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Biotic aspects involved in the control of *damping-off* producing agents: The role of the thermotolerant microbiota isolated from composting of plant waste

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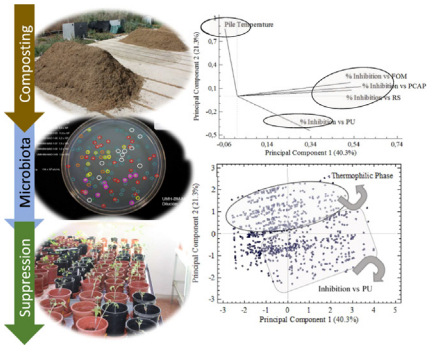
GRAPHICAL ABSTRACT



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ABSTRACT

Along with the high temperatures generated during the composting process, the microbial community of a compost pile collaborates actively in the control of *damping-off* producing agents. Such collaboration could be justified on the basis of the ability of the microbiota to produce fungicide substances that are involved in the control of *damping-off* or other bioactive compounds that affect the growth promotion of plants. Thanks to *ex situ* experiments, a remarkable suppressive effect was corroborated against different agents producing damping-off. The microbial strains involved in this effect were detected mainly in the bio-oxidative and maturation phases of the composting process. However, only 3% of the total collection of strains proved to have a multipotential character with respect to its spectrum of action against the *damping-off* producing agents, as well as in relation to its capacity to produce substances of agronomic interest. It is worth mentioning the presence of two thermophilic isolates identified as *Geobacillus thermodenitrificans* and *Bacillus aerius* that showed the ability to inhibit the symptoms caused by *Phytophthora capsici* in vivo. Both strains were able to produce siderophores, salicylic acid and chitinase enzymes in vitro, but only *G. thermodenitrificans* was able to stimulate the development of the root in pepper seedlings. In this sense, it is suspected that the production of cyanide by this thermophilic bacteria could be related to this latter effect.

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1. Introduction

One of the main risks arising from the massive generation of plant remains is that they can serve as a source of entry and spread of potentially phytopathogenic microbial agents (Noble and Roberts, 2004). Exposing contaminated material to environmental conditions is a suitable process to eliminate phytopathogenic microbial agents as suggested by several authors (Mansoori and Jaliani, 1996). However, in this case, the complete elimination of dangerous microorganisms is only possible over a long period. On the other hand, although composting can be considered a powerful method of sanitizing plant remains (Moreno and Mormeneo, 2008), unfortunately there is evidence that many of these pathogens can survive during the composting process or other waste treatment procedures (Noble and Roberts, 2004).

While it is true that high temperatures generated inside a compost pile greatly influence the eradication of plant pathogens (Ryckeboer et al., 2003), other factors should not be ruled out, such as the presence of phenolic compounds, nutritional competition, natural loss of viability of the pathogen after a certain period or the action of a variety of antagonistic microorganisms inside the composting piles (Noble and Roberts, 2004).

Numerous studies have demonstrated the potential of composted organic waste not only as a substitute for peat as a growth substrate, but also to stimulate plant growth and suppress soilborne diseases (Avilés et al., 2011). Composts have proven effective against soil-borne diseases including *damping-off*, root rots (*Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora* spp.) and wilts (*Fusarium oxysporum*), though the mechanisms involved in the disease suppression are not fully understood. Broadly speaking, the sterilization of the composts results in a loss in the disease suppressiveness (Noble and Roberts, 2004), which indicates the predominant involvement of biological suppression mechanisms.

Biological control using beneficial microorganisms has been considered a viable alternative strategy for the replacement of chemical methods. The most common antagonistic agents that have been used to control *damping-off* include bacteria such as *Pseudomonas*, *Bacillus* and *Burkholderia* isolates (Ramarathnam et al., 2011; Sallam et al., 2013), whose presence in composted materials is very significant. Their involvement both in the compost suppressive abilities and plant growth promotion is widely known (Haruta et al., 2005). The biocontrol efficacy of these bacteria is most likely related to their numerous antagonistic mechanisms of action. These mechanisms include production of antibiotics (Prasanna et al., 2009), siderophores, cell wall degrading enzymes or hydrogen cyanide (Voisard et al., 1989).

Therefore, in addition to the high temperatures reached during the composting process, the microbial community of a composting pile actively collaborates in the control of *damping-off* producing agents. Such collaboration could be justified on the basis of the capacity of the microbiota to produce fungicidal substances involved in the control of *damping-off* or other bioactive compounds affecting plant growth promotion.

Based on the comments indicated above, the main objectives of this work were (i) characterize the typical microbiota of the different thermal stages of a plant waste composting process, according to its spectrum of action against producing agents of *damping-off*; (ii) identify *ex situ* the capacity of this group of microorganisms to produce bioactive substances such as siderophores, salicylic acid and cyanide, as well as enzymes involved with the degradation of chitin and the solubilization of phosphates, also establishing a possible relationship with the antagonist spectrum against agents that produce *damping-off*; (iii) finally, evaluate *in vivo* the effect of two thermophilic strains isolated from a composting process, identified as *Geobacillus thermodenitrificans* and *Bacillus aerius*, as control agents against the oomycete *Phytophthora capsici* and as biostimulants of the root development in pepper seedlings.

2. Material and methods

2.1. Composting process and sampling protocol

Composting of lignocellulosic materials (sun-dried tomato plant waste and pine woodchips at a 1:1 (w/w) ratio was performed. The mixture was composted in trapezoidal piles (1.5 m width × 3 m length × 1 m height) with a total weight of 500 kg for 42 days with forced aeration (7.5–9.0 L kg^{-1}, every 4 h) during the bioxidative stage, and then allowed to cool and cure for an additional 147-day period. The heaps were shovel four times throughout the process. Approximately one kg of compost sample from nine sampling sites was collected from the piles at 19 different times, corresponding to distinct stages of the composting: Mesophilic, Thermophilic, Cooling and Maturation phases (Supplementary Material 1). All samples were freshly processed for the microbial analyses.

2.2. Isolation of potentially antagonistic microorganisms and plant growth promoters: collection of microorganisms

Mesophilic and thermophilic bacteria were isolated as described by López-González et al. (2013). Ten grams of compost was suspended in 90 mL of sterile saline solution (0.9% w/v NaCl) and shaken for 30 min at room temperature. Ten-fold serial dilutions were made in sterile saline solution (0.9% w/v NaCl) and 100 μL of the diluted suspensions was inoculated in plates containing count agar medium APHA (Cultimed, Spain). The plates were incubated at 30 °C (mesophiles), and 50 °C (thermophiles) for 24 and 48 h, respectively. Pure cultures of the different colonial morphotypes were isolated on APHA plates. In order to avoid duplicating the strains, they were characterized and grouped according to the typical morphology of the colonies in petri dishes with APHA medium. For comparison between colonial morphotypes, the plates were photographed with a Canon EOS 450D camera, fitted with an EF-S18-55 mm f/3.5–5.6 IS zoom lens. Additional bacterial differentiation was carried out by using Gram-staining, oxidase and catalase tests (data not shown). Finally, around 600 strains were preserved on agar slants at 4 °C and cryogenized (Cryoinstant, Deltalab, Spain).

2.3. Phytopathogenic agents

Phytopathogenic strains were supplied by the Spanish Type Culture Collection (CECT). Cultures of the fungi *Fusarium oxysporum* f.sp. *melonis* CECT 20474 (FOM) and *Rhizoctonia solani* CECT 2824 (RS), as well as the oomycetes *Pythium ultimum* CECT 2365 (PU) and *Phytophthora capsici* CECT 20433 (PCAP), were kept on Potato Dextrose Agar (PDA, Oxoid Ltd., UK) at 4 °C.

2.4. Production of biological control substances/activities: chitinase-like enzymes, cyanide hydrogen, siderophores, salicylic acid and solubilization of phosphates

600 microbial strains were isolated on agar plates for chitinolytic activity containing 0.5% colloidal chitin, 0.8% Nutrient Broth (NB, Oxoid Ltd., UK), 1% malt extract, 1% Peptone, 0.1% NaCl and 2% bacteriological agar (Zarei et al., 2010). The cultures were incubated for seven days at 30 °C. The clear zones produced by the chitinolytic bacteria were measured to identify the best chitinolytic strains.

Colloidal chitin was prepared according to Roberts and Selitrennikoff (1988). The acid hydrolysis of chitin was performed in concentrated HCl by constant stirring using a magnetic stirrer at 4 °C overnight, followed by the extraction of colloidal chitin in 2 L of ice cold 95% ethanol maintained at 4 °C overnight and then centrifuged at 3000 rpm for 20 min at 4 °C. The pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4 °C until the alcohol was completely removed. The colloidal chitin obtained had a soft pasty consistency at 90–95% moisture and was stored at 4 °C until use.

Bacterial strains were grown in nutrient broth for 48 h and the released HCN was estimated by a modification of the picrate/Na_2CO_3 method (Lorck, 1948). For each 5 mL of culture broth (NB, Oxoid Ltd., UK), 165 μL of saturated picric acid (neutralized with Na_2CO_3) was added and the reaction mixture was incubated for 5 h to allow color development. Changes in color from yellow to light brown, moderate brown or reddish brown showed the presence of HCN production.

The protocol used to detect microorganisms producing siderophores was a modified method of Schwyn and Neilands (1987). In a preliminary selection step, cultures of all strains were prepared in 3 mL of modified King B medium (KBM: glycerol, 8.7 mL, peptone, 20 g, K 2 HPO 4, 1.5 g, MgSO 4, 0.1 g, tryptophan, 0.1 g, distilled water, 1 L, pH 7.2). After the incubation period, the cultures were centrifuged at 5000 rpm for 15 min and 0.5 mL of the supernatant was mixed with 0.5 mL of CAS (Chrome Azurol S solution). CAS solution: 6 mL of a solution of HDTMA (hexadecyltrimethylammonium bromide) was passed into a 100 mL volumetric flask and diluted with a small volume of water. A mixture of 1.5 mL of Fe (III) (Fe III: 1 mM FeCl 3 .6H 2O, 10 mM HCl) and 7.5 mL of a 2 mM aqueous CAS solution (SIGMA 199532) was prepared and slowly added to a stirring flask. 4.307 g of anhydrous piperazine (SIGMA 80621) was dissolved in the lowest possible volume of water and 6.25 mL of 12 M hydrochloric acid was carefully added. This buffer solution was added to the starting flask and made up to 100 mL. The mixture was incubated at room temperature. The presence of siderophores was detected by a change in color from blue to orange-brown.

The methodology used for the study of salicylic acid-producing microorganisms was based on the modified Trinder universal test (Gil and Martínez-Merino, 2011). Bacterial cultures were prepared as mentioned above. After the incubation period, the cultures were centrifuged at 5000 rpm for 15 min and the extracts were acidified to pH 2 with 2 N HCl. Thereafter, 1 mL of the microbial extract was mixed with 1 mL of distilled water and 2 mL of a 0.1% ferric chloride solution. The formation of a purple complex was observed for producers of salicylic acid, caused by the reaction that occurs between this molecule and iron.

The phosphate solubilizing activity was demonstrated by growing the microbial strains in culture medium containing 2.5% tricalcium phosphate. A zone of clearance of the medium was detected in the case of phosphate solubilizing colonies (Nautiyal, 1999) after 14 days of incubation at 30 °C.

2.5. Spectrum of in vitro inhibition: dual cultures

Each potentially antagonistic bacterial strain was cultured in nutrient broth (NB, Oxoid Ltd., UK) for 24 h at 30 °C prior to use. Suppressive effect was demonstrated using the modified techniques of Landa et al. (1997). First, 2% water agar (WA) plates were prepared. After the agar solidified, four 8-mm-diameter steel cylinders were placed equidistantly from the edge. A second layer of PDA was added on the WA plates. Once the cylinders were removed, the wells were filled with 50 μL liquid culture of the antagonist to be assayed and a plug of 5-day-old phytopathogen agent (PA) culture, removed from a PDA plate, was placed at the center of the assay plate. Four replicates for each antagonist-PA combination were prepared. Plates were incubated at 30 °C for 5 days and the inhibition index (I) was expressed as the percentage of PA inhibition in the presence of the antagonistic strain using the formula:

$$I = [(C - T)/C] \times 100$$

where I: Inhibition Index (%); C: growth of the phytopathogenic agent in absence of the antagonistic strain (mm); T: growth of the phytopathogenic agent in presence of the antagonistic strain (mm).

2.6. In vivo suppression of damping-off caused by P. capsici in pepper seedlings

For experiments with pepper seedlings susceptible to *P. capsici*, bacterial strains were grown in Nutrient Agar (NA, Oxoid Ltd., UK) and incubated at 28 °C for 48 h. Bacterial cells were suspended in 10 mM MgSO4 and the final inoculum was adjusted to 10^8 bacterial cells/mL. To prepare the pathogen inoculum as described by Kim et al. (1997), *P. capsici* CECT 20433 was cultured on oat agar for 7 days at 28 °C. The cultures were then flooded with 20 mL of sterile distilled water and incubated under continuous fluorescent light for an additional 7 days at 28 °C to induce the production of zoosporangia. Zoospores were released from the zoosporangia when the culture was flooded with sterile cold water, stored at 4 °C for 30 min and then at room temperature for 30 min. The mycelium and other remains were separated from the zoospores by filtration through cheesecloth. The concentration of zoospores was determined with the Neubauer chamber.

Pepper seeds (*Capsicum annuum* L., var. Sweet Italian) were germinated at 26 °C in the dark in 77-well trays filled with a steam-sterilized substrate mixture from organic substrate (VLC Horticultura, CB, E98581028) and vermiculite (Projar, SA) at a ratio of 3: 1 (v/v).

Greenhouse experiments were performed in duplicate for each antagonist-pathogen combination. A randomized experimental design was established consisting of 3 different treatments, each using 20 plants. Three weeks after sowing, the seedlings were transplanted in 10-cm-diameter pots. One week later, the pots were soaked with 25 mL of the prepared bacterial suspensions. These plants were inoculated with zoospores of *P. capsici* (25 zoospores/g substrate) one week after bacterial treatments. For inoculation, zoospore suspensions were injected into four holes (1 cm in diameter × 1 cm deep) around the root zone of each plant. Inoculated or uninoculated plants treated with 10 mM MgSO4 buffer served as positive or negative controls, respectively. After inoculation, the plants were watered for 4–5 days to avoid drying of the substrate. The plants were kept in a greenhouse at a controlled temperature of 25 ± 1 °C and photoperiod of 12 h over 40 days. The severity of the disease was evaluated every day after inoculation with the pathogen on a scale of 0 (asymptomatic) – 5 (dead plants), as described by Kim et al. (1989).

2.7. Promotion of root development in pepper seedlings

The effects of bacterial inoculation on plant growth were evaluated as previously described by Santoro et al. (2011). Greenhouse experiments were performed in duplicate for each treatment using a randomized design of 20 plants each: negative control (uninoculated) plants and plants inoculated with the biological control agent. The temperature and dark/light conditions were similar to those used in the experiments cited above.

After collection of the pepper plants from the containers, the roots were rinsed with water to remove the substrate, and the following standard growth parameters were evaluated for each plant: leaf number, stem and root length (cm), fresh plant weight (g) and root/stem length ratio.

2.8. Molecular identification of biocontrol agents

The identities of the specific isolates were determined based on partial or nearly full length 16S rRNA gene sequence analysis. For bacterial genomic DNA extraction, freshly grown colonies were suspended in 500 μL of sterile milliQ water, heated at 97 °C for 5 min followed by exposure to ice bath for 5 min. The 1/10 diluted supernatant was used as the template. The amplification was carried out using a MyCycler thermal cycler (Biorad). The 16S rRNA bacterial genes were amplified using universal primers: 27F (5′-AGAGTTTGAT CATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The reaction mixture (20 μL) contained: 10 μL kit Mastermix 2,0X Taq DNA

Polymerase 2.0 mM MgCl2 (VWR), 0.06 μM of each primer, and 2 μL of template DNA. The following thermal profile was used for the PCR: 95 °C for 2 min, 30 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min), 72 °C for 10 min. The PCR products were purified using Diffinity RapidTips (Sigma–Aldrich) and the clean DNA was sequenced by capillary sequencer ABI Hitachi 3500 Genetic Analyzer (Applied Biosystems). The forward and reverse sequences were edited, assembled and aligned using the programs Sequence Scanner v1.0 (Applied Biosystem), Reverse Complement (www.bioinformatics.org/sms/rev_comp.html), Clustal X v2.0.11, and MEGA 5 v5.2. The partial or nearly full-length sequences were compared for similar nucleotide sequences with the Identify utility at the EzTaxon server http://www.ezbiocloud.net/eztaxon/identify.

2.9. Statistical analyses

Data obtained were subjected to statistical analysis using Statgraphics Centurion XVII. A multifactorial analysis of variance (ANOVA) and a multiple comparison test (Fisher's Least Significant Difference) were performed to compare mean values for different levels of sampling ($P < 0.05$). To select the most effective strains from a suppressive point of view, dispersion diagrams were obtained. To identify groups of interrelated variables, a principal components analysis was performed. Finally, between-groups linkage was used as clustering method and the interval measured was the squared Euclidean distance.

3. Results and discussion

3.1. Screening of anti damping-off strains isolated from different phases of the composting process

The traditional method of screening antagonistic bacteria *in vitro* consists of isolating single colonies from the studied materials and evaluating their antagonistic ability using dual culture assays (Kavroulakis et al., 2010; Cuesta et al., 2012; Suárez-Estrella et al., 2013). Though this massive screening could represent some drawbacks *a priori*, it is the most suitable procedure when a huge microbial collection must be widely tested in the search for biological control agents. Thanks to this methodology, the antagonistic spectrum of a large microbial collection against four *damping-off* producing pathogens has been revealed in this work.

Results derived from *in vitro* antagonism tests using the dual culture technique are shown below. These tests were performed once strains were isolated in pure culture from the different compost samples. However, it is more interesting to express the results according to the temporal origin of the strains, that is, days since the beginning of the composting process (Fig. 1).

Results shown in Fig. 1 indicate that, for both mesophilic and thermophilic microbiota (Fig. 1a and b, respectively), the numbers of antagonistic strains (strains considered as biological control agents) against *F.o. melonis* and *P. capsici* were higher than those found against *P. ultimum* and *R. solani*, which were less susceptible to the suppressive effect caused by the microbiota isolated throughout the composting process.

Consequently, microbiota present during the composting process could significantly favor the hygienization process. Thus, when the influence of the mesophilic microbiota (strains isolated at 30 °C in the laboratory) of the composting process was analysed on the elimination of *damping-off* producing fungi and oomycetes (Fig. 1a), it could be noted that the largest proportion of biological control agents came about between days 1 and 14 after the beginning of the process (bioxidative phase) and later during the maturation phase. In contrast, data derived from the group of thermophilic bacteria (strains isolated at 50 °C in the laboratory, Fig. 1b) showed that the largest proportion of potentially antagonistic strains was detected right at the first week after the beginning of the process (1–9 days) and later during the cooling phase (approximately 42 days later).

A Principal Component Analysis (PCA) was carried out for several of the parameters analyzed in this work (Fig. 2). The parameters considered in the statistical analysis were Pile Temperature (°C), and percentage of *in vitro* growth inhibition against each of the phytopathogenic fungi and oomycetes. In this case, it was measured the inhibition strength or degree that the microbial strains could exert on the pathogen (see quantitative analysis in Section 2.5). Establishing two principal components (PCs), the model explained more than 60% of the data variability, with the following contributions of each PC: PC1 40.3% and PC2 21.3%. PC1 was mainly associated with biotic factors involved in the hygienization process, while PC2 was directly associated with the temperature inside the composting pile (abiotic factor). Regarding PC1, the variables concerning the percentage of growth inhibition against FOM, PCAP and RS were grouped, while the percentage of growth inhibition against PU was less influenced (Fig. 2a). In Fig. 2b, inhibition indexes against FOM, PCAP, RS, PU and sampling times (Mesophilic, Thermophilic, Cooling and Maturation phases) were analyzed on the basis of a PCA (scatter plot graph). In this case, PCA also revealed a clear differentiation of groups of objects, mainly detected between Thermophilic Phase and Inhibition against *P. ultimum* (Fig. 2b). This discovery is surprising as it could reveal a greater weight of biotic control mechanisms against *P. ultimum* as opposed to high temperatures, traditionally considered the main factor involved in the sanitization of the composting process. This phenomenon was not so clearly observed in the case of the other three phytopathogenic agents.

From the initial microbial collection only 3% of the isolated bacteria showed potential to act against multiple phytopathogenic agents. In order to select those microbial strains which showed greater potential as control agents, a dispersion graph was made. The most useful graphical representation to describe the joint behavior of two variables is the dispersion diagram, where each case is represented as a point in the plane defined by the two variables. Fig. 3 shows that some of the strains tested were able to inhibit the *in vitro* growth of *R. solani* and *P. capsici* at a rating of over 75%.The least susceptible phytopathogenic agent to the biocontrol strains tested was *P. ultimum* (data not shown). In addition, the growth inhibition range was narrower (30–60%) for *P. ultimum* than those observed for *F. oxysporum* f.sp. *melonis*, *R. solani* and *P. capsici*. Those strains that showed capacity to inhibit fungal growth by over 75% were *Bacillus siamensis* 2727 (*versus R. solani*), *Cellulosimicrobium cellulans* 2653, *Bacillus aerius* 3358, *Bacillus mojavensis* 3654 and *Geobacillus thermodenitrificans* 3750 (*versus P. capsici*).

Composting is a bioxidative transformation process that requires a rich and varied microbiota and it has already been proposed as an important means of plant disease control when applied to plant waste (EPA625-R-92-013, 2013). Several plant pathogens and seeds are killed by the heat generated during the high-temperature phases throughout composting (Noble and Roberts, 2004). However, in addition to heat, two main factors are involved in the elimination of plant pathogens during the process: (i) the production of antimicrobial substances such as phenolic compounds, generated during lignocellulosic material decay and (ii) the colonization of compost by many different thermotolerant organisms that compete with pathogens for nutrients and produce general antibiosis phenomena which reduce pathogen survival and growth (Hoitink and Boehm, 1999). All these factors are desirable to ensure a high-quality final compost.

To date, the high temperatures generated during the bioxidative phase have been considered the main factor involved in the disinfecting capacity of the composting process. However, the thermotolerant capacity of the typical microbiota of composting has previously been highlighted by other authors (López-González et al., 2014).

The first section of this work has sought to highlight the fact that the control of plant pathogens during the composting process could take place not only during the thermophilic stages, but throughout the entire process, occurring continuously even in those phases in which

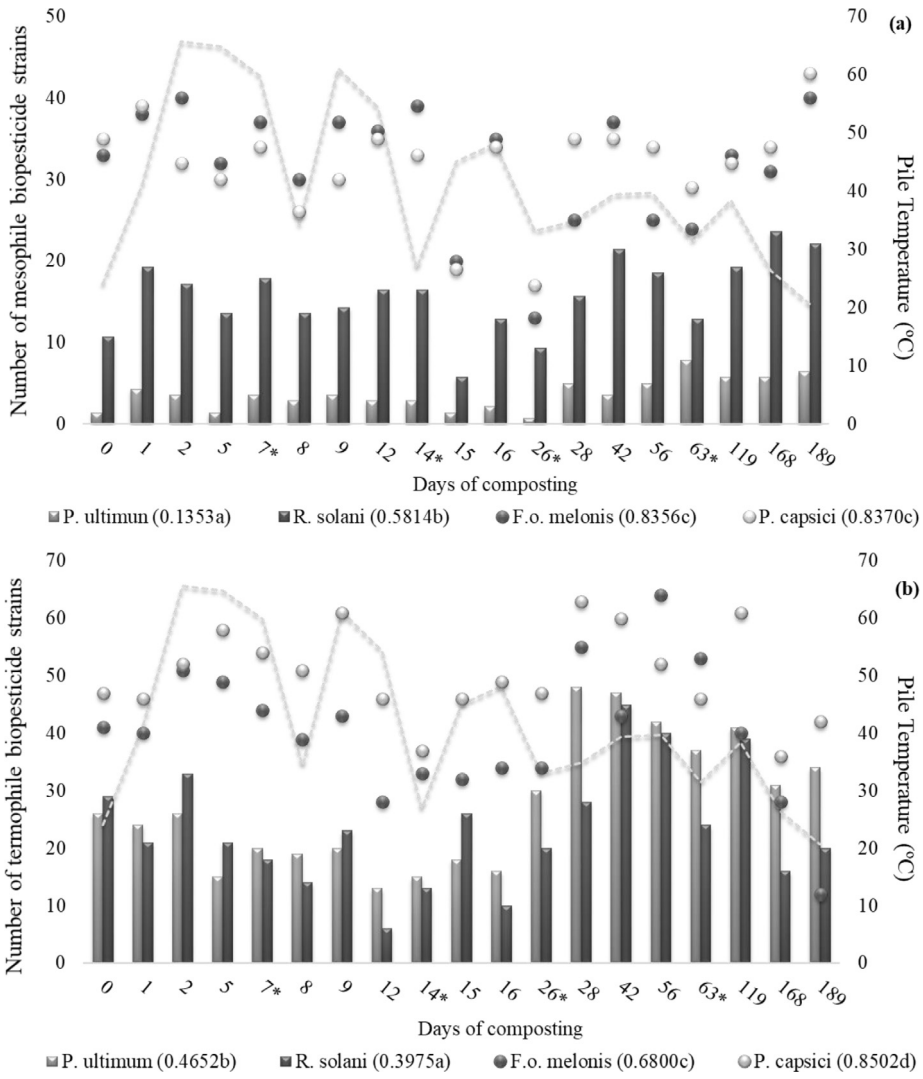

Fig. 1. Number and evolution of bacterial morphotypes isolated throughout a composting process that were effective against *in vitro* growth of fungi (*Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *melonis*) and oomycetes (*Pythium ultimum* and *Phytophthora capsici*) producing *damping-off* (a: number of mesophilic biocontrol agents isolated at 30 °C; b: number of thermophilic biocontrol agents isolated at 50 °C). Temperature evolution throughout the composting process are shown by a doted line. *Samplings at which turning operations were carried out. Least Significant Means are indicated in parentheses (values labelled with different letters are significantly different according to Fisher's LSD test at P = 0.05).

mesophilic temperatures govern the composting pile.

The results shown in Fig. 1 have confirmed by *ex situ* experiments the presence of a microbiota that could be considered globally involved in the biological control of plant diseases. On the other hand, these results corroborate the thermal tolerance of bacteria isolated from a composting pile of plant waste (López-González et al., 2014; 2015).

3.2. Production of siderophores, salicylic acid, chitinase-like enzymes, cyanide and phosphate solubilization

Inside the composting pile there is a great number of microbial strains capable of producing siderophores, salicylic acid, chitinase-like enzymes and cyanide, as well as phosphate solubilization. As it has been shown in Fig. 4, that microbiota is present during the whole composting process. In both groups of isolates (mesophilic and thermophilic bacteria), the proportion of siderophore-producing strains was higher in relation to the proportion of strains producing other substances or activities. In contrast, the least represented activities in the mesophilic and thermophilic groups were salicylic acid production and phosphate solubilization, respectively (Fig. 4a and b).

Diánez et al. (2006) detected the presence of siderophores in grape marc aerated compost tea and assessed their suppressive effect on nine selected soilborne pathogens. Commonly, beneficial microorganisms present in compost (including bacteria, actinobacteria, and fungi) can induce all those mechanisms associated with plant disease suppression. *Bacillus* spp. and *Pseudomonas* spp. are the most frequently used plant growth-promoting rhizobacteria, and many strains are also able to suppress the growth of harmful microbiota present in most soils (Mehta et al., 2014). In fact, fourteen of the most important strains isolated in this work (Table 1) were identified as *Bacillus* spp. and *Pseudomonas* spp., corroborating the potential effect of these genera as biological control agents and plant growth stimulating microorganisms.

Under the criteria of broad spectrum of inhibition against plant pathogens and the versatility to produce substances of agronomic interest, 18 of the tested strains were selected as potential biocontrol agents and promoters of plant growth (Table 1). They represented around 3% of the total. Molecular identification of the 18 strains up to species level is shown in Table 1. The strains identified as *Bacillus tequilensis* 2167 and *B. subtilis* subsp. *subtilis* 2265 stood out due to their broad spectrum antagonism against soil pathogenic fungi and oomycetes (Table 1). The potential of the strains identified as *Pseudomonas rhizosphaerae* 2082, *Cellulosimicrobium celllulans* 2653, *Bacillus aerius* 3358 and *Geobacillus thermodenitrificans* 3750 to produce substances and enzymatic activities (phosphate solubilization) of agronomic interest should also be highlighted. These last three strains also demonstrated high thermal tolerance and the ability to inhibit the growth of *P. capsici* at a level of over 75%.

Lytic enzymes excreted by several antagonistic bacteria are among the most powerful factors that degrade fungal cell walls (Leelasuphakul et al., 2006), and chitinase-like enzymes are a clear example of these. Other microbial byproducts, such as cyanide hydrogen may also contribute to pathogen suppression. Though cyanide blocks the

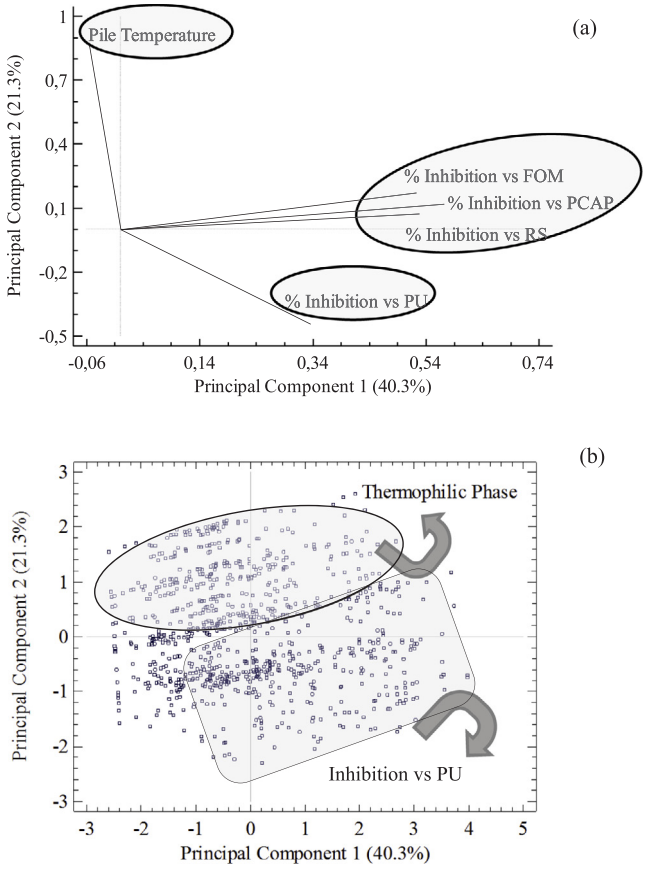

Fig. 2. Results of principal component analysis (PCA) based on microbial inhibition (%) *versus* (vs) the growth of different phytopathogenic fungi and oomycetes (PU: *P. ultimum*; FOM: *Fusarium oxysporum* f.sp. *melonis*; RS: *Rhizoctonia solani*; PCAP: *Phytophthora capsici*). Temperatures inside composting piles were also considered as a variable in this study (a: Graph of Principal Components; b: Scatterplot Graph). All results are means (n = 4 repetitions).

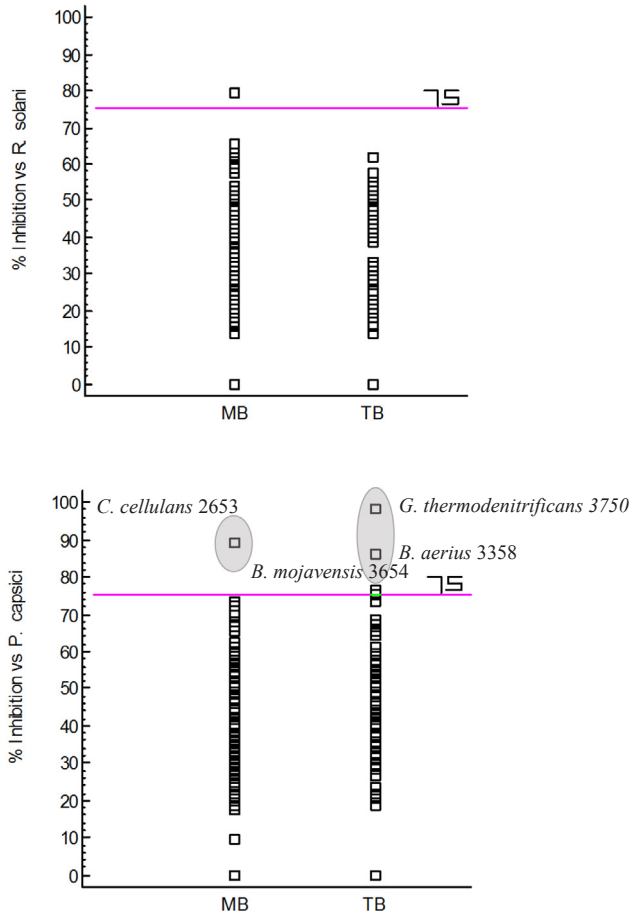

Fig. 3. Dispersion Graph for the biocontrol ability of microbial strains (MB: Mesophilic bacteria; TB: Thermophilic bacteria) to inhibit *Rhizoctonia solani* and *Phytophthora capsici*. Values are indicated as the inhibition percentage of fungal growth *in vitro*. The horizontal lines highlight the most potent antagonistic strains (inhibition of over 75% was produced by *Bacillus siamensis* 2727, *Cellulosimicrobium cellulans* 2653, *Bacillus aerius* 3358, *Bacillus mojavensis* 3654 and *Geobacillus thermodenitrificans* 3750). All results are means (n = 4 repetitions).

cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations, the production of this compound by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens (Pal and McSpadden-Gardener, 2006).

On the other hand, many studies have reported that some bacteria have the ability to solubilize phosphates, produce siderophores, salicylic acid, or other substances involved in plant promotion mechanisms, as well as in the induction of plant defence responses (Nagarajkumar et al., 2003). Phosphate solubilizing bacteria can increase P uptake by plants and increase crop yield (Nautiyal et al., 2006). Siderophores synthetized and released by some bacteria not only strongly chelate Fe^{3+}, but also can form stable complexes with other metals which are no longer available for the growth of other phytopathogenic microorganisms (Räjkumar et al., 2010). Salicylic acid is also known to inhibit the growth and conidia germination of phytopathogenic fungi (Wu et al., 2008).

In the present study, several of the strains isolated from plant based compost exhibited one or more of the previously described activities (Table 1). When the results related to the production of substances of agronomic interest were analyzed, together with the capacity to solubilize phosphates and the potential to inhibit the growth of soil pathogenic agents, interesting relationships between different pairs of variables were obtained. The linkage between groups was used as clustering method, and the squared euclidean distance was used to link the clusters (Fig. 5). The cluster analysis was carried out based on the results derived from the preliminary *in vitro* tests. The entire microbial collection was taken into account, and not only the finally selected strains. This analysis revealed the presence of four different clusters: microbial production of siderophores and salicylic acid (Cluster I), microbial production of cyanide and chitinase-like enzymes (Cluster II), phosphate solubilization and inhibition of *R. solani* (Cluster III), and finally the *in vitro* multiple inhibition of the oomycetes *P. ultimum* and *P. capsici* (Cluster IV). In the case of the latter, the production of difusible microbicide substances may be inferred as the main mechanism involved in the antagonistic effect against *P. ultimum, P. capsici*, and *F. oxysporum melonis* by the method of dual cultures. In this sense, the capacity of several biological control agents to produce different antifungal substances has been widely described (Pliego et al., 2011; Mehta et al., 2014).

In conclusion, the results shown in Figs. 4 and 5 have confirmed by *ex situ* experiments the presence of a microbiota producing agronomic metabolites of interest and which could be considered globally involved in the biological control of plant pathogens inside and outside the composting pile.

3.3. *In vivo* suppression of damping-off caused by *P. capsici* in pepper seedlings: antibiosis and plant growth promotion

In this work, *G. thermodenitrificans* strain 3750 and *B. aerius* 3358

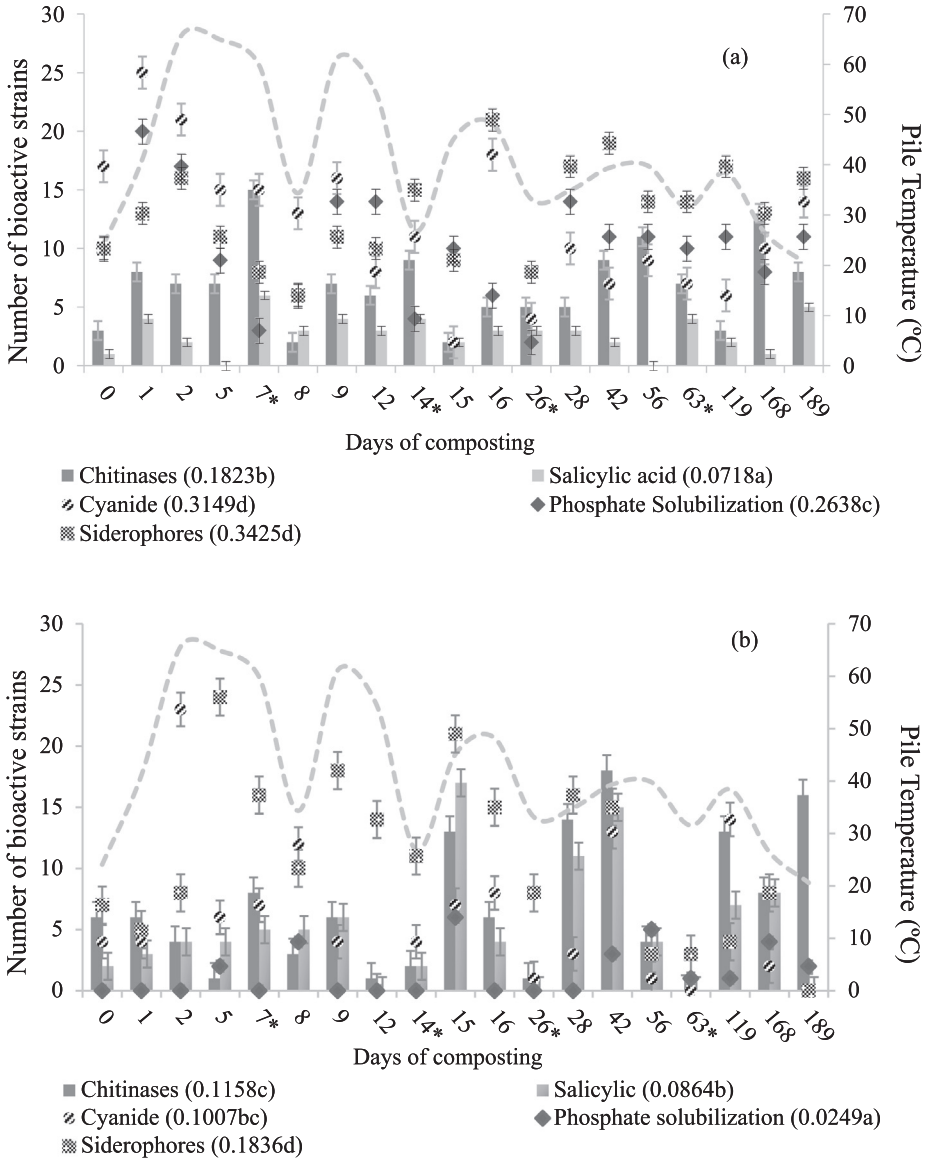

Fig. 4. Number and evolution of bacterial morphotypes isolated throughout a composting process producing chitinase like-enzymes, hydrogen cyanide, siderophores, salicylic acid, and phosphate solubilization (a: number of mesophilic biocontrol agents isolated at 30 °C; b: number of thermophilic biocontrol agents isolated at 50 °C). Temperature evolution throughout the composting process are shown by a doted line. *Samplings at which turning operations were carried out. Least Significant Means are indicated in parentheses (values labelled with different letters are significantly different according to Fisher's LSD test at P = 0.05).

were finally selected on the basis of their ability to act as strong biological control agents against the *in vitro* growth of the plant pathogen *P. capsici*, as well as their thermal tolerance and potential to act as plant growth promoting agents. As seen in Fig. 6, both strains were able to decrease symptoms caused by *P. capsici* on artificially inoculated pepper seedlings. The minimal significant difference analysis of Fisher indicates that disease severity values in infected and previously treated pepper plants (Pcap+ 3750 and Pcap+ 3358) were similar to uninfected plants (negative controls).

However, with regard to the capacity to promote plant growth, results corroborated the stimulating effect of the strain *G. thermodenitrificans* 3750 on the radicular development of pepper seedlings (Table 2). This capacity directly affected the increase of the root/stem ratio in plants previously treated with strain 3750.

Like strain 3750, strain 3358 showed capacity to produce substances such as siderophores, salicylic acid and chitinase enzymes, but not cyanide, which was considered the main differential factor between them. Both strains showed an important suppressive effect of the symptomatology caused by *P. capsici* in pepper seedlings. This effect could be quantified in more than 60% with respect to the group of plants infected and not treated with the biological control agents (Fig. 6). However, the application of strain 3358 apparently did not show any improvement in the stimulation of the growth of a group of pepper seedlings, a fact that was effectively revealed after applying the strain 3750 (Table 2). Depending on the preliminary characterization of both strains, one of the most notable differences between them was the ability to produce cyanide. Therefore, could some kind of relationship be established between the production of cyanide and the stimulation of plant growth? Until recently, this statement would have been somewhat absurd since, although the production of cyanide is closely related to the biopesticide effect of certain microbial strains, for years it has also been considered a compound that could cause problems of root toxicity (Blom et al., 2011). However, recently some authors have found that the microbial production of cyanide is closely related to iron sequestration and solubilization of phosphates, which favors the development of the root of the plant (Rijavec and Lapanje, 2016). Our results

Table 1
Characterization of biocontrol strains against *damping-off* producing phytopathogen agents and production of metabolites of interest.

Code	Identity	Microbial Group[1]	% Identity	EzTaxon[2]	Substance Production[3]					Phytopathogen Fungi[4]			
					CHIT	CIAN	SID	SAL	PHO	PU	FOM	RS	PCAP
2082	*Pseudomonas rhizosphaerae*	MB	99.13	AY152673	+	+	+	+	+	–	+	+	–
2092	*Pseudomonas xanthomarina*	MB	98.76	AB176954	–	–	+	–	–	–	+	+	+
2124	*Corynebacterium stationis*	MB	99.76	AJ620367	–	–	+	–	–	–	+	+	+
2167	*Bacillus tequilensis*	MB	98.64	HQ223107	+	–	–	+	–	+	+	+	+
2265	*Bacillus subtilis* subsp. *subtilis*	MB	100	ABQL01000001	–	–	–	+	–	+	+	+	+
2296	*Bacillus aerophilus*	MB	100	AJ831844	–	+	+	–	+	–	+	–	+
2298	*Bacillus megaterium*	MB	98.67	D16273	–	–	+	–	+	–	+	+	+
2303	*Bacillus aerophilus*	MB	99.86	AJ831844	–	–	+	–	+	–	+	+	+
2416	*Pseudomonas xanthomarina*	MB	98.35	AB176954	–	–	–	+	–	–	+	–	+
2449	*Staphylococcus succinus*	MB	99.48	AF004220	–	+	+	–	–	–	+	–	+
2624	*Bacillus safensis*	MB	99.48	AF234854	–	–	+	–	–	–	–	+	+
2653	*Cellulosimicrobium cellulans*	MB	99.16	CAOI01000359	–	+	+	–	+	–	+	+	*
2668	*Pseudomonas xanthomarina*	MB	98.69	AB176954	+	+	–	–	–	–	–	+	+
2727	*Bacillus siamensis*	MB	97.8	AJVF01000043	–	–	+	–	–	–	+	*	+
2788	*Bacillus aerophilus*	MB	98.79	AJ831844	–	–	+	–	+	–	–	+	–
3358	*Bacillus aerius*	TB	99.32	AJ831843	+	–	+	+	–	+	+	–	*
3654	*Bacillus mojavensis*	TB	99.92	JH600280	–	+	–	–	–	+	+	+	*
3750	*Geobacillus thermodenitrificans*	TB	99.77	CP000557	+	+	+	+	–	+	–	+	*

+ Positive; – negative; * antagonist effect of over 75% against *in vitro* growth of phytopathogen fungi.
[1] MB: mesophile bacteria; TB: thermophile bacteria.
[2] EzTaxon: web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences.
[3] CHIT: Chitinases; CIAN: Cyanide; SID: Siderophores; SAL: Salicylic Acid; PHO: Phosphate Solubilization.
[4] PU: *P. ultimum* CECT 2365; FOM: *F.o. melonis* CECT 20474; RS: *R. solani* CECT 2824; PCAP: *P. capsici* CECT 20433.

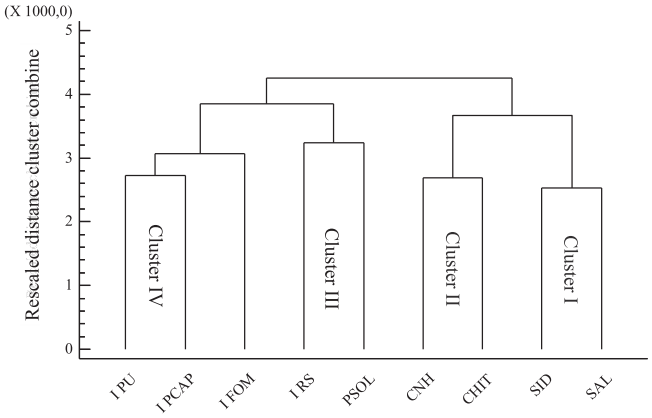

Fig. 5. Cluster analyses showing differentiation based on antagonistic spectrum toward the different phytopathogenic fungi and oomycetes (IPU: Inhibition against *P. ultimum*; IFOM: Inhibition against *Fusarium oxysporum* f.sp. *melonis*; IRS: Inhibition against *Rhizoctonia solani*; IPCAP: Inhibition against *Phytophthora capsici*), production of biofungicidal metabolites and growth promoting substances (PSOL: phosphate solubilization; CNH: hydrogen cyanide; CHIT: chitinase like-enzymes; SID: siderophores; SAL: salicylic acid). Between-group linkage was used as the clustering method.

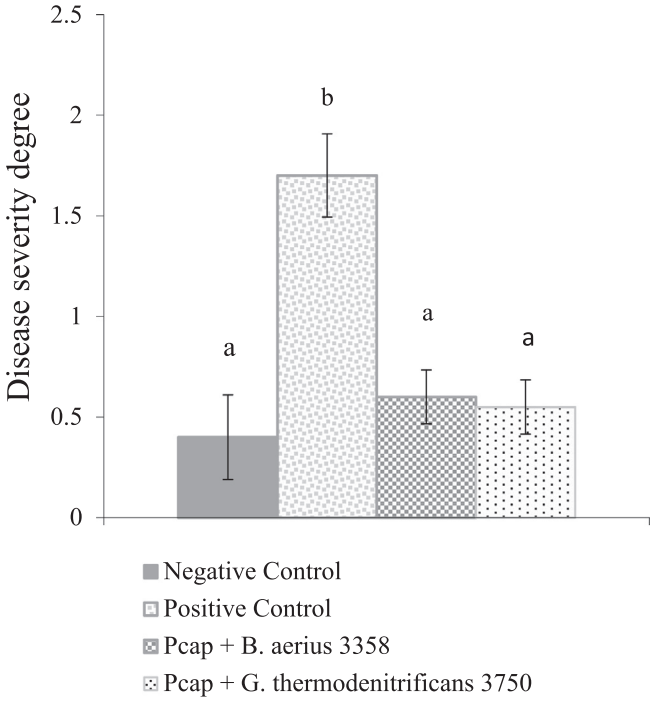

Fig. 6. Suppressive effect of *Geobacillus thermodenitrificans* 3750 and *Bacillus aerius* 3358 on symptoms caused by *Phytophthora capsici* in pepper seedlings (0: healthy plant; 1: slightly reduced aerial growth; 2: remarkable growth deficit; 3: dead plant). Negative control: non-inoculated plants; Positive control: plants inoculated only with *P. capsici*; Pcap+ 3750: plants inoculated with *G. thermodenitrificans* 3750 and *P. capsici*; Pcap+ 3358: plants inoculated with *B. aerius* 3358 and *P. capsici*. Significantly different homogenous groups are indicated with capital letters (Fisher's Least Significant Difference at a significance level of 95%). All results are means (n = 20 repetitions).

corroborate what was indicated by these authors, since the main effect detected after the application of strain 3750 in pepper seedlings is related to root stimulation, whereas this effect was not observed after the application of strain 3358 (Table 2).

On the other hand, compost is a very important source for the isolation of thermophilic and thermotolerant species (Sung et al., 2002; López-González et al., 2015). The observed abundance of the genera *Bacillus* and *Geobacillus* (notably *G. thermodenitrificans*) among the thermophilic phases of composting has been previously reported (Ryckeboer et al. 2003; Li et al., 2014; Bosma et al., 2015). One innovative biotechnological application of several thermophilic strains, such as *Geobacillus* spp., is the manipulation of the crop rhizosphere for the biocontrol of plant pathogens (Chung et al., 2011). However, the capacity of *G. thermodenitrificans* to inhibit the growth of phytopathogen agents and stimulate plant growth have not been widely described (Hussein et al., 2015).

In this work, the root/stem ratio has been used as a measure of root size (weight, length, etc.) versus stem size. This parameter estimates the overall health of the plants. Any changes in the normal ratio in non-

Table 2
Plant promoting effects of *Geobacillus thermodenitrificans* 3750 and *Bacillus aerius* 3358 on pepper seedlings. Significantly different homogenous groups are indicated with asterisks (Fisher's Least Significant Difference at a significance level of 95%).

Treatment	Leaf number	Stem length (cm)	Root length (cm)	Root/ Stem Ratio	Plant fresh weight (g)
Control	3.42a	9.72a	11.98a	1.18a	0.13a
G. thermodenitrificans 3750	3.99a	10.87a	15.04b	1.46b	0.14a
B. aerius 3358	3.93a *ns*	10.21a *ns*	10.68a *	1.07a *	0.14a *ns*

treated control plants would be an indication of a change in overall plant health. Thus, an increase in the root/stem ratio is considered a sign of healthier plants when due greater root size as opposed to a decrease in stem size (Rajendran et al., 2012). In this work, plant inoculation with the strain *G. thermodenitrificans* 3750 showed a significant increase in root/stem ratio due to a proportional increase in root length when compared to uninoculated plants (Table 2). In paralel, this strain showed a noticeable control of the damages caused by the oomycete *P. capsici* in pepper plants. Both effects corroborate a better plant growth and confirm this strain as a promising biological agent in agriculture.

4. Conclusions

During the composting of plant remains, an important community of thermotolerant bacteria is globally involved in the control of phytopathogenic organisms that may be present in the plant material. The mechanisms that such organisms use are very varied and range from substrate competition, the production of siderophores or the solubilization of phosphates, to the release of antifungal substances such as cyanide, salicylic acid, chitinase-like enzymes and/or lipopeptide production. In view of the results obtained, it is evident that during the composting of plant remains, the performance of such microorganisms could favor the elimination or deactivation of harmful agents. Although temperature has traditionally been considered of the utmost importance for the correct sanitization of the compost pile, the influence of other biotic factors must not be neglected.

The present study has proven successful in highlighting the close relationship between those strains from a composting process capable of inhibiting *R. solani* and solubilizing phosphates. Likewise, an important link was established between the microbial production of siderophores and salicylic acid. It is worth mentioning the presence of two thermophilic isolates identified as *Geobacillus thermodenitrificans* 3750 and *Bacillus aerius* 3358 that showed the ability to inhibit the *damping-off* caused by *Phytophthora capsici in vivo*. Both strains were able to produce siderophores, salicylic acid and chitinase enzymes in vitro, but only *G. thermodenitrificans* 3750 was able to stimulate the development of the root in pepper seedlings. It is suspected that the production of cyanide could be related to this latter effect. Therefore, this strain could be considered an excellent biotechnological tool for innovative practices of biological control in the agricultural sector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocontrol.2018.04.015.

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