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Isolation of bio-protective microbial agents from eco-composts

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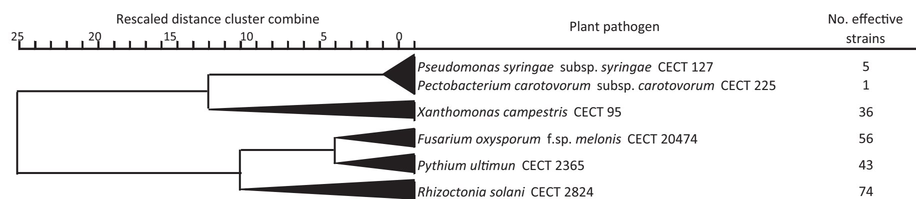
HIGHLIGHTS

- Selection of some broad spectrum biocontrol agents from plant-based compost.
- Two strains identified as *B. subtilis* and *P. chrysogenum* were effective against Fom.
- The best biocontrol agent showed a disease reduction range near to 50%.
- The best biocontrol agent weakly affected plant health in the absence of phytopathogen.

GRAPHICAL ABSTRACT



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ABSTRACT

Although increasing soil fertility is the main use of compost, the presence of bio-protective microorganisms against plant pathogens confers it an added value.

Here we review a microbial collection from plant waste based composting piles, and we tested the selected microbiota towards phytopathogenic bacteria and fungi. The raw material used for composting mixtures was vegetable waste from organic agriculture. Compost samples were collected at different stages of the composting process and one hundred and twenty-six microbial strains were selected. Phytopahtogenic strains were supplied by the Spanish Type Culture Collection: *Fusarium oxysporum* f.sp. *melonis* CECT 20474, *Rhizoctonia solani* CECT 2824, *Pythium ultimum* CECT 2365, *Pectobacterium carotovorum* subsp. *carotovorum* CECT 225, *Pseudomonas syringae* subsp. *syringae* CECT 127 and *Xanthomonas campestris* CECT 95.

Forty out of all tested isolates showed *in vitro* antagonistic activity against at least three out of the six phytopathogenic agents investigated. Six strains were then selected and *in vivo* tested to induce systemic resistance in melon plants towards the fungus *Fom.* In the presence on antagonistic strains, plants exhibited an enhanced defensive capacity against the pathogenic fungus as compared with non-inoculated control plants. Two strains identified as *Bacillus subtilis and Penicillium chrysogenum* showed a higher antagonistic capacity against Fom. These biocontrol agents showed a disease reduction range near to 50% and weakly affected plant health in the absence of phytopathogen.

On the basis of the results here shown, this study was successful in selecting some biocontrol agents which showed to be effective against important and devastating phytopathogen microorganisms. According to this research work, these microorganisms could potentially be formulated and used as biopesticide products, avoiding the adverse environmental effects of chemical hazardous pesticides.

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1. Introduction

Several studies have shown the suppression of numerous plant diseases by the use of organic amendments from heterogenic sources such as agro-industrial wastes including fresh plant, grape or winery wastes, manure, rubbish and sludge (Ntougias et al., 2008; Suárez-Estrella et al., 2007) however, this suppressive capacity is highly variable, depending on composted materials, application doses or compost aging (Bonanomi et al., 2007; Noble and Coventry, 2005).

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The production of fungitoxic compounds such as organic acids or ammonia from some organic amendments contributes to the observed antagonistic effect (Bollen et al., 1989). In addition, the presence of typical microorganisms in these substrates could enhance the antagonistic mechanisms involved in plant pathogen suppression. In fact, suppressive capacity of composts has been attributed to complex interactions between abiotic and biotic factors (Pérez-Piqueres et al., 2006; Van Loon, 2007). On the other hand, the use of compost in agriculture and horticulture as a natural nutrient source can contribute to waste recycling and reduce the use of other more harmful (chemically-synthesized) fertilizers.

Fusarium oxysporum f.sp. *melonis* (Fom) is one of the most harmful phytopathogenic agents detected along the Mediterranean coast. The mild climatic conditions favor the occurrence of this phytopathogen (González et al., 1988; Tamietti and Valentino, 2006) so its suppression is now considered an urgent need for Mediterranean agriculture. Currently, chemical methods used to control vascular wilt caused by Fom are inefficient or imply environmental damage, both of which are detrimental to human health. In this sense, alternatives to chemical pest control are being extensively investigated (Pascual et al., 2002; Suárez-Estrella et al., 2007, 2012).

In contrast to the general suppression of phytopathogenic oomycetes that seems 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 oxysporum* and *Rhizoctonia solani* (Ntougias et al., 2008).

Various studies related to biological control of Fusarium wilt by suppressive compost or by antagonistic microorganisms from compost have helped to diminish the incidence of these diseases around the world (Kavroulakis et al., 2005; Suárez-Estrella et al., 2007). In this sense, a disease reduction up to 90% has been achieved in several cases.

The main objective of this work was the isolation and identification of novel, native and effective broad-spectrum biocontrol agents from eco-compost. This objective was achieved by evaluating their *in vitro* antagonistic activity against major bacterial and fungal soilborne and foliar pathogens and their effectiveness to suppress *in vivo* the melon plant pathogen *Fusarium oxysporum* f.sp. *melonis.*

2. Material and methods

2.1. Plant pathogens

Phytopathogenic strains were supplied by the Spanish Type Culture Collection (CECT). Bacterial cultures of *Pectobacterium carotovorum* subsp. *carotovorum* CECT 225 (Pcc), *Pseudomonas syringae* subsp. *syringae* CECT 127 (Pss) and *Xanthomonas campestris* CECT 95 (Xc), were kept in slants on nutrient agar (NA; CM0003, Oxoid Ltd. UK) at 4 °C, while fungal cultures of *Fusarium oxysporum* f.sp. *melonis* CECT 20474 (Fom), *Rhizoctonia solani* CECT 2824 (Rs) and *Pythium ultimum* CECT 2365 (Pu) were kept on potato dextrose agar (PDA; CM0139B, Oxoid Ltd. UK) at 4 °C.

2.2. Compost material used

Three composting piles (3 m × 1.5 m × 1 m) were made of various vegetable materials to be used as the source of strains. The raw materials for the mixtures were pea and cucumber plant waste and pruning waste in a proportion of 50:25:25 (by volume). Plant material came from organic agriculture facilities. Aeration and moisture were controlled. Piles were periodically aerated by turning (approximately every 15 days) while there were fluctuations in temperature. During turning operations, water was added when necessary. The whole process including maturation lasted for 150 days.

2.3. Isolation of potential antagonistic microorganisms

Compost samples were collected at different stages of the composting process. Samples were taken at 0, 14, 28, 45, 73, 100 and 150 days after the beginning of the process. Actinobacteria were recovered using Sodium Caseinate Agar (SCA: sodium caseinate, 0.20 g; K_2HPO_4, 0.50 g; $MgSO_4$, 0.20 g; $FeCl_3$, 0.01 g; agar, 16 g; distilled water, 1 L; pH 6.5) and incubated at 30 °C for 72 h. Bacteria were isolated using Nutrient Agar (NA) plates and incubated at 30 °C for 24 h. In both cases, representative colonies growing on plates were selected, isolated and kept in NA at 4 °C. Fungal strains were isolated using Rose-Bengal Chloramphenicol agar plates (RB CM0549B, Oxoid Ltd. UK) and incubated at 30 °C for 96 h. In this case, representative colonies growing on plates were selected, isolated and kept in PDA at 4 °C.

The final strain collection consisted of 35 actinobacterial, 46 bacterial and 45 fungal strains. The different strains selected were named with a code indicating the compost pile source (PI, PII or PIII), the sampling time (T0–T150) and the microbial type (B, A or F: Bacterium, Actinobacterium or Fungus).

2.4. In vitro evaluation of antagonistic activity against plant pathogens

2.4.1. Antagonistic activity of isolated bacteria (AB) and actinobacteria (AA) against phytopathogenic bacteria (PB)

Each potentially antagonistic bacterial and actinobacterial strain was cultured on nutrient broth (NB; CM0001, Oxoid Ltd. UK) for 24 and 96 h respectively at 30 °C prior to use. Suppressive effect of cultures was demonstrated using a slightly modified version of the technique described by de Boer et al. (1999). First, 2% water agar (WA) plates were prepared and, after its solidification, two 8-mm-diameter steel hollow cylinders were placed equidistantly from the edge of the plate. A second layer of NA was added on the WA plates. Once NA had solidified, the cylinders were removed and two empty wells were obtained. A PB (Xc, Pss or Pcc) liquid culture was streaked on the plate surface with a sterile swab. The wells were then filled with 50 ml of the antagonist liquid culture to be assayed and the plates were incubated at 30 °C for 48 h. The plates were observed for clear (inhibition) zones around the wells. Two replicated plates were used for each antagonist-PB combination. Inhibition index (I) was expressed as percentage of PB growth inhibition in the presence of the antagonistic strain.

2.4.2. Antagonistic activity of isolated bacteria (AB) and actinobacteria (AA) against phytopathogenic fungi (PF)

Potentially antagonistic cultures were prepared as described previously. In this case, suppressive effect was demonstrated using the modified techniques of Landa et al. (1997). First, 2% water agar (WA) plates were prepared. After the agar was 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 ml liquid cultures of the antagonist to be assayed and a plug of 5-days-old PF (Fom, Rs or Pu) culture was removed from a PDA plate and placed at the center of the assay plate. There were two replicated plates for each antagonist-PF combination. Plates were incubated at 30 °C for 5 days and the inhibition index (I) was expressed as percentage of PF growth inhibition in the presence of the antagonistic strain.

2.4.3. Antagonistic activity of isolated fungi (AF) against phytopathogenic bacteria (PB)

Suppressive effect of fungal cultures was demonstrated using the modified technique of de Boer et al. (1999). A PB (Xc, Pss or

Pcc) liquid culture was streaked on the surface of a NA plate with a sterile swab. For each potentially antagonistic fungal (AF) strains, two plugs were removed from a PDA culture previously incubated at 30 °C for a week, and placed equidistantly from the edge on the same NA plate. The plates were incubated at 30 °C for 48 h and then observed for clear (inhibition) zones. Inhibition index (I) was expressed as percentage of PB growth inhibition in the presence of the antagonistic strain. There were two replicated plates for each antagonist-PB combination.

2.4.4. Antagonistic activity of isolated fungi (AF) against phytopathogenic fungi (PF)

Suppressive effect of fungal cultures was demonstrated using the modified technique of Mónaco et al. (1994). Antagonistic and phytopathogenic fungi were cultured on PDA plates for 1 week at 30 °C. After incubation, four plugs of the antagonistic fresh culture were removed and placed equidistantly from the edge on a PDA plate. A plug of the selected phytopathogenic fungus culture (Fom, Rs or Pu) was placed in the center of the same plate and incubated at 30 °C for 5–7 days. The plates were then observed for clear (inhibition) zones. There were two replicated plates for each antagonist-PF combination. Inhibition index (I) was expressed as percentage of PF growth inhibition in the presence of the antagonistic strain.

2.5. In planta evaluation of antagonistic activity of isolates

The most significant or representative microorganisms selected from preliminary *in vitro* tests were *in vivo* assayed against Fom infection in susceptible melon plants. Plants only treated with antagonistic strains were used as control.

In vivo suppressive effect against Fom growth was demonstrated using the modified technique of Larkin and Fravel (1998). Initially, *Cucumis melo* (cultivar "Amarillo Canario") seedlings planted in pots (1.5 L) with commercial substrate were inoculated with microbial suspensions of antagonists. Fungal inocula were prepared from 7 days old cultures in potato dextrose broth (PDB 254920, Difco, Voigt Global Distribution Inc. USA) while bacteria and actinobacteria were cultured in NB during 48 h and 5 days respectively. In both cases the inocula were added to pots at a dose of 10^8 colony forming units (CFU) per pot. After 10 days of incubation a Fom culture grown in PDB for 1 week was added to pots containing plants and antagonist microorganisms. Pathogen inoculum was made up of a mixture of conidia and chlamydospores and added to substrate at a dose of 10^4 propagules/g of substrate.

The experimental design consisted of 4 different treatments: (i) uninfected plants (no antagonist, nor phytopathogen) served as negative control; (ii) plants inoculated only with the phytopathogen were used as positive control; (iii) plants inoculated only with antagonist informed on possible plant damage caused by the antagonistic strain and (iv) finally, plants inoculated with both the antagonistic and the phytopathogen strains were used to prove suppressiveness. Twenty plants were employed for each treatment. After 30 days post-inoculation, the evaluation of symptoms was assessed on treated plants using a semi-quantitative rating scale from 1 to 4 as follows: 1 = no symptoms; 2 = weak yellowing; 3 = wilting and 4 = death of plant. After disease evaluation, stem sections of all plants were surface-disinfected and plated on PDA to confirm the presence of the wilt pathogen.

Greenhouse experiments were repeated twice and disease reduction was evaluated in the presence of antagonists by using two parameters: efficacy and consistency. Efficacy was considered as the percentage of mean disease reduction and consistency (i.e. the variability in the level of control provided) was derived from the standard deviations of the means. The ratio between efficacy and consistency was called Biological Control Index (BCI) according to Byrne et al. (2005), and was used to integrate these two attributes and facilitate comparisons among antagonistic strains.

2.6. Molecular characterization of antagonistic agents

Molecular characterization of the antagonist bacterial and fungal strains was conducted by DNA extraction, amplification (universal primers), sequencing and finally, analyses of DNA sequences. For this purpose, the fungal strains were grown for 120 h at 30 °C in PDB on a rotary shaker at 120 rpm. The mycelium was then harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground. Bacterial (and actinobacterial) strains were grown for 24–72 h in NB under the same conditions previously stated. After incubation, 1 mL of culture was centrifuged for 2 min at 13000*g* and the supernatant was discarded. Once cultures were processed, DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA) following the manufacturer's indications. The purified DNA was either used directly or stored at −20 °C. One mL of the diluted genomic DNA was used for PCR amplification (PERKIN ELMER, 9600 GeneAmp PCR System). The fungal specific amplification was achieved using the universal primers NS1 and NS2 + 10, annealing to the 18S rDNA of fungi (Kowalchuk et al., 1997; White et al., 1990). PCR amplification conditions were 94 °C for 5 min, 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min for 35 cycles, with 10 min extension at 72 °C used for the final cycle. On the other hand, the 16S rDNA targeted primer pair consisting of 341F and 907R was used for bacterial and actinobacterial strains amplification (Muyzer et al., 1993). In this case, PCR amplification conditions were 94 °C for 7 min, 94 °C for 45 s, 49 °C for 45 s and 72 °C for 1 min 30 s for 32 cycles, with 10 min extension at 72 °C used for the final cycle. A standard PCR mix was used in both cases. PCR amplifications were performed with 25 µL volumes containing 12.5 µL of PCR Mix (ReadyMixTMTaq PCR, Sigma–Aldrich™, Inc., P.O. Box 14508, St. Louis, MO 63178), 10–50 ng of template DNA, 150–300 nM of each primer and 10 µL of ultrapure water. Finally, the NCBI Nucleotide Sequence Database was used to identify the analyzed strains (National Center for Biotecnology Information, 2001).

2.7. Statistical analysis

Statistical analyses were carried out to study the microbial suppressive effect on Fom-infected plants in relation to infected seedlings non-inoculated with antagonists. Data were analyzed by multifactorial analysis of variance (ANOVA) and compared using Fisher's protected least significant difference test (LSD) at 95% confidence interval.

Additionally, hierarchical cluster analyses were performed for pathogens and antagonists to identify homogeneous groups or profiles. Pathogens were grouped according to the antagonistic strains by which they were inhibited. Antagonistic strains were grouped according to which plant pathogens they were effective against. Between-groups linkage was used as clustering method and the measured interval was squared Euclidean distance.

All statistical analyses were performed using SPSS 19.0 (IBM SPSS Statistics).

3. Results

3.1. Isolation of potential antagonistic strains

One hundred and twenty-six microbial strains were isolated from compost samples obtained at different stages of the composting process (45 bacteria, 35 actinobacteria and 46 fungi). Forty-two

Table 1
Distribution of tested strains and *in vitro* effective strains (inhibition percentage ⩾20%) against plant pathogens, according to microbial type, pile from where they were isolated and samplig time.

Factor	Level	Tested strains	Number of antagonistic strains against[c]					
			Fom	Pu	Rs	Xc	Pss	Pcc
Microbial type[a]	B	46	13	6	32	9	4	1
	F	45	40	36	37	6	0	0
	A	35	3	1	5	21	1	0
Pile[b]	Rm	7	4	2	2	1	0	0
	PI	42	15	13	23	16	3	1
	PII	38	20	13	24	10	1	0
	PIII	39	17	15	25	9	1	0
Composting stage (d)	0	7	4	2	2	1	0	0
	14	19	9	5	9	4	0	0
	28	16	6	4	10	6	1	0
	45	16	3	2	8	5	0	0
	73	19	10	9	11	5	0	0
	100	25	15	11	19	10	3	1
	150	24	9	10	15	5	1	0
	Total	126	56	43	74	36	5	1

[a] B: bacteria; F: fungi; A: actinobacteria.
[b] Rm: raw materials; PI, PII, PIII: piles I, II and III.
[c] Fom: *fusarium oxysporum* f.sp. *melonis*; Pu: *pythium ultimum*; Rs: *rhizoctonia solani*; Xc: *xanthomonas campestris*; Pss: *pseudomonas syringae* subsp. *syringae*; Pcc: *pectobacterium carotovorum* subsp. *carotovorum.*

out of the 126 strains were isolated from Pile 1 (14 bacteria, 13 actinobacteria and 15 fungi), while 38 and 39 came from Pile 2 (15 bacteria, 14 actinobacteria and 9 fungi) and Pile 3 (15 bacteria, 10 actinobacteria and 14 fungi), respectively (Table 1). In relation to sampling time, a total of 68 potential antagonistic strains were isolated during the maturation phase (T73, T100 and T150), while 51 were obtained from the bio-oxidative phase (T14, T28 and T45). Seven strains were isolated from the raw materials (T0) (see third column in Table 1).

3.2. In vitro evaluation of antagonistic activity of microbial strains against plant pathogens

The potential antagonistic strains previously isolated were assayed against the selected phytopathogenic bacteria and fungi above indicated, using *in vitro* confrontations described previously. For each paired confrontation an Inhibition Index (I) expressed as percentage, was obtained. Only antagonistic strains showing I equal or greater than 20% were considered as true antagonists in order to set a high enough inhibition threshold. Distribution of tested strains and *in vitro* effective strains (I ⩾ 20%) against each plant pathogen, according to microbial type, pile from where they were isolated and samplig time is shown in Table 1. According to data presented in this table, antagonistic strains effective against pathogenic fungi (56 *vs.* Fom, 43 *vs.* Pu and 74 *vs.* Rs) were more numerous than those against pathogenic bacteria (36 *vs.* Xc, 5 *vs.* Pss and 1 *vs.* Pcc).

When assayed against antagonistic bacteria, the pathogenic fungi Fom and Rs showed a higher susceptibility than the other pathogenic agents. In this sense, 28% and 69% of the total bacteria tested were efficient, against Fom and Rs respectively. On the other hand, when assayed against antagonistic fungi, the pathogenic fungi were much more susceptible than the pathogenic bacteria. So around 80% of the total fungi tested were efficient toward Fom, Pu and Rs while only Xc was susceptible toward a low number of fungi. Finally, Xc was the most susceptible agent when assayed against antagonistc actinobacteria, since 60% of them were effective in this case (Table 1). In general, Rs was the most susceptible phytopathogenic fungus while the bacteria Pss and Pcc were not significantly affected by the antagonistic strains.

Efficiency against at least three plant pathogens was the criteria selected to choose the best antagonistic strains. Forty out of the 126 isolated strains showed antagonistic activity against at least three of the plant pathogens. These forty strains were divided into 8 bacteria, 1 actinobacterium and 31 fungi (approximately 30% of the total strains tested). The antagonistic spectrum corresponding to each selected strain is indicated in Table 2.

Several of these strains were antagonistic against four or five pathogenic agents. However, the strain PI-T100-B3, which was efficient against five plant pathogens, was not selected due to the loss of viability during *in vitro* culturing. On the other hand, strains PI-T100-H2 and PIII-T14-B3 were rejected due to the low inhibition indexes they exhibited (data not shown).

Fig. 1 shows the distribution of the antagonistic strains according to the number of plant pathogens against which they were effective (I ⩾ 20%). None of the strains tested were effective against all six of the selected phytopathogenic agents. Only one of them (<1%) was antagonist against five out of the total of pathogens, while 21 (16%) strains did not show any effect.

To establish similarity relations between phytopathogenic behavior toward bio-protective agents tested, a cluster analysis was carried out calculating the distances using between-groups linkage as clustering method. In this sense, antagonistic strains were divided into fifteen groups on the basis of their antagonistic spectrum (Fig. 2). One hundred and five strains are clustered in Fig. 2. The groups showing the closest antagonistic spectrum were 1, 10 and 14 (Fig. 2). Eighteen out of the twenty strains included in group 1 were classified as actinobacteria and were effective toward Xc, while fifteen out of the seventeen strains included in group 14 were classified as bacteria and effective toward Rs. Both groups are very far from each other in the dendrogram and they could be used as specific treatments. Number 7 was a very interesting group too, because it shows a clear antifungal spectrum. In this sense, 90% of the total strains included in this group were classified as fungi.

Dendrogram shown in Fig. 3 indicates the susceptibility degree of the six pathogenic agents towards the total effective strains. In general, phytopathogenic bacteria and fungi were clustered into 2 independent groups. In the first stage, Pss was combined with Pcc, and both were grouped with Xc at a farther level. On the other hand, Fom and Pu were closely clustered and both were grouped with Rs at a higher stage. Therefore, on the basis of phytopathogen susceptibility, we could obtain 5 different clusters, though Fom and Pu appear very closely related in the dendrogram (Fig. 3).

Table 2
Antagonistic strains effective (I ⩾ 20%) against at least three plant pathogens. Strains written in bold were selected for *in vivo* assays.

Antagonistic strain	Phytopathogen					
	Fom[a]	Pu	Rs	Xc	Pss	Pcc
PI-T100-B3	+	+	+	–	+	+
PIII-T100-B3	+	+	+	+	–	–
PI-T100-H1	+	+	+	+	–	–
PI-T150-H2	+	+	+	+	–	–
PII-T73-H2	+	+	+	+	–	–
PIII-T28-H3	+	+	+	+	–	–
PIII-T45-H3	+	+	+	+	–	–
PIII-T73-H2	+	+	+	+	–	–
PI-T100-B1	+	+	+	–	+	–
PII-T28-B2	+	–	+	+	+	–
PII-T73-B3	+	+	+	–	–	–
PIII-T14-B3	+	+	+	–	–	–
PII-T150-A2	+	+	+	–	–	–
AE-To-H2	+	+	+	–	–	–
AE-To-H3	+	+	+	–	–	–
PI-T28-H3	+	+	+	–	–	–
PI-T73-H1	+	+	+	–	–	–
PI-T73-H2	+	+	+	–	–	–
PI-T100-H2	+	+	+	–	–	–
PI-T100-H3	+	+	+	–	–	–
PI-T150-H3	+	+	+	–	–	–
PII-T14-H1	+	+	+	–	–	–
PII-T14-H3	+	+	+	–	–	–
PII-T28-H1	+	+	+	–	–	–
PII-T73-H3	+	+	+	–	–	–
PII-T100-H2	+	+	+	–	–	–
PII-T100-H3	+	+	+	–	–	–
PII-T150-H1	+	+	+	–	–	–
PII-T150-H2	+	+	+	–	–	–
PII-T150-H3	+	+	+	–	–	–
PIII-T14-H1	+	+	+	–	–	–
PIII-T73-H1	+	+	+	–	–	–
PIII-T100-H1	+	+	+	–	–	–
PIII-T100-H2	+	+	+	–	–	–
PIII-T100-H3	+	+	+	–	–	–
PIII-T150-H1	+	+	+	–	–	–
PIII-T150-H2	+	+	+	–	–	–
PIII-T150-H3	+	+	+	–	–	–
PII-T100-B3	+	–	+	+	–	–
PIII-T100-B2	+	–	+	+	–	–

[a] Fom: *fusarium oxysporum* f.sp. *melonis*; Pu: *pythium ultimum*; Rs: *rhizoctonia solani*; Xc: xanthomonas campestris; Pss: pseudomonas syringae subsp. syringae; Pcc: *pectobacterium carotovorum* subsp. *carotovorum*.

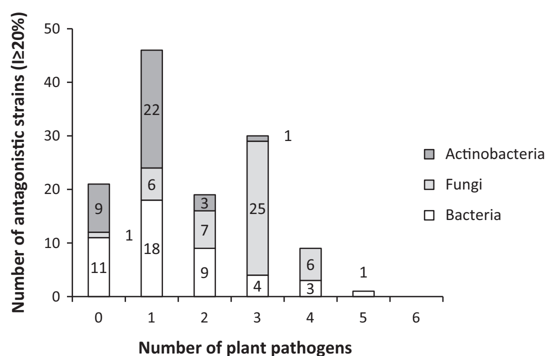

Fig. 1. Distribution of the antagonistic strains according to the number of plant pathogens against which they were effective (I ⩾ 20%).

3.3. In planta evaluation of antagonistic activity of selected strains

Six strains were finally selected to evaluate their suppressive activity towards Fom in melon plants. These strains are bolded in Table 2. In all cases, excepting PII-T28-B2, these strains showed an extended antifungal spectrum due to their efficiency against all pathogenic fungi tested. Additionally, four out of the six strains selected (PI-T100-B1, PII-T28-B2, PIII-T100-B3 and PI-T100-H1) showed inhibitory activity towards the bacterial plant pathogens Pss and/or Xc (Table 2).

Infective capacity expressed as a percentage of Fom isolation from infected plants is shown in Fig. 4. Those plants inoculated exclusively with Fom (Control) had an infectivity level near 85%. Meanwhile, a percentage of disease reduction around 50% was observed by the most suppressive microorganism assayed (Table 3). Statistical analyses showed that isolate PI-T100-B1 gave the highest level of protection in relation to the control plants, followed by isolates PI-T100-H1, PIII-T100-B3 and PII-T73-B3. Pre-treatment with strain PII-T28-B2 increased the infective capacity of Fom showing values near to 95% (Fig. 4).

On the other hand, from the plant health point of view, the influence of the treatment with the bio-protective agents was evaluated. In this sense, the disease progression in antagonistic-treated plants in presence/absence of Fom was measured (Table 3). Excepting strain PII-T28-BII, plants pre-treated with the biological control agents and then infected with Fom showed a similar health degree to control plants only infected with Fom (Table 3, third column). Surprisingly, plants pre-treated with the PI-T100-B1 and later infected with Fom showed a better health degree than the rest of plants, even considering control plants. When plants were only pre-treated with the biocontrol agents in absence of Fom, the health degree fluctuated between 1 and 2, being the control plants and those pre-treated with PI-T100-B1 the healthiest (Table 3). BCI (Biological Control Index) ranged from 0.23 to 2.56 with PI-T100-B1 showing the highest BCI value. In this case, a decrease of the pathogen isolation was observed in relation to the disease reduction (Fig. 4, Table 3).

Therefore, on the basis of the combined results from antagonistic capacity and disease progression (Fig. 4 and Table 3), strain PI-T100-B1 was considered the most bio-protective microorganism.

3.4. Molecular characterization of the antagonistic microorganisms selected from in planta experiments

All six strains assayed by *in vivo* experiments were finally identified by traditional and molecular methods. Microbial identification of these potential antagonist strains was previously achieved by macroscopical and microscopical observations. Strains named PII-T28-B2, PII-T73-B3, PI-T100-B1, PIII-T100-B3 and PII-T150-A2, were confirmed by Gram staining as Gram positive sporulated rods. In the case of fungal strain (PI-T100-H1), macroscopical growing and microscopical structures confirmed a great similarity with *Penicillium* spp.

Finally, molecular methods showed a great homology between the fungal strain PI-T100-H1 and *Penicillium chrysogenum* and other closely related species. In the case of bacterial strains named PII-T28-B2, PII-T73-B3, PI-T100-B1 and PIII-T100-B3, these were identified as *Bacillus subtilis,* while the strain PII-T150-A2 showed the maximum homology with *B. licheniformis.*

4. Discussion

In the last 30 years interest in biological control practices has increased due to the need to eliminate chemical residues on vegetable cultures. In relation to this subject, compost, the final product of the aerobic biodegradation of organic matter, exhibits marked disease suppressive activity (Hoitink et al., 1993).

Composts are used as a soil amendment to provide nutrients and organic matter, and to improve soil structure. They are generally made up of waste products, such as tree bark, animal waste,

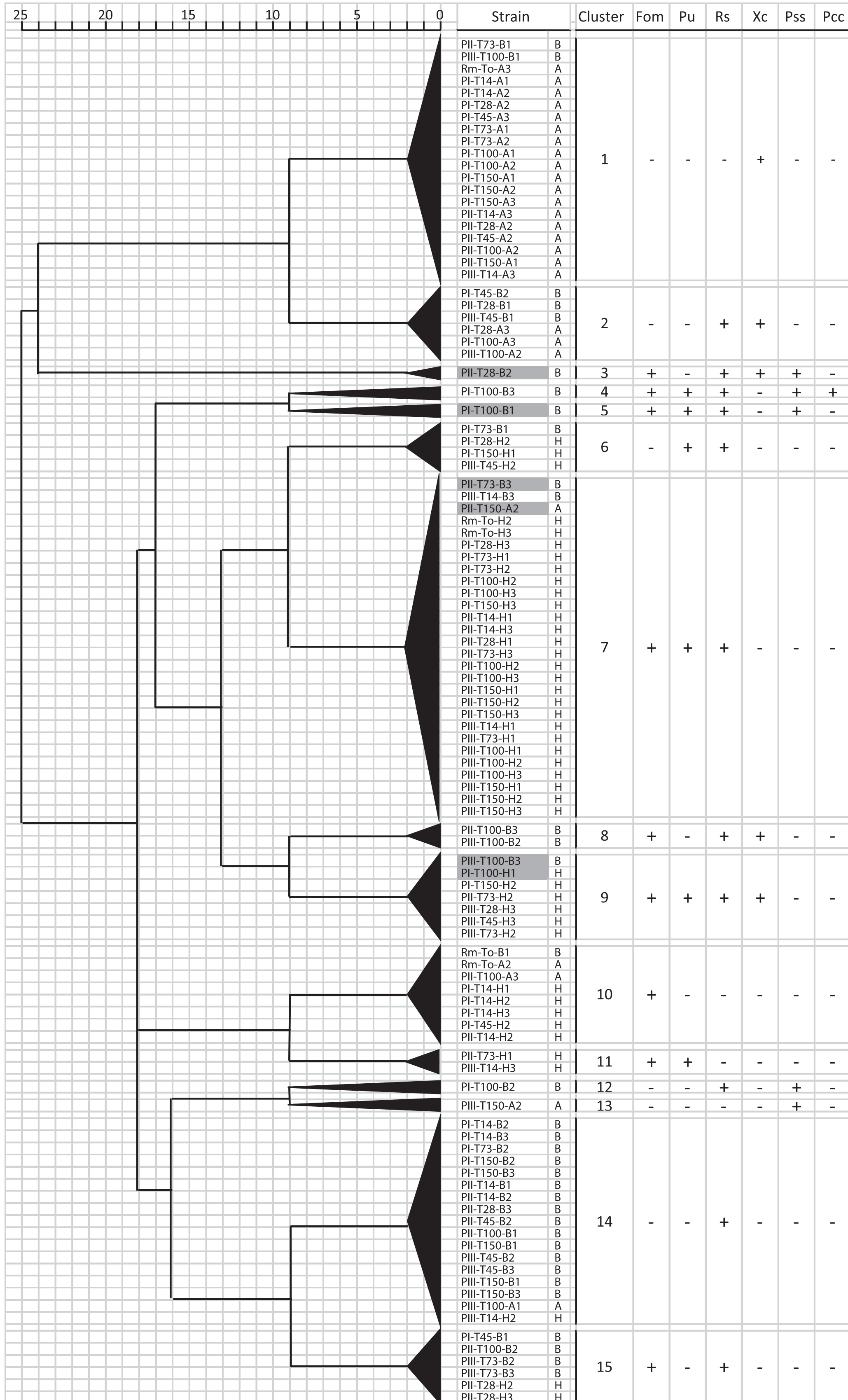

Fig. 2. Cluster analyses showing differentiation based on antagonistic spectrum toward the six pathogenic agents.

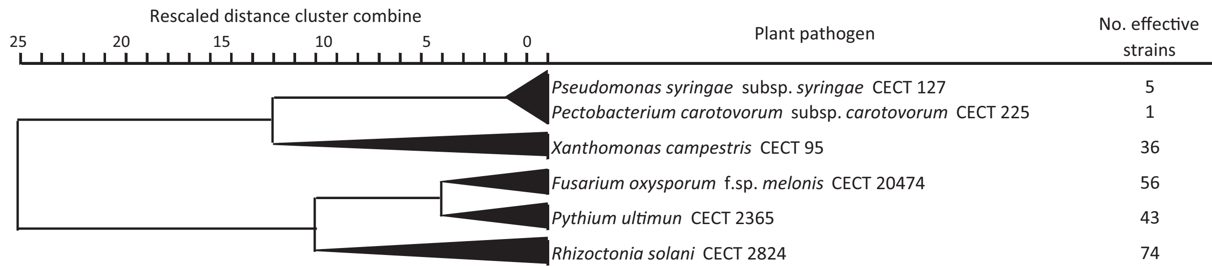

Fig. 3. Cluster analyses showing differentiation based on pathogenic susceptibility toward the total of effective antagonistic strains.

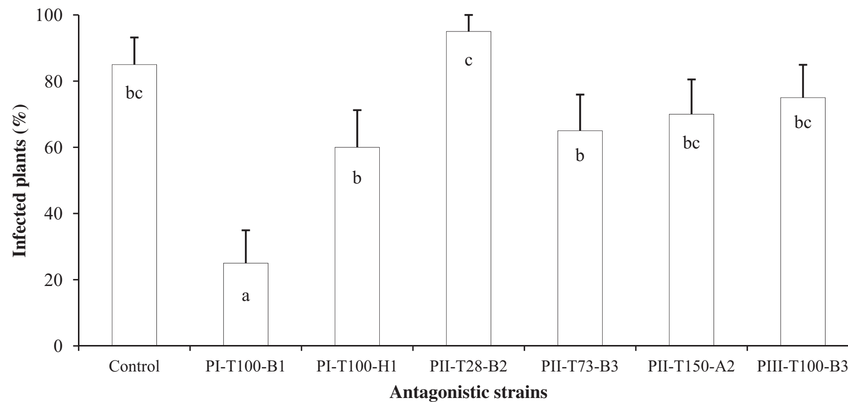

Fig. 4. Infective capacity as percentage of positive isolations of *Fusarium oxysporum* fsp. *melonis* from melon plants in the presence of the selected antagonistic strains. Bars with the same letter are not significantly different ($P < 0.05$).

Table 3
Health status of melon plants in the presence of antagonistic strains (with and without Fom), disease reduction and BCI. Values with different letters are significantly different ($P < 0.05$).

Strain	Plant health[a]		Disease reduction (mean percentage ± SD)[b]	BCI[c]
	−Fom	+Fom		
Control	1.10a	2.25b	0.00 ± 0.00b	–
PI-T100-B1	1.10a	1.20a	46.67 ± 18.24c	2.56
PI-T100-H1	2.00c	1.90b	15.56 ± 23.47b	0.66
PII-T28-B2	1.60b	2.73c	−21.11 ± 39.14a	−0.54
PII-T73-B3	1.50b	1.98b	12.22 ± 33.41b	0.37
PII-T150-A2	2.00c	2.10b	6.67 ± 29.38b	0.23
PIII-T100-B3	1.90c	1.93b	14.44 ± 28.18b	0.51

[a] Values ranged from 1 (healthy plants) to 4 (dead plants). See materials and methods for a detailed explanation.
[b] Mean of percent disease reduction achieved in greenhouse assays. Data shown are for strains assayed in two or more experiments. SD, standard deviation of the mean.
[c] Biological control index (Mean disease reduction/SD).

municipal solid waste, green waste and sewage sludge. Some compost suppress soilborne plant pathogens (Hoitink et al., 1997) although the exact mode of action is not clear. However, several mechanisms seem to be involved, such as enhanced plant growth vigor, presence of microbial antagonists in the composts and induction of plant resistance. Our results confirm the potential of some composted ecological plant waste as an eco-friendly management option for suppression of phytopathogenic bacteria and fungi.

This suppression capacity could be due to compost recolonization by effective biocontrol agents after peak heating has occurred in the composting process (Kwok et al., 1987). To date, *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp., other bacterial groups and *Streptomyces* spp., as well as *Penicillium* spp., several *Thichoderma* spp., isolates of *Gliocladium virens* and other fungi have been identified as biocontrol agents in compost-amended substrates (Hoitink et al., 1996, 1997). Results obtained throughout this work show at least 40 microbial agents isolated from eco-compost whose antagonistic capacities were at least corroborated toward three phytopathogenic agents (Table 2). Those strains tested *in vivo* were identified as *B. subtilis* (PII-T28-B2, PII-T73-B3, PI-T100-B1 and PIII-T100-B3), *B. licheniformis* (PII-T150-A2) and *P. chrysogenum* (PI-T100-H1).

In most cases, effective bacteria used as biocontrol agents of plant diseases belong to the genera *Bacillus*, *Pseudomonas* and *Streptomyces* (Edwards et al., 1994). Landa et al. (1997) showed that approximately 32% of 74 bacterial isolates from the chickpea rhizosphere inhibited *in vitro* growth of *F. oxysporum* f.sp. *ciceris* in dual cultures. More recently, 36% of strains isolated from plant based-compost have shown to be antagonistic toward Fom by *in vitro* tests (Suárez-Estrella et al., 2007). In contrast, Myatt et al. (1992) obtained a lower proportion of antagonistic bacteria toward *Phytophthora megasperma* Dreschsler f.sp. *medicaginis* from a similar environment. Our results and those obtained by Myatt et al. (1992) corroborate that the general suppression of phytopathogenic oomycetes appears not to be related to the specific

antagonist capacity of microbial agents but to broad microbial consortia (Hoitink et al., 1996). Bearing in mind the previously cited data, the degree of disease suppression probably depends on the strength of the antagonistic microorganisms and the hosts, as well as the pathogen inoculum concentration (Landa et al., 1997).

According to this research work, 18%, 22% and 6% of the total bacteria, actinobacteria and fungi, respectively, isolated from eco-compost appeared to be effective against at least one of the plant pathogens tested (approximately 36% from the total of strains tested). Several works have been carried out in order to screen the potential antagonistic bacteria and fungi isolated from the rhizosphere, plant waste and composts (Landa et al., 1997; Suárez-Estrella et al., 2007). In most cases the main objective was to search for interesting strains showing an antagonistic broad spectrum. Suárez-Estrella et al. (2007), detected antagonistic actinobacteria and fungi isolated from plant waste compost which were tested against different phytopathogenic agents. As in the present work, the behavior of the different strains was variable with respect to the phytopathogenic agent tested toward more than 150 potential antagonistic agents. From results obtained in this work, we can infer that the bacterial susceptibility profile was nearer between Pcc and Pss rather than these and Xc. When phytopathogenic fungi were clustered, the nearest distance was found between Fom and Pu. The observations here described confirm the difficulties to find "universal" antagonistic microorganisms showing an antimicrobial broad spectrum.

On the other hand, our results support the importance of the maturation phase respect to the isolation of antagonistic agents. More than 60% of microorganisms selected on the basis of their broad antagonistic spectrum (Table 1) were isolated from a composting advanced stage, after the bioxidative phase (>45 days). Stability of composts must be considered in biological control and this concept has been previously defended by other authors (Kwok et al., 1987; Suárez-Estrella et al., 2007). However, excessively stabilized organic matter does not support the activity of biocontrol agents since the most relevant/abundant microorganisms in it are incapable of providing biological control (Workneh et al., 1993).

The most interesting antagonistic agents selected in this work were two strains identified as *B. subtilis* and one fungal strain identified as *P. chrysogenum*. *B. subtilis* and other *Bacillus* spp. are frequently found both in fresh plant material and in composted plant waste. The antagonistic effect of *B. subtilis* has been previously described against phytopathogenic fungi and bacteria (Czaczyk et al., 2000; Zanón and Jordá, 2008). However, on the basis of results here obtained, the antagonistic capacity of different strains of *B. subtilis* against a given phytopathogenic agent showed to be variable. Strains named PII-T28-B2, PII-T73-B3, PI-T100-B1 and PIII-T100-B3, all identified as *B. subtilis,* showed different behavior when they were tested *in vitro* and *in vivo*. While the *in vitro* antagonistic profile was similar for PII-T73-B3, PI-T100-B1 and PIII-T100-B3 (Table 2), the profile of strain PII-T28-B2 was very different from other *B. subtilis*. In addition, PII-T28-B2 did not show a significant *in vivo* suppressive efficiency (Fig. 2). On the contrary, it favored Fom infectivity of the melon plants tested. Though the procedence of these four strains was the same (horticultural waste from organic activities), they were isolated at different stages of the composting process. While the strains PII-T73-B3, PI-T100-B1 and PIII-T100-B3 were isolated during maturation phase, the strain PII-T28-B2 was isolated during the bioxidative phase (28 days). This fact could support the importance of maturation phase with respect to the isolation of effective biological control agents. Anyway, the strain PI-T100-B1, showed the most interesting suppressive efficiency against Fom, around 50%. The effect of this strain could be based on its capacity to act both as a systemic resistance inducer and a plant growth promoting bacterium (PGPB), supporting data previously obtained by other authors (Ji et al., 2006). In this case, the value of *Biological Control Index* (BCI) was similar to those obtained by Byrne et al. (2005). The best data observed by these authors were around 1.59–2.98 corresponding to biocontrol bacterial agents such as *Pseudomonas syringae*, *P. putida* or *Pantoea agglomerans*. Assuming that some strains may be inherently more variable than others in the level of control provided, BCI (mean percentage reduction divided by standard deviation) was considered as a selection criterion. Therefore, consistency was also an important attribute to bear in mind. In this sense, consistency data (standard deviation) obtained in this work were similar to those obtained by Byrne et al. (2005) (Table 3). On the basis of the results obtained, the effect of a biocontrol agent on uninfected plants is also another important aspect to consider. This factor could help to decide the best bioncontrol agent among different strains showing a similar BCI.

The strain PI-T100-H1, identified as *P. chrysogenum*, showed a very weak suppressive effect against Fom. However, previous data have corroborated an important suppressive activity when several strains of this species have been tested. Dry mycelium of *P. chrysogenum*, a waste product of the pharmaceutical industry, is used as an organic fertilizer in agriculture. *P. chrysogenum* induces significant resistance against *Fusarium* and *Verticillium* wilts in potted cotton plants under glasshouse conditions (Dong et al., 2006). Other earlier studies conducted under greenhouse or shade-house conditions showed that application of *P. chrysogenum* could protect melon plants against *Fusarium oxysporum* f.sp. *melonis* (Dong et al., 2006), supporting the data here obtained. Recently, Gotlieb et al. (2003) reported that *P. chrysogenum* protected cucumber and tomato plants against the root-knot nematode *Meloidogyne javanica* in a shade house.

Enhancement of natural plant-defense mechanisms in order to provide resistance against pathogens is a newly developed approach for plant protection. Induced resistance can be achieved by using biotic inducing agents (Siddiqui and Shaukat, 2004). In our work, the suppression of Fusarium wilt when PI-T100-H1 was applied on melon plants reached a protection level around 15%. These results are supported by previous investigations in which protection percentages fluctuated between 14% and 50% (Chen et al., 2006; Dong et al., 2006), since the disease-control efficiency of *P. chrysogenum* is also very dependent on application methods and rates in field experiments (Dong et al., 2006). Some rhizospheric species of *Penicillium.* spp., *Trichoderma* spp., *Fusarium* spp., and *Phoma* spp. are able to promote plant growth upon root colonization. These fungal strains are functionally designated as 'plant-growth-promoting-fungi (PGPF)' (Hyakumachi, 1994), and several of them have been shown to trigger systemic resistance against various pathogens in cucumber plants (Shoresh et al., 2005). In this sense, Hossain et al. (2008) suggested that several species of *Penicillium* spp. may produce one or more elicitors that induce resistance towards the foliar pathogen *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana* plants.

5. Conclusions

This study was successful in selecting some broad spectrum biocontrol agents which showed to be effective against several plant pathogens. According to this research work, the strains named PII-T73-B3 and PI-T100-B1, identified as *B. subtilis*, and the fungus PI-T100-H1, identified as *P. chrysogenum*, were effective toward Fusarium wilt of melon, an important and devastating disease very usual in organic agriculture. These microorganisms could therefore be used as biocontrol agents, avoiding the adverse environmental effects of hazardous pesticides.

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