bacterial extracellular compounds *per se* most probably cannot induce plant defense responses. It cannot be excluded that, in the concurrent presence of the pathogen, plant responses could also be triggered, although this hypothesis requires further investigation. Nevertheless, the level of protection conferred by the extracellular compounds produced by *Enterobacter* sp. AR1.22 (>40%) could account for almost all the antagonistic activity of the strain observed when plants were inoculated with the bacterial cells. We have shown that *Enterobacter* sp. AR1.22 produces lytic extracellular enzymes in vitro, but it is important to investigate which other antifungal compounds may be produced by this highly antagonistic bacterial strain and whether the production of lytic enzymes takes indeed place in the rhizosphere.

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## References

Ajithkumar B, Ajithkumar VP, Iriye R, Doi Y, Sakai T (2003) Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank. Int J Syst Evol Microbiol 53:253–258

Amaral C, Lucas MS, Coutinho J, Crespi AL, Anjos MD, Pais C (2008) Microbiological and physicochemical characterization of olive mill wastewaters from a continuous olive mill in Northeastern Portugal. Bioresour Technol 99:7215–7223

Arlorio M, Ludwig A, Boller T, Bonfante P (1992) Inhibition of fungal growth by plant chitinases and $\beta$-1, 3-glucanases. Protoplasma 171:34–43

Ben Sassi A, Boularbah A, Jaouad A, Walker G, Boussaid A (2006) A comparison of Olive oil Mill Wastewaters (OMW) from three different processes in Morocco. Process Biochem 41:74–78

Bonanomi G, Antignani V, Pane C, Scala F (2007) Suppression of soilborne fungal diseases with organic amendments. J Plant Pathol 89:311–324

Borrero C, Ordovás J, Trillas MI, Avilés M (2006) Tomato Fusarium wilt suppressiveness. The relationship between the organic plant growth media and their microbial communities as characterised by Biolog®. Soil Biol Biochem 38:1631–1637

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Bruce A, Srinivasan U, Staines HJ, Highley TL (1995) Chitinase and lamarinase production in liquid culture of *Trichoderma* spp and their role in biocontrol of wood decay fungi. Int Biodeterior Biodegrad 35:337–353

Brurberg MB, Nes IF, Eijsink VGH (1996) Comparative studies of chitinases A and B from *Serratia marcescens*. Microbiology 142:1581–1589

Castric PA (1975) Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can J Microbiol 21:613–618

Chae DH, De Jin R, Hwangbo H, Kim YW, Kim YC, Park RD, Krishnan HB, Kim KY (2006) Control of late blight (*Phytophthora capsici*) in pepper plant with a compost containing multitude of chitinase-producing bacteria. Biocontrol 51:339–351

Chet I, Ordentlich A, Shapira R, Oppenheim A (1990) Mechanisms of biocontrol of soil-borne plant pathogens by rhizobacteria. Plant Soil 129:85–92

Choudhary DK, Johri BN (2009) Interactions of *Bacillus* spp. and plants—with special reference to induced systemic resistance (ISR). Microbiol Res 164:493–513

Chung YR, Hoitink HAH, Lipps PF (1988) Interactions between organic-matter decomposition level and soilborne disease severity. Agric Ecosyst Environ 24:183–193

Coorevits A, De Jonghe V, Vandroemme J, Reekmans R, Heyrman J, Messens W, De Vos P, Heyndrickx M (2008) Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. Syst Appl Microbiol 31:126–140

Dahiya N, Tewari R, Tiwari R, Hoondal G (2005) Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. World J Microbiol Biotechnol 21:1611–1616

De la Cruz J, Rey M, Lora JM, Hidalgo-Gallego A, Domınguez F, Pintor-Toro JA, Llobell A, Benitez T (1993) Carbon source control on $\beta$-glucanases, chitobiase and chitinase from *Trichoderma harzianum*. Arch Microbiol 159:316–322

Dunn AK, Stabb EV (2005) Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis* (Neuroptera: Myrmeleontidae). Appl Environ Microbiol 71:8784–8794

Erhart E, Burian K, Hartl W, Stich K (1999) Suppression of *Pythium ultimum* by biowaste composts in relation to compost microbial biomass, activity and content of phenolic compounds. J Phytopathol 147:299–305

Fernandez-Caballero C, Romero I, Goi O, Escribano MI, Merodio C, Sanchez-Ballesta MT (2009) Characterization of an antifungal and cryoprotective class I chitinase from table grape berries (*Vitis vinifera* Cv. Cardinal). J Agric Food Chem 57:8893–8900

Garbeva P, van Veen JA, van Elsas JD (2004) Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiol Ecol 47:51–64

Grimont PAD, Grimont F (1984) Genus *Serratia* Bizio 1823, 288$^{AL}$. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. The Williams & Wilkins Co, Baltimore, pp 477–484

Gupta R, Saxena RK, Chaturvedi P, Virdi JS (1995) Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. J Appl Bacteriol 78:378–383

Hoffmann H, Stindl S, Stumpf A, Mehlen A, Monget D, Heesemann J, Schleifer KH, Roggenkamp A (2005) Description of *Enterobacter ludwigii* sp. nov., a novel *Enterobacter* species of clinical relevance. Syst Appl Microbiol 28:206–212

Hoitink HAJ, Boehm MJ (1999) Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. Annu Rev Phytopathol 37:427–446

Hoitink HAL, Stone AG, Grebus ME (1996) Suppression of plant diseases by composts. In: de Bertoldi M (ed) The science of composting. Blackie Academic and Professional, Glasgow, pp 373–381

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic, New York, pp 21–132

Jung WJ, An KN, Jin YL, Park RD, Lim KT, Kim KY, Kim TH (2003) Biological control of damping-off caused by *Rhizoctonia solani* using chitinase-producing *Paenibacillus illinoisensis* KJA-424. Soil Biol Biochem 35:1261–1264

Karpouzas DG, Rousidou C, Papadopoulou KK, Bekris F, Zervakis GI, Singh B, Ehaliotis C (2009) Effect of continuous olive mill wastewater applications, in the presence and absence of N fertilization, on the structure of rhizosphere-soil fungal communities. FEMS Microbiol Ecol 70:56–69

Kavroulakis N, Ehaliotis C, Ntougias S, Zervakis GI, Papadopoulou KK (2005) Local and systemic resistance against fungal pathogens of tomato plants elicited by a compost derived from agricultural residues. Physiol Mol Plant Pathol 66:163–174

Kersters K, De Ley J (1984) Genus *Alcaligenes* Castellani and Chalmers 1919, 936$^{AL}$. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. The Williams & Wilkins Co., Baltimore, pp 361–373

Lim H-S, Kim Y-S, Kim S-D (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism

against *Fusarium solani*, an agent of plant root rot. Appl Environ Microbiol 57:510–516

Lorito M, Di Pietro A, Hayes CK, Woo SL, Harman GE (1993) Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. Mol Plant Pathol 83:721–728

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combination of chitinase and $\beta$-1, 3 glucanase. Plant Physiol 88:936–942

McKellar ME, Nelson EB (2003) Compost-induced suppression of *Pythium* damping-off is mediated by fatty-acid-metabolizing seed-colonizing microbial communities. Appl Environ Microbiol 69:452–460

Morrissey RF, Dugan EP, Koths JS (1976) Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. Soil Biol Biochem 8:23–28

Nagarajkumar M, Bhaskaran R, Velazhahan R (2004) Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol Res 159:73–81

Noble R, Coventry E (2005) Suppression of soil-borne plant diseases with composts: a review. Biocontrol Sci Technol 15:3–20

Ntougias S, Zervakis GI, Kavroulakis N, Ehaliotis C, Papadopoulou KK (2004) Bacterial diversity in spent mushroom compost assessed by amplified rDNA restriction analysis and sequencing of cultivated isolates. Syst Appl Microbiol 27:746–754

Ntougias S, Zervakis GI, Ehaliotis C, Kavroulakis N, Papadopoulou KK (2006) Ecophysiology and molecular phylogeny of bacteria isolated from alkaline two-phase olive mill wastes. Res Microbiol 157:376–385

Ntougias S, Papadopoulou KK, Zervakis GI, Kavroulakis N, Ehaliotis C (2008) Suppression of soil-borne pathogens of tomato by composts derived from agro-industrial wastes abundant in Mediterranean regions. Biol Fertil Soils 44:1081–1090

Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84–88

Perez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C (2006) Response of soil microbial communities to compost amendments. Soil Biol Biochem 38:460–470

Priest FG, Aquino De Muro M, Kaji D (1994) Systematics of insect pathogenic bacilli: uses in strain identification and isolation of novel pathogens. In: Priest FG, Ramos-Cormenzana A, Tindall BJ (eds) Bacterial diversity and systematics. Plenum, New York, pp 275–296

Raddadi N, Cherif A, Ouzari H, Marzorati M, Brusetti L, Boudabous A, Daffonchio D (2007) *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. Ann Microbiol 57:481–494

Roberts DP, JrNM S, Maloney AP, Nelson EB, Schaff DA (1994) Role of colonization in biocontrol: studies with *Enterobacter cloacae*. Plant Sci 101:83–89

Robinson SP, Jacobs AK, Dry IB (1997) A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol 114:771–778

Saitou N, Nei M (1987) The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK, Annapurna K (2009) Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. Curr Microbiol 58:288–293

Shoebitz M, Ribaudo CM, Pardo MA, Cantore ML, Ciampi L, Curá JA (2009) Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* rhizosphere. Soil Biol Biochem 41:1768–1774

Siddiqui Y, Meon S, Ismail MR, Ali A (2008) *Trichoderma*-fortified compost extracts for the control of choanephora wet rot in okra production. Crop Prot 27:385–390

Someya N, Nakajima M, Akutsu K (2005) Potential of an antagonistic bacterium *Serratia marcescens* strain B2 for the biological control of cucumber damping-off disease. Biocontrol Sci 10:101–104

Streichsbier F, Messner K, Wessely M, Rohr M (1982) The microbiological aspects of grape marc humification. Eur J Appl Microbiol Biotechnol 14:182–186

Strohl WR (1997) Industrial antibiotics: today and the future. In: Strohl WR (ed) Biotechnology of antibiotics. Marcel Dekker Ins, New York, pp 3–35

Termorshuizen AJ, van Rijn E, van der Gaag DJ, Alabouvette C, Chen Y, Lagerlöf J, Malandrakis AA, Paplomatas EJ, Rämert B, Ryckeboer J, Steinberg C, Zmora-Nahum S (2007) Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. Soil Biol Biochem 38:2461–2477

Trillas MI, Casanova E, Cotxarrera L, Ordovás J, Borrero C, Avilés M (2006) Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biol Control 39:32–38

Vaidya RJ, Shah IM, Vyas PR, Chhatpar HS (2001) Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol. World J Microbiol Biotechnol 17:691–696

Van de Peer Y, De Wachter R (1993) TREECON: a software package for the construction and drawing of evolutionary trees. Comput Appl Biosci 9:77–182

van Dijk K, Nelson EB (2000) Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Appl Environ Microbiol 66:5340–5347

Van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Eur J Plant Pathol 119:243–254

Vierheilig H, Alt M, Neuhaus JM, Boller T, Wiemken A (1993) Colonization of transgenic *Nicotiana sylvestris* plants expressing different forms of *Nicotiana tabacum* chitinase by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant Microbe Interact 6:261–264

Voisard C, Keel C, Haas D, Defago G (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J 8:351–358

Walsh GA, Murphy RA, Killeen GF, Headon DR, Power RF (1995) Technical note: detection and quantification of supplemental fungal beta-glucanase activity in animal feed. J Anim Sci 73:1074–1076

Weller DM, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309–348

Wilson K (1992) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Short protocols in molecular biology. Wiley, New York, pp 2-10–2-11

Zhang W, Han DY, Dick WA, Davis KR, Hoitink HAJ (1998) Compost and compost water extract-induced systemic acquired resistance in cucumber and Arabidopsis. Phytopathology 88:450–455