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Research Papers

Evaluation of microbial products for the control of zucchini foot and root rot caused by *Fusarium solani* f. sp. *cucurbitae* race 1

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Summary. Microbial products containing bacteria (Cedomon [*Pseudomonas chlororaphis* MA342, PC-MA342], Mycostop [*Streptomyces* sp. K61, SG-K61], Proradix®Agro [*Pseudomonas* sp. DSMZ13134, PS-DSMZ13134]) and fungi (Clonotry [*Trichoderma harzianum* and *Clonostachys rosea*, TH+CR], Remedier [*T. asperellum* ICC012 and *T. gamsii* ICC080, TA-ICC012+TG-ICC080], Rootshield WP [*T. harzianum* T22, TH-T22]) were tested for efficacy against *Fusarium solani* f. sp. *cucurbitae* race 1 (FSC7 strain) on zucchini. They were applied to seeds (S), plant growth substrate (PGS) and both (S+PGS) in a growth chamber experiment, and to PGS, transplantation soil mixture (TSM) and both (PGS+TSM) in a greenhouse experiment. FSC7 was inoculated in PGS at sowing time in the growth chamber and in TSM at transplant in the greenhouse. In the growth chamber, the most effective products were Cedomon (S and S+PGS treatments), Rootshield (PGS treatment) and Proradix (S+PGS treatment), reducing the disease by 39.7, 43.1, 25.8 and 36.4%, respectively. In the greenhouse, all tested products applied to PGS reduced the disease severity and more markedly when applied to PGS+TSM. In the PGS and PGS+TSM treatments, Cedomon was the most effective product showing a disease decrease by 42.4 and 59.5%, respectively. The data obtained *in vivo* were consistent with the ability of the antagonists to colonize zucchini rhizosphere and with their inhibitory effects on the growth of the pathogen in *in vitro* assays. The bacteria caused the greatest growth inhibition of FSC7 showing abnormal morphology, while *Trichoderma* spp. parasitized FSC7 hyphae. Bacteria were the most active in reducing pathogen colony growth through antibiotic metabolites. All antagonists produced exo- and endochitinase enzymes. *Trichoderma* strains showed greater levels of β-*N*-acetylhexosaminidase and endochitinase, whereas SG-K61 was the most active producer of chitin 1,4-β-chitobiosidase. These results indicate that the studied bioproducts have potential for an effective management of zucchini *Fusarium* foot and root rot through rhizosphere competence and several mechanisms exerted by their microbial ingredients.

Key words: biological control, *Cucurbita pepo*, seed treatment, soil treatment.

Introduction

Fusarium solani (Mart.) Sacc. (teleomorph *Nectria haematococca* Berk. and Br.) is a cosmopolitan soilborne fungus with saprotrophic and pathogenic strains. Several *formae speciales* exhibiting host specificity have been described (Booth, 1971). Among these, *Fusarium solani* f. sp. *cucurbitae* W.C. Snyder and H.N. Hansen is the causal agent of cucurbit diseases described by various names, including Fusarium crown and foot rot, wilt, root rot, foot rot, root and stem rot and cortical rot (Martyn, 1996). Two races of this pathogen have been identified on the basis of tissue specificity. Race 1 causes rot of roots, stems and mature fruits and occurs worldwide, while race 2 causes only fruit rot and has been occasionally reported (Tousson and Snyder, 1961; Fantino *et al.*, 1989; Martyn, 1996). On the basis of their sexual compatibility, these two races are separated into different mating populations: strains of race 1 belong to mating population I, and strains of race 2

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belong to mating population V (Snyder *et al*., 1975). These two races most probably represent reproductively isolated biological species with independent evolutionary origin (O'Donnell, 2000).

Fusarium solani f. sp. *cucurbitae* was first reported on squash in 1930 in South Africa and then in United States, Europe, Australia, New Zealand, Japan and Africa (Snyder *et al*., 1975; Sherf and MacNab, 1986; Hawthorne *et al*., 1992; Champaco *et al*., 1993; Nagao *et al*., 1994; Armengol *et al*., 2000, Boughalleb *et al*., 2005). In Italy, *F. solani* f. sp. *cucurbitae* race 1 was first described on zucchini (*Cucurbita pepo* L.) by Vannacci and Gambogi (1980) and subsequently reported by Stravato and Cappelli (1996), by Roberti *et al.* (2008a) and also in association with *Plectosporium tabacinum* by Vitale *et al.* (2007). In recent years, attacks of the pathogen have caused severe losses of yield in the Emilia Romagna region, Northern Italy, particularly in greenhouses where zucchini monoculture is often practiced. The pathogen survives for 2–3 years in the soil, in the absence of susceptible hosts, and in plant debris, and can infect plants and fruits at any stage, favoured by high humidity and temperatures. Most of cucurbits are susceptible to infection. The pathogen can also be seedborne, but infection does not appear to affect seed viability or germination (Martyn, 1996).

Nowadays, there are no highly effective controls for the disease, since all chemicals, including those for soil disinfestation such as methyl bromide, are subjected to restrictions in their use both in European countries and elsewhere. In Italy, very few chemicals are allowed for use on cucurbit crops; among these are fumigants with some fungicide effect, such as chloropicrin (only Tripicrin is currently authorized), metham sodium and metham potassium (for essential uses), dazomet (until 31/12/2012), and non-fumigant fungicides including thiram, etridiazole and chlorothalonil. These non-fumigant fungicides are claimed by the manufacturers to control Fusarium diseases in the soil, but they are not applied under highly conducive conditions for the disease. Moreover, there are no commercial cultivars resistant to this disease.

Among other disease management strategies, microbial products represent an attractive method to control numerous soilborne diseases (Sivan and Chet, 1993; Inbar *et al*., 1996; Harman, 2000; Naseby *et al*., 2000). There are few reports of the *F. solani* biocontrol. The application of microorganisms such as *Trichoderma harzianum* controlled *Fusarium solani* root rot on peanut both in naturally infested and artificially contaminated fields (Rojo *et al*., 2007) and on common bean in greenhouse pot experiments (Abeysinghe, 2007). Also bacteria like *Pseudomonas aeruginosa* and *Pochonia chlamydosporia* suppressed *F. solani* infection on tomato (Siddiqui and Shaukat, 2003). To our knowledge, the potential of microorganisms for control of foot rot of cucurbits has been tested only on watermelon in Egypt (El-Sheshtawi *et al*., 2007).

According to the currently available literature, no studies have been carried out on the mechanism of action of antagonists specifically against *F. solani* f. sp. *cucurbitae*.

The aim of the present research was to examine the defensive role of microbial products applied by different strategies to seed, plant growth substrates or transplantation soil mixtures against foot and root rot of zucchini caused by *F. solani* f. sp. *cucurbitae* race 1. A second aim of this study was to evaluate rhizosphere and root colonization by the bioproduct antagonists, since the efficacy of antagonists has been related to their ability to grow after application. Finally, we have investigated possible mechanisms of action of the antagonists, such as competition, antibiosis and production of chitinolytic enzymes, likely to be involved in the pathogen control.

Materials and methods

For biocontrol activity, commercial microbial products (bioproducts) were used, while for all the other experiments, the active microbial ingredients of the products (antagonists) were tested.

Bioproducts, antagonists and pathogen

The bioproducts employed were supplied by the manufacturers and are listed in Table 1.

The first four products are biofungicides registered by Italian Health Ministry for the application against phytopathogens on various crops, while Proradix®Agro and Clonotry are not registered as biofungicides in Italy, but commercialized as fertilizers. The biofungicides are claimed by the manufacturers to control some soilborne pathogens, but not specifically *F. solani*, and their recommended application methods include seed and/or soil treatment. All of the microbial products claim to improve plant growth or to stimulate plant resistance against pathogens.

For the experiments on rhizosphere and root colonization and on the mechanisms of action, each antagonist was isolated from the bioproduct and cultured on potato dextrose agar (PDA, 3.9%, Difco) for fungi and on King's B medium for bacteria. The fungal antagonists were identified through microscopic observation of cell morphology and by sequencing the internal transcriber spacers 1 (ITS1) and 2 (ITS2) and 5.8S ribosomal RNA gene according to White *et al.* (1990). The nuclear rDNA region was amplified using the primer combinations ITS1 and ITS4 for TA-ICC012 and TG-ICC080, and with the primer combinations SR6R and LR1 for TH-T22. The sequences were aligned with those deposited in GeneBank. Concerning SG-K61, the identification was carried out only through microscopic observation of cell morphology, since contamination tests proved that K61 was the sole streptomycete occurring in the Mycostop batch we have used (Lahdenperä M., Verdera OY, personal communication). The strain isolated from Proradix® was identified as PS-DSMZ13134 by the manufacturer (Sourcon-Padena, Germany), whereas certification of isolated bacteria from Cedomon as PC-M342, was warranted by BioAgri-Blue Line, Sweden.

A pathogenic strain of *Fusarium solani* f. sp. *cucurbitae* race 1 (FSC7), was used throughout this study. It was chosen among the more virulent strains isolated from zucchini showing symptoms of *Fusarium* disease, cultivated in greenhouses in the province of Bologna, Emilia Romagna region, Northern of Italy (Veronesi *et al*., 2009); these strains were identified on the basis of morphological features, and host specificity (Booth, 1971; Nelson *et al.*, 1983), and of molecular methods amplifying the translation elongation factor 1-α region (O'Donnell *et al*. 1998).

Biocontrol assays

The effects of the bioproducts against FSC7 were studied in a growth chamber experiment, simulating the nursery phase, and in a greenhouse experiment, simulating practical conditions of transplantation in infected soil. Bioproducts were applied at manufactures' recommended doses, through different methods, in the two separate experimental conditions (Table 1 and 2).

In order to standardize the experimental conditions, the plant growth substrate (PGS) was a mixture of peat moss, sand and vermiculite (2:1:1 v:v:v), while the transplantation soil mixture (TSM) was a mixture of loamy soil, peat moss and sand (2:1:1 v:v:v). These substrates were heat-treated at 120°C for 30 min on two consecutive days then cooled. The substrates were inoculated with FSC7 at the rate of 60 cfu g^{-1} of PGS at sowing time (growth chamber experiment), and at the same rate for TSM at transplantation time (greenhouse experiment). The rate of 60 cfu g-1 was chosen after preliminary experiments, since it caused a high disease pressure, allowing at the same time an evaluation of the different efficacy of bioproducts. The inoculum was obtained by growing FSC7 in flasks containing moistened seeds (90–95% relative humidity) of wheat and millet (2:1 w:w). The flasks were autoclaved at 120°C for 30 min on two consecutive days, then inoculated separately with 5-cm disks cut from 9-day-old cultures of FSC7. After 2 weeks of incubation at 25±2°C in the dark, the content of the flasks was air-dried in a sterile flow cabinet at room temperature, finely ground with a hand mixer and maintained at 4°C until use. Seeds of zucchini cv. Giambo F1 (Semencoop s.r.l, Italy) were first surface-disinfected with sodium hypochlorite solution (NaOCl, 2% available chlorine) for 5 min, rinsed twice in sterile distilled water and dried on sterile paper in a laminar flow cabinet. Experiments were laid out in completely randomized designs with four replicates of each treatment. Each experiment was repeated twice.

Growth chamber experiment

The effects of bioproducts against FSC7 were studied in a growth chamber at 25±1°C, 84–88% relative humidity, 12-h photoperiod (5000 lux). Bioproducts were applied at sowing time by three methods, to seed (S), to PGS or a combination of both (S+PGS) (Table 2). Seeds were sown in 22 × 10 × 10 cm trays (260 g of PGS each) at the rate of 20 seeds per tray. For seed treatment, seeds were immersed in a suspension of each product in sterile distilled water for 24 h; this period was sufficient for microorganism spores to germinate and for seeds to pre-germinate absorbing all the water. Seeds immersed in water for 24 h served as control. Non-inoculated controls did not show any disease symptom. For PGS treatment, 100 mL of each product suspended in water were applied to each tray. For seed plus PGS treatments, a combination of the two described treatments was carried out.

Table 1. Bioproducts, active microbial ingredients (antagonists) and their abbreviations, and dosages used in biocontrol activity and rhizosphere colonization assays.

Bioproduct (Company)	Active microbial ingredient	Abbreviation	Bioproduct dosage	
			per g of seed	in PGS and TSM[a]
Cedomon, (BioAgri-Blu Line, Sweden)	*Pseudomonas chlororaphis* MA342, 10^9–10^{10} cfu mL^{-1}	PC-MA342	0.05 mL	10%
Mycostop (Verdera OY, Finland)	*Streptomyces* sp. K61[b], 10^8 cfu g^{-1}	SG-K61	8 mg	20 mg m^{-2}
Remedier (Isagro, Italy)	*Trichoderma asperellum* ICC012, 5×10^7 cfu g^{-1} and *Trichoderma gamsii* ICC080, 5×10^7 cfu g^{-1}	TA- ICC012 + TG- ICC080	1 mg	0.25 g m^{-2}
Rootshield WP (Bioworks, USA)	*Trichoderma harzianum* T22, 1×10^7 cfu g^{-1}	TH-T22	3 mg	1.5 g m^{-2}
Proradix®Agro (Sourcon-Padena, Germany)	*Pseudomonas* sp. DSMZ13134, 5×10^5 cfu g^{-1}	PS-DSMZ13134	0.2 mg	0.02%
Clonotry (Elep, Italy)	*Trichoderma harzianum* and *Clonostachys rosea*, total for both 1×10^9 cfu mL^{-1}	TH+CR	0.01 mL	0.2 mL m^{-2}

[a] PGS, plant growth substrate; TSM, transplantation soil mixture, as specified in Table 2.
[b] Formerly *S. griseoviridis* K61.

Table 2. Description and abbreviations of treatments with bioproducts applied in growth chamber and greenhouse experiments against *Fusarium solani* f. sp. *cucurbitae* race 1 (FSC7).

Experimental condition	Method of treatment with bioproduct	Time of treatment with bioproduct	Time of inoculation of FSC7[a]
Growth chamber[b]	Seed (S)	Sowing	Sowing
	Plant growth substrate[c] (PGS)	Sowing	Sowing
	Seed + plant growth substrate (S+PGS)	Sowing	Sowing
Greenhouse[c]	Plant growth substrate (PGS)[d]	10 days after sowing	Transplant
	Transplantation soil mixture (TSM)[e]	17 days after sowing (time of transplant)	Transplant
	Plant growth substrate + transplantation soil mixture (PGS+TSM)	10 + 17 days after sowing	Transplant

[a] FSC7 was inoculated in PGS or TSM. Inoculation consisted in 60 cfu g^{-1} PGS or TSM.
[b] Plants were grown for 21 days then evaluated for disease symptoms.
[c] Plants were grown in nursery for 17 days, then transplanted and maintained for further 21 days until disease symptom evaluation.
[d] Plant growth substrate (PGS): peat moss, sand and vermiculite (2:1:1 vol/vol/vol).
[e] Transplantation soil mixture (TSM): loamy soil, peat moss and sand (2:1:1 vol/vol/vol).

Three weeks after sowing/inoculation, the plants were carefully removed from PGS and washed under tap water. The severity of disease was determined by evaluating foot and root rot visually on a scale of 0 to 100, where: 0 = no symptoms; 5 = slight browning of roots; 20 = moderate browning of foot and roots; 50

= severe browning of foot and roots; 100 = plants totally wilted and dead (modified from Srinivastan *et al*., 2009). Samples of root/foot 1–2 cm long of the most representative plants were surface disinfected with 5% NaOCl, rinsed in sterile distilled water and transferred to Petri dishes containing PDA supplemented with 60 mg L^{-1} of streptomycin sulphate (Sigma - Aldrich Co.). After incubation at 25°C in the dark for 4 d, then for 6 d at 12 h under fluorescent lamps and 12 h darkness, the presence or absence of FSC from zucchini tissues was examined using a light microscope (Carl Zeiss mod. ZM, Germany) at ×500 magnification.

Greenhouse experiment

The microbial products were applied to the PGS in nursery phase, to the TSM at transplantation time and in both ways. Seeds were sown in alveolate polystyrene trays containing the PGS, placed in a growth room at 25±1°C, 85–90% relative humidity, 12 h photoperiod (5000 lux) and watered as required. Trays were assembled in two groups: one received treatments with each product in PGS 10 d after sowing and the other received water only. Products were applied at the same doses used in growth chamber experiment. Seven d after the treatment, all plants were transplanted in 40 × 15 × 17 cm plastic containers each containing a drainage layer 1 cm depth of expanded clay. The TSM inoculated with FSC7 was placed on the layer and treated or not with bioproducts. Thus, three groups of plants were established: 1) plants treated with single bioproducts only in PGS; 2) plants treated with single bioproducts only in TSM; or 3) plants treated with bioproducts both in PGS and TSM (Table 2). Inoculated controls received the same volume of sterile distilled water. In non-inoculated controls, plants did not show any disease symptom. Each treatment and the controls consisted of 25 plants per container, with four replicates. Plants were placed in a greenhouse at 25±2°C, 70% relative humidity, under daylight conditions. Three weeks after planting, the symptoms of disease caused by the pathogen were evaluated by examining the intensity of browning on foot and roots, using the procedure and severity score described above.

Rhizosphere and root colonization

Rhizosphere colonization ability of antagonists was tested by the method of Ahmad and Baker (1987), with modifications. Seeds were first surface-disinfected with NaOCl for 5 min and rinsed twice in sterile distilled water. PGS was sterilized as above. The treatments with antagonists were performed on seed or PGS at the doses shown in Table 2. Sterile water was applied to the untreated controls. Three experiments were carried out: in the first, treated seeds were sown in sterile PGS; in the second, seeds were sown in PGS which received treatment at seeding time; and in the third, seeds were seeded in PGS which received the treatment (5 mL of a suspension of antagonist spores in sterile water) 7 d after seeding. All seeds were sown in sterile plastic tubes 10 cm long (1 cm diam.) longitudinally sliced, fastened with rubber bands, and filled with moistened PGS. Tubes were placed vertically, five per each plastic pot containing moistened sterile sand. The pots, four for each treatment and for the untreated controls, were covered with sterile plastic bags and incubated in a growth chamber (25±1°C, 12 h photoperiod, 5000 lux). After 19 days of incubation, all tubes were opened and roots were carefully removed from the PGS. Loosely adhering substrate was shaken off, then the roots with their adhering rhizosphere PGS were air-dried under a 100 W lamp for 40 min. Each root was transferred to a 50 mL flask containing 10 mL of sterile distilled water. Flasks were vigorously shaken and the numbers of cfu g^{-1} rhizosphere soil of each inoculated microorganism were determined by a dilution-plating method, on PDA for fungi and KMB for bacteria. Ten replicates per treatment were made and experiments were repeated twice. The inoculum density applied was determined per g of seed on treated seeds before the sowing and per g of PGS as soon as treatment was applied, by plating serial dilutions of seeds and PGS on PDA and KB medium, for fungi and bacteria respectively.

The soil-free roots of seedlings grown for 12 days after treatments were also tested to check whether the microorganisms colonized root tissues. Roots were washed and disinfected with NaOCl and ethanol (Petrini *et al*., 1992) then plated and incubated as described above. Plates were microscopically observed for the development of each microorganism for fungi and SG-K61, and using UV trans-illumination (312 nm (Bio-Rad)) for PC-MA342 and PS-DSMZ13134 since these two organisms produced fluorescent pigments.

Dual culture assays

The effect of each antagonist, PC-MA342, PS-DSMZ13134, SG-K61, TA-ICC012, TG-ICC080 and

TH-T22, on the growth of FSC7 was studied in dual culture assay on Petri dishes (90 mm diam.) containing 20 mL PDA. In each dish, two 2 mm diam. colonial disks, one from antagonistic colonies and the other from FSC7 colony, were placed on the agar surface 40 mm apart on opposite sides, with control consisting of the pathogen strain alone. Plates were incubated in darkness at 25±1°C. Antagonistic efficacy was ascertained by the presence of a zone of inhibition between the two microorganisms, by the percent inhibition of radial growth (PIRG) of the pathogen and by the degree of overgrowth of the antagonist on the pathogen colony. Zones of inhibition, i.e. clear zones between the margins of the two microorganisms, were measured after 5 d. PIRG was calculated using the formula: PIRG = $[(R_1–R_2) / R_1] \times 100$, where R_1 and R_2 are the largest and the smallest radii of FSC7 colony, respectively (Manandhar *et al.*, 1987); in detail, R_2 is the radius, along the line which connects the two inoculated disks and R_1 is the radius which forms an angle of 90° with R_2. For each antagonist-pathogen combination, the colonization of substrate by antagonists was also evaluated using the method of Camporota (1985) with the formula $(D_1 \times 100)/D_2$, where D_1 is the distance covered by antagonist along the line which connects the two inoculated disks and D_2 is the distance between the two disks (40 mm). This measure represents the competitive capacity of an antagonist. Five replicates were prepared for each pairing. The experiment was repeated twice.

Antibiosis assays

The antagonists were also tested for their capacity to produce non-volatile metabolites effective against mycelial growth of FSC7, using the method of Dennis and Webster (1971). They were grown on a sterile cellophane disc on PDA (4%) in 9 cm diam. Petri dishes for 48 h, then the cellophane with the fungal mycelium or bacterial colony was removed and in the same position where each antagonist was grown, a mycelial plug 4 mm diam. of FSC7 was inoculated. Radial growth of the pathogen colonies was determined after 24 and 72 h of incubation in darkness at 25±1°C, and compared with those of the pathogen grown on PDA without metabolites (controls). Five replicates for each combination antagonist-pathogenic strain and for the controls were considered. The experiment was repeated twice.

Enzyme assays

In order to assess the production of enzymes by the antagonists involved in the degradation of chitin, the main component of fungal pathogen cell wall, the activities of endochitinase and of two exochitinases, β-N-acetylhexosaminidase and chitin 1,4-β-chitobiosidase, were assayed. FSC7 was inoculated (10^6 cfu mL^{-1}) into 500 mL flasks with 200 mL of 2% malt extract and incubated at 25±2°C on a horizontal shaker for 7 d. The mycelium was then collected by filtration through coarse muslin, washed under tap water until the filtrate was clear, lyophilized and ground to fine powder with mortar and pestle to obtain a crude cell wall powder (Chet *et al.*, 1967; Elad *et al.*, 1982). Crude cell wall powder (30 mg d wt) was suspended in 250 mL of synthetic medium containing (g l^{-1}): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002; Mn^{2+}, 0.002; Zn^{2+}, 0.002; in sterile distilled water, pH 6.3, prepared according to Okon *et al.* (1973) and autoclaved at 121°C for 30 min. The antagonists, grown on PDA and KMB plates for fungi and bacteria respectively, were inoculated (10^8 conidia mL^{-1}) into the flasks with cell wall powder of the pathogen and incubated on a horizontal shaker at 180 rpm for 4 d at 25°C (Harman *et al.*, 1993). Cultures from each flask were filtered, using a vacuum pump, through Whatman No. 1 filter paper and the filtrates were supplemented with various specific substrates to test enzymatic activities. To quantitify β-*N*-acetylhexosaminidase, chitin 1,4-β-chitobiosidase and endochitinase, the release of *p*-nitrophenol from the chromogenic substrates *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-*N*,*N'*-diacetylchitobiose and *p*-nitrophenyl-β-D-*N*,*N'*,*N''*-triacetylchitotriose (all from Sigma), respectively, was determined spectrophotometrically in 50 mM potassium phosphate buffer (pH 6.7) at 50°C, following methods of Ohtakara (1988), Roberts and Selitrennikoff (1988) and Tronsmo and Harman (1993). These enzyme activities were expressed as units (U) mL^{-1} of growth culture medium. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol min^{-1} under the specified conditions. Each enzyme activity assay was performed twice in triplicate.

Statistical analyses

Data were first examined for distribution normal before analysis of variance (ANOVA), and percent-

age values were arcsine transformed before ANOVA. Two-way ANOVA was applied to test the main effects and interaction of bioproduct and method of treatment against the disease scores, both from the growth chamber and greenhouse experiments. One-way ANOVA was applied to test the effect of antagonists against FSC7 in dual culture and for antibiosis and enzyme assays. Duncan's Multiple Range Test was used to separate the means. The software Statgraphics Plus 2.1, and statistical significance at $P<0.05$ was used. Since repeated experiments showed similar trends and there were no significant differences between them, the data were combined for analyses.

Results

Biocontrol activity

Growth chamber experiment

The effects of the bioproducts applied with three methods on the severity of disease caused by FSC7 in the growth chamber experiment are shown in Table 3. Non inoculated control did not show any disease symptom (data not shown). Disease symptoms were observed as browning of foot and roots of plants. The 2-way analysis of variance showed a significant interaction ($P<0.05$) between bioproducts and methods of treatment. All products, except Clonotry applied by all methods of treatment, and Cedomon and Mycostop applied to PGS, decreased disease severity compared with inoculated controls. The most effective products were Cedomon from seed application (39.7% disease reduction), Rootshield in PGS treatment (25.8% reduction), Cedomon (43.1% reduction) and Proradix (36.4% reduction) in S+PGS treatment. Concerning the comparison of methods, with the exception of Clonotry, disease severity values obtained from the S+PGS treatments were less than those in each single treatments, although only for Proradix and Remedier were these values statistically significant. A synergistic effect was noted from the combination of the two methods of treatment for these two products. On the contrary, no significant difference among methods of treatment was noted for Rootshield. A similar result was observed from the repeat experiment.

Greenhouse experiment

The effects of bioproducts against FSC7 in the greenhouse experiment are shown in Table 4. Clonotry was not included in this experiment, because it was ineffective in growth cabinet experiment. Signif-

Table 3. Effect of bioproducts applied to seed (S), to plant growth substrate (PGS) and to both (S+PGS) on the disease severity [a] of zucchini caused by *Fusarium solani* f. sp. *cucurbitae* strain 7 (FSC7) inoculated in PGS, in growth chamber experiment.

Bioproduct	S	PGS	S+PGS
Cedomon (PC-MA342)	24.1a A	38.7 c B	23.0 a A
Clonotry (TH + CR)	39.7 e A	39.6 c A	39.7 c A
Mycostop (SG-K61)	29.8 bc A	39.0 c B	28.6 b A
Proradix (PS-DSMZ13134)	28.7 ab B	35.1 b C	25.7 a A
Remedier (TA-ICC012 + TG-ICC080)	35.0 d B	34.7 b B	29.6 b A
Rootshield (TH-T22)	31.0 cd A	29.3 a A	28.9 b A
Inoculated control [b]	40.0 e A	39.5 c A	40.4 c A

[a] Disease severity was calculated on a scale of 0 to 100. Each value represents the mean of two independent experiments. Means within columns followed by the same lowercase letters are not significantly different, Duncan's Multiple Range Test ($P<0.05$). Means within rows followed by the same uppercase letters are not significantly different, Duncan's Multiple Range Test ($P<0.05$).
[b] Control and bioproduct-treated plants were inoculated with 60 cfu g^{-1} PGS of FSC7 at sowing time.

icant interactions were found between bioproducts and methods of treatment ($P<0.05$). Plants were very sensitive to FSC7 as shown in the controls (disease severity 62.3–64.0%). All bioproducts reduced the disease severity when applied to PGS and to PGS+TSM with respect to the inoculated controls. The Cedomon PGS treatment (42.4% disease reduction cf. inoculated controls) and Cedomon PGS+TSM (59.5% reduction) were the most effective against the disease. In the TSM treatment, only Mycostop significantly reduced the disease (4.1% reduction). All products were more effective when applied both in PGS and TSM.

Rhizosphere and root colonization

All antagonists applied to seed and to PGS were recovered from the rhizosphere at the end of experiments, at different concentrations depending on the microorganisms (Table 5). In all experiments colonies of TH+CR, the microbial ingredients of Clonotry, were found at very low levels. From seed treatment, the population densities of PS-DSMZ13134 and PC-MA342 were greater ($+0.9_{Log}$ and $+0.7_{Log}$ cfu g rhizosphere PGS^{-1} respectively) than the inoculum density applied to the seed. Small decreases in population density were observed for SG-K61, TA-ICC012+TG-ICC080 and TH-T22. In PGS treatment, increases in population densities of PS-DSMZ13134, TA-ICC012+TG-ICC080 and TH-T22 were observed, whereas decreases of population densities of PC-MA342 and SG-K61 were noted; the decrease was particularly consistent for PC-MA342 (-4.3_{Log} g rhizosphere PGS^{-1}). From the PGS treatment 7 d after sowing, all microorganisms, except TH+CR, were recovered in greater amounts in the rhizosphere at the end of the experiment (from +1.1 to $+1.7_{Log}$ g^{-1} rhizosphere PGS).

Microscopic observations of portions of roots plated on WA from PGS treatments, which received the treatment 7 d after sowing, plated on water agar (1.6% Difco) revealed the presence of abundant mycelia and conidia of *Trichoderma* spp., the microbial ingredients of Remedier and Rootshield, and of SG-K61. Roots that received treatments with microbial products based on PS-DSMZ13134 and PC-MA342 showed the presence of bacterial colonies on their surfaces, evidenced by fluorescent pigments (data not showed).

Dual culture assays

All the antagonists significantly reduced the radial growth of FSC7 ($P<0.05$). The greatest growth inhibition was obtained with the bacterial strains SG-K61 (PIRG 77.6%), PS-DSMZ13134 (66.5%) and PC-MA342 (59.5%); *Trichoderma* strains inhibited FSC7

Table 4. Effect of bioproducts applied to plant growth substrate (PGS), to transplantation soil mixture (TSM) and to both (PGS+TSM) on the disease severity[a] of zucchini caused by *Fusarium solani* f. sp. *cucurbitae* strain 7 (FSC7) inoculated in TSM, in greenhouse experiment.

Bioproduct	PGS	TSM	PGS+TSM
Cedomon (PC-MA342)	35.9 a B	62.0 ab C	25.9 a A
Mycostop (SG-K61)	57.3 d B	60.5 a B	46.5 c A
Proradix (PS-DSMZ13134)	47.8 b B	63.0 b C	36.1 b A
Remedier (TA-ICC012 + TG-ICC080)	52.3 c B	62.1 ab C	47.6 c A
Rootshield (TH-T22)	57.5 d B	63.2 b B	53.0 d A
Inoculated control[b]	62.3 e A	63.1 b A	64.0 e A

[a] See Table 3
[b] Control and bioproduct-treated plants were inoculated with 60 cfu g^{-1} TSM of FSC7 at transplant.

Table 5. Inoculum density (ID) and population density (PD) of the antagonists applied to seed (S treatment) and to plant growth substrate (PGS treatment). In S treatment, seeds were planted in non treated PGS; in PGS treatment, non treated seeds were planted in PGS that received the treatment at sowing time and seven days after sowing.

Antagonist[a]	S treatment		PGS treatment			
			Sowing time		Seven days after sowing	
	ID g seeds^{-1} [b]	PD g rhizosphere PGS^{-1} [c]	ID g non-rhizosphere PGS^{-1} [b]	PD g rhizosphere PGS^{-1} [c]	ID g non-rhizosphere PGS^{-1} [b]	PD g rhizosphere PGS^{-1} [c]
PC-MA342	8.2 ± 1.4 [d]	8.9 ± 1.3	7.6 ± 2.1	3.3 ± 1.9	7.5 ± 1.9	8.9 ± 2.0
PS-DSMZ13134	1.9 ± 1.1	2.8 ± 1.9	1.3 ± 0.9	1.7 ± 0.7	1.5 ± 0.7	3.2 ± 1.3
SG-K61	4.9 ± 2.3	4.7 ± 1.1	2.0 ± 0.7	1.1 ± 1.0	2.0 ± 1.4	3.2 ± 1.9
TH-T22	4.2 ± 1.9	4.1 ± 1.5	2.9 ± 1.3	3.2 ± 1.4	2.9 ± 1.3	4.0 ± 1.8
TH + CR	1.7 ± 1.0	0.1 ± 0.4	1.6 ± 2.0	0.8 ± 1.5	1.4 ± 1.1	0.7 ± 1.0
TA-ICC012 + TG-ICC080	4.9 ± 2.6	3.7 ± 1.5	2.4 ± 1.6	2.8 ± 1.3	2.7 ± 1.1	3.9 ± 1.3

[a] Active microbial ingredients of Cedomon, Proradix, Mycostop, Rootshield, Clonotry and Remedier, respectively.
[b] ID was determined as colony forming units (cfu) of antagonists by serial dilutions from treated S and PGS soon after the treatment.
[c] PD was determined as for ID, but at the end of the experiment.
[d] All data are expressed as Log cfu g seed^{-1} or Log cfu g PGS^{-1}, and represent means of two independent experiments ± standard deviation.

colonies from 31.6 (TH-T22) to 53.4% (TA-ICC012). Moreover, clear zones of inhibition between antagonistic bacterial strains SG-K61 (mean zone size = 28.2 mm), PS-DSMZ13134 (26.0 mm) and PC-MA342 (22.8 mm) and FSC7 were observed. No inhibition zones between fungal antagonists and the pathogen strain were noted. Observations by eye revealed that the inhibiting activity of *Trichoderma* spp. (TA-ICC012, TG-ICC080, TH-T22) consisted of an initial deadlock at mycelial contact followed by partial or complete replacement of pathogen by the antagonistic fungi. TA-ICC012 and TG-ICC080 partially overgrew the colonies of the pathogen, while TH-T22 completely overgrew the pathogen. Furthermore, as shown in Table 6, the three strains of *Trichoderma* spp. exhibited the greatest substrate colonization: 70.0, 58.9 and 53.0% for TH-T22, TA-ICC012 and TG-ICC080, respectively. The substrate colonization by bacteria was very scarce compared to that by fungi: 18.0% for SG-K61 and 10.0% for both PS-DSMZ13134 and PC-MA342.

Microscopic observations (magnification of ×250) of the interaction areas between antagonistic fungi and pathogen revealed some coilings and hooks produced by the antagonists on pathogen hyphae. Antagonistic bacteria caused abnormal morphology of the pathogen, such as swollen hyphal tips and lysis of hyphae in the deadlock area (not shown).

Antibiosis assays

In the antibiosis assays, all antagonists showed the production of non-volatile metabolites which significantly inhibited the mycelial radial growth of the pathogen with respect to the control (P<0.05). Metabolites of bacteria PC-MA342, PS-DSMZ13134 and SG-K61 reduced the radial growth of the pathogen significantly and similarly (67.3%, 70.7% and 70.2%, respectively). The fungal metabolites from TA-ICC012, TG-ICC080 and TH-T22 were less effective than those of bacteria; nevertheless, they caused considerable reductions of pathogen radial growth: TA-ICC012 reduced radial growth by 42.7%, TH-T22 by 29.8% and TG-ICC080 by 29.6%. Microscopic observations of margin areas of pathogenic hyphae, grown in the presence of bacterial metabolites, revealed abnormal morphology (swollen hyphal tips and lysis), as noted in dual culture assays. This effect was also observed for fungi, although to a far lesser extent.

Moreover, bacteria and fungi completely inhibited macro- and micro-conidium formation of FSC7 in our experimental conditions. However, the reversibility of these effects depended on the antagonist. The pathogen was able to grow and to produce conidia again, as disks from colonies grown in the presence of metabolites of fungi and of PS-DSMZ13134 bacterium were transferred to fresh PDA plates. However, the pathogen transferred from colonies grown in the presence of PC-MA342 and SG-K61 metabolites, was able to grow, but did not produce conidia.

Enzymatic assays

All the antagonists produced enzymes with both exochitinase (β-N-acetylhexosaminidase and chitin 1,4-β-chitobiosidase) and endochitinase activity at various levels (Figure 1). Overall, values of exochitinase activities were greater than those of endochitinase activity. *Trichoderma* strains produced significantly greater β-N-acetylhexosaminidase activities than bacteria strains (Figure 1a). The bacterium SG-K61 was the most active producer of chitin 1,4-β-chitobiosidase among all the microorganisms. TG-ICC080 exhibited the least β-N-acetylhexosaminidase activity among the *Trichoderma* strains and the least chitin 1,4-β-chitobiosidase activity among all the microorganisms. PC-MA342 and PS-DSMZ13134 produced comparatively low activities of both enzymes. All *Trichoderma* strains and the bacterium SG-K61 showed appreciable endochitinase activities (Figure 1b). TA-ICC012 and TH-T22 produced the greatest endochitinase activity, whereas PC-MA342 and PS-DSMZ13134 gave the least.

Discussion

The present report is the first comparing the bioproducts Cedomon, Clonotry, Mycostop, Proradix®, Remedier and Rootshield in the control of *Fusarium* crown and root rot on cucurbits. Although the inoculation pressure used in the present experiments may have been much greater than that found in typical greenhouse conditions or in the field, the products exerted various levels of efficacy. The data obtained *in vivo* were consistent with the clear inhibitory effects exhibited by the antagonists on the growth of the pathogen in the *in vitro* assay, and with their colonization of the rhizosphere of zucchini plants. Rhizosphere colonization is an important characteristic of antagonists of soilborne pathogens. These pathogens, growing around host roots, constitute stable populations able to compete and act against pathogens through various mechanisms (Harman, 2000; de Weert and Bloemberg, 2007).

In the growth chamber experiment, products based on bacteria were more effective when applied to seed than to PGS. Our data suggest that under the experimental conditions used, the seed treatment gave greater rhizosphere colonization than that obtained from the PGS treatment at sowing time. Some reports on antagonists applied to seeds have stated that antagonists not only can protect the seeds but may also protect plants against root infecting pathogens, because they are initial colonizers of roots (Ahmand and Baker, 1987; Weller, 1988; Kortemaa *et al.*, 1994). The bacteria PC-MA342, SG-K61 and PS-DSMZ13134 applied to seeds showed good rhizosphere colonization and exerted some control of crown and root rot, but when applied to PGS at sowing time, they colonized the plant rhizospere only very scarcely, and were poorly or non-effective against the disease, under our experimental conditions. Our results from Mycostop (SG-K61) are in accordance with those of Kortemaa *et al.* (1997), who showed that the population density of SG-K61 in the rhizospheres of carrot and turnip rape, was greater than that in non-rhizosphere and in root-free sand. We deduce that the tested bacteria survived poorly in the PGS used in our experiments in the absence of plant roots. The secretion of organic compounds by plant root favours the growth of microbial populations in the rhizosphere, as emphasized by Montesinos *et al.* (2002) for plant growth promoting rhizobacteria. Moreover, different plant species are known to produce different quality and quantity of root exudates (Curl and Truelove, 1986), which influence root colonization (Weller, 1988). We think that the PGS used in our study probably had too little carbon, which is necessary for growth of bacteria and to allow competitive activity with the pathogen already present in the substrate at seeding time. On the contrary, Remedier and Rootshield were effective both after seed and PGS applications, probably because their fungal components can survive in PGS enough to colonize the zucchini rhizosphere. Moreover, the strains of *Trichoderma* contained in Remedier and Rootshield showed parasitization of pathogen hyphae *in vitro*, and maybe they were able to parasitize the pathogen also in the PGS before it

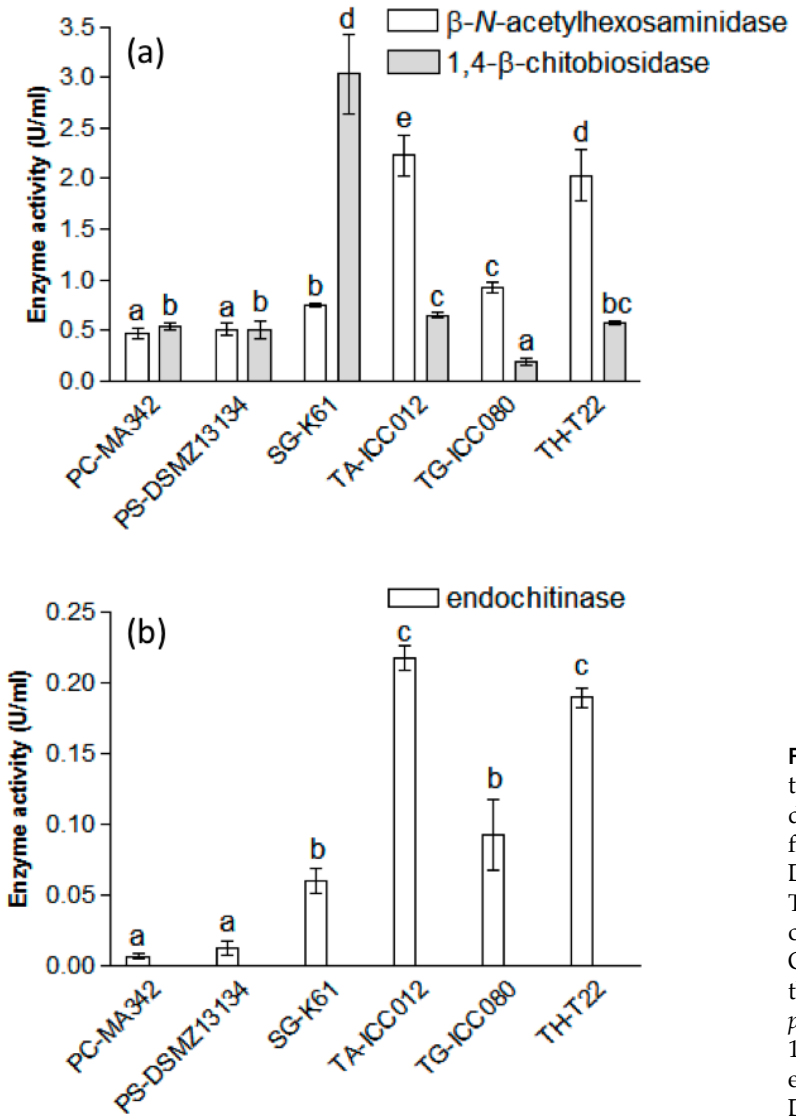

Figure 1. *β*-N-acetylhexosaminidase and chitin 1,4-*β*-chitobiosidase activities (a) and endochitinase activity (b), determined in culture filtrates of each antagonist, PC-MA342, PS-DSMZ13134, SG-K61, TH-T22, TA-ICC012 and TG-ICC080, grown for 7 d in synthetic medium containing cell wall of FSC as carbon source. One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1 ηmol of *p*-nitrophenol per minute. Data are averages ± 1SD. Columns with different letters within each enzymatic activity are significantly different by Duncan's Multiple Range Test (P<0.05).

attacked the plant roots. The antagonists contained in Clonotry also showed inhibition of FSC7 mycelial growth and parasitization of pathogen hyphae *in vitro* (data not shown), but they colonized rhizosphere scarcely. We can hypothesize that the lack of efficacy of Clonotry against the disease could be due to the poor rhizosphere colonization of zucchini by GR+TH in PGS. The synergistic effects of the two methods of application of Proradix or Remedier against the disease caused by FSC7 could be ascribed to the high concentration of antagonistic microorganisms required by the heavy disease pressure occurring in our experimental conditions.

In the greenhouse experiment, the bioproducts showed efficacy if applied in the PGS or in PGS+TSM. Poor or no effectiveness was obtained in the case of the TSM treatment; we emphasize that plants were not treated, thus not colonized by antagonists, before the transplantation and that the TSM was already infected by the pathogen at the time of transplantation. Our data showed that all the microorganisms contained in the bioproducts were able to colonize host rhizospheres when they were applied to the substrate in the presence of plants with roots, 7 d after sowing. Therefore, we think that young plants should be already colonized by antagonists before

pathogen inoculation in order to obtain effective disease control in our experimental conditions,. Since greenhouse experiments reflect practical conditions more than growth chamber experiments, we suggest that plants should be treated by drenching PGS at least 1 week before transplanting to better protect the plants from pathogen attack, which may occur after the transplantation.

Several mechanisms of action play essential roles in the biocontrol activity of microorganisms. Our study demonstrated the involvement of competition for space, parasitism and antibiosis due to non-volatile metabolites as modes of action of the organisms in the products tested. In dual plate assays, *Trichoderma* strains competed for space better than bacteria, growing first to the edges of FSC7 colonies and then overgrowing and parasitizing the pathogen hyphae with coilings and hooks, as frequently reported for *Trichoderma* spp. against other pathogens (Chet, 1987; Pisi *et al*., 2001; Innocenti *et al*., 2003). Bacteria, on the contrary, competed poorly for space, but produced zones of inhibition indicating that metabolites were secreted in the substrate, able to block pathogen colony growth. The specific test used to assay antibiosis revealed that all antagonists were able to produce metabolites which were active in blocking the development of pathogen hyphae, but bacteria produced much more effective metabolites than fungi. The antagonists also caused several morphological changes of pathogen hyphae, such as swollen tips, vacuolization and lysis. However, these effects were only fungistatic, since FSC7 colonies were able to grow when transferred in non-treated substrate. This suggests that the growth of antagonists in host rhizospheres and/or on root surfaces is important in order to continue the suppression of pathogen development. Therefore, in high disease pressure situations, such as that used in our experiments, two treatments with the bioproduct are necessary to give consistent antagonist populations and thus greatly reducing disease. According to Harman (2000), rhizosphere competent biocontrol agents can provide long-term protection, since they can establish on roots, grow with the developing root system, and remain functional for at least the life of annual crop plants.

The inhibition zone observed in dual culture experiment and the reduction of radial growth in the antibiosis assay suggest that PC-MA342 produces metabolites active against FSC7. Actually Linser *et al*. (2006) reported that the main mechanism of action of PC-MA342 is related to the production of the fungitoxic compound 2,3-deepoxy-2,3-didehydrorhizoxin. Moreover, Hohlneicher *et al*. (1995) proved that this bacterium, as other plant growth promoting rhizobacteria, produces siderophores that chelate iron. SG-K61 also produced metabolites very active in inhibiting FSC7 mycelial growth. It is known that *Streptomyces* species are producers of many metabolites (Anderson et al., 1956) and that the strain SG-K61 generates an aromatic heptaene polyene antibiotic involved in biocontrol of soil-borne fungi (Raatikainen *et al*., 1994). The antibiosis showed by PS-DSMZ13134 cannot be related to any specific metabolite; the literature focuses the role of this bacterium only in plant induced resistance and growth promotion. Several antifungal compounds, such as 2,4-diacetylphloroglucinol and hydrogen cyanide, were instead characterized as products of other species of fluorescent *Pseudomonas* and correlated with their biocontrol activity (Haas and Défago, 2005). Species of *Trichoderma* also produce a wide range of antibiotic compounds belonging to different chemical groups, such as alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines, sesquiterpenes and steroids, with roles in the biocontrol (Howell, 1998). It has been suggested that antibiosis and production of hydrolytic enzymes work together sinergistically in the parasitic action of *Trichoderma* (Di Pietro *et al*., 1993; Schirmböck *et al*., 1994).

Hydrolytic enzymes are thought to be closely related to the mycoparasitism. Chitinases have an important role in degrading pathogen cell walls, which are mainly composed by chitin (Viterbo *et al*., 2002). In the present study, the greatest levels of endochitinase and β-N-acetylhexosaminidase were produced by *Trichoderma* species, particularly TH-T22 and TA-ICC012. *Trichoderma* is a genus widely studied in biological control for the effectiveness of many of its members against several phytopathogens, due to their different mechanism of action that have been investigated by many researchers (Harman *et al*., 2004; Druzhinina *et al*. 2011). The roles of the various lytic enzymes of *Trichoderma* appear to be different, and enzymes with different or complementary modes of action appear to be required for maximal antifungal effects on different pathogens (Lorito *et al*., 1993, 1994; Roberti *et al*., 2002). Among *Trichoderma* strains tested, the level of β-N-acetylhexosaminidase was always greater than chitin 1,4-β-chitobiosidase, in accordance with the results obtained by Tronsmo and

Harman (1993) with cultures of *T. harzianum*. On the contrary, the bacterium SG-K61 produced the greatest chitin 1,4-β-chitobiosidase activity and among bacteria the greatest levels of the all three chitinases tested. Species of *Streptomyces* are able to produce chitinases with important roles both in degrading fungal cell walls (Beyer and Diekmann, 1985) and in decomposition of chitin in soil, and a medium based on chitin has been used to isolate *Streptomyces* strains from soil (Baxby and Gray, 1968). A study by Valois *et al*. (1996) established that the ability to degrade *Phytophthora* cell walls, as well as the production of fungitoxic metabolites are good markers. Since the two bacteria PC-MA342 and PS-DSMZ13134 secreted low levels of chitinases, we presume that the antibiosis discussed above may be an important mechanism against FSC7, even if other mechanisms of action cannot be excluded. In addition, rhizobacteria, such as *Pseudomonas* spp., are able to induce systemic resistance in plants against pathogens (Van Loon *et al*., 1998), as well as *Trichoderma* or other antagonistic fungi (Harman *et al*., 2004; Roberti *et al*., 2008b).

In conclusion, we have found that Cedomon, recommended for seed dressing only, is also effective if applied by drench PGS in the plant nursery phase, 1 week before the transplantation of seedlings in soil already infected by the pathogen, as demonstrated in the greenhouse experiment; it is important that growth of bacterium occurs in the rhizosphere where it produces antifungal metabolites able to inhibit pathogen growth. Also we have demonstrated efficacy of Proradix against the disease caused by FSC7, and we propose antibiosis as a mechanism of action against this pathogen in addition to the known induced systemic resistance (Von Rad *et al*., 2005). Finally, we have demonstrated the possibility to control FSC7 infection under experimental conditions very conducive for disease development. The decrease in disease severity from biological treatments appears to result from rhizosphere colonization by the microorganisms and from some mechanisms they exerted against the pathogen, such as competition, parasitism, production of chitinolytic enzymes and antifungal metabolites. We cannot exclude other mechanisms of biocontrol activity, such as production of glucanase enzymes, inactivation of the pathogen's enzymes and induction of plant resistance. Although further studies are necessary to verify the efficacy of the biocontrol agents under different management practices, this research establishes the potential of using the tested products for controlling root rot caused by *F. solani*, reducing the potentially adverse environmental effects of hazardous pesticides.

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Literature cited

Abeysinghe S., 2007. Biological control of *Fusarium solani* f. sp. *phaseoli* the causal agent of root rot of bean using *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01. *Ruhuna Journal of Science* 2, 82–88.

Ahmad J.S. and R. Baker, 1987. Rhizosphere competence of *Trichoderma harzianum*. *Phytopathology* 77, 182–189.

Anderson L.E., J. Ehrlich, S.H. Sun and P.R. Burkholder, 1956. Strains of *Streptomyces* the sources of azaserine, elaiomycin, griseoviridin and viridiogrisein. *Antibiotics and Chemotherapy* 6, 100–115.

Armengol J., C.M. José, M.J. Moya, R. Sales, A. Vicent and J. Garcia-Jiménez, 2000. *Fusarium solani* f. sp. *cucurbitae* race 1, a potential pathogen of grafting watermelon production in Spain. *OEPP Bulletin* 30, 179–183.

Baxby P. and T.R.G. Gray, 1968. Chitin decomposition in soil I: Media for isolation of chitinolytic microorganisms from soil. *Transactions of the British Mycological Society* 51, 287–292.

Beyer M. and H. Diekmann, 1985. The chitinase system of *Streptomyces* sp. ATCC 11238 and its significance for fungal cell wall degradation. *Applied Microbiology and Biotechnology* 23, 140–146.

Booth C., 1971. *The genus* Fusarium, Commonwealth Mycological Institute, Kew, Surrey, UK, 237 pp.

Boughalleb N., J. Armengol and M. El Mahjoub, 2005. Detection of races 1 and 2 of *Fusarium solani* f. sp. *cucurbitae* and their distribution in watermelon fields in Tunisia. *Journal of Phytopathology* 153, 162–168.

Camporota P., 1985. Antagonisme in vitro de *Trichoderma* spp. vis-à-vis de *Rhizoctonia solani* Kühn. *Agronomie* 5, 613–620.

Champaco E.R., R.D. Martyn and M.E. Miller, 1993. Comparison of *Fusarium solani* and *F. oxysporum* as causal agents of fruit rot and root rot of muskmelon. *Hortscience* 28, 1174–1177.

Chet I., 1987. *Trichoderma* - Application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: *Innovative Approaches to Plant Disease Control* (I. Chet, ed.), John Wiley Sons, New York, NY, USA, 137–160.

Chet I., Y. Henis and R. Mitchell, 1967. Chemical composition of hyphal and sclerotial walls of *Sclerotium rolfsii* Sacc. *Ca-*

nadian Journal of Microbiology 13, 137–141.
Curl E.A. and B. Truelove, 1986. *The Rhizosphere*, 1st edition. Springer-Verlag, Berlin, Heidelberg, Germany, 288 pp.
de Weert S. and G.V. Bloemberg 2007. Rhizosphere competence and the role of root colonization in biocontrol. In: *Plant Associated Bacteria* (S.S. Gnanamanickam, ed.), Springer, Dordrecht, Germany, 317–334.
Dennis C. and J. Webster, 1971. Antagonistic properties of species of groups of *Trichoderma*. I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society* 57, 25–48.
Di Pietro A., M. Lorito, C.K. Hajers, R.M. Broadway and G.E. Harman, 1993. Endochitinase from *Gliocladium virens*: isolation, characterization and synergistic antifungal activity in combination with gliotoxin. *Phytopathology* 83, 308–313.
Druzhinina I.S., V. Seidl-Seiboth, A. Herrera-Estrella, B.A. Horwitz, C.M. Kenerley, E. Monte, P.K. Mukherjee, S. Zeilinger, I.V. Grigoriev and C.P. Kubicek, 2011. *Trichoderma*: the genomics of opportunistic success. *Nature Reviews Microbiology* 9, 749–759.
Elad Y., I. Chet and Y. Henis, 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Canadian Journal of Microbiology* 28, 719–725.
El-Sheshtawi M., S. El-Afifi and M. El-Kahky, 2007. Efficacy of some biological agents in controlling pathogenic soilborne fungi infesting watermelon in Egypt. *IOBC/wprs Bulletin* 6, 27–32.
Fantino M.G., F. Fantuz and S. Gennari, 1989. Ricerche sul marciume della zucca causato da *Fusarium solani* f. sp. *cucurbitae*. *La difesa delle piante* 1/2, 147–154.
Haas D. and G. Défago, 2005. Biological control of soilborne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* 3, 307–319.
Harman G.E., 2000. Myths and dogmas in biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* 84, 377–393.
Harman G.E., C.K. Hayes, M. Lorito, R.M. Broadway, A. Di Pietro, C. Peterbauer and A. Tronsmo, 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology* 83, 313–318.
Harman G.E., C.R. Howell, A, Viterbo, I. Chet and M. Lorito, 2004. *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nature Reviews* 2, 43–56.
Hawthorne B.T., J. Rees-George and P.G. Broadhurst, 1992. Mating behavior and pathogenicity of New Zealand isolates of *Nectria haematococca* (*Fusarium solani*). *New Zealand Journal of Crop Horticultural Science* 20, 51–57.
Hohlneicher U., R. Hartmann, K. Taraz and H. Budzikiewicz, 1995. Bacterial constituents 62. Pyoverdin, ferribactin, azotobactin - a triad of siderophores from *Pseudomonas chlororaphis* ATCC 9446 and its relation to *Pseudomonas fluorescens* ATCC 13525. *Zeitschrift fur Naturforschung* 50, 337–344.
Howell C.R., 1998. The role of antibiosis in biocontrol. In: *Trichoderma and Gliocladium* (G.E. Harman, C.P. Kubicek, ed.), Taylor and Francis Ltd, London, UK, vol 2, 173–184.
Inbar J., A. Menendez and I. Chet, 1996. Hyphal interactions between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. *Soil Biology and Biochemistry* 28, 757–763.
Innocenti G., R. Roberti, M. Montanari and E. Zakrisson, 2003. Efficacy of micro-organisms antagonistic to *Rhizoctonia cerealis* and their cell wall degrading enzymatic activities. *Mycological Research* 107, 421–427.
Kortemaa H., H. Rita, K, Haahtela and A. Smolander, 1994. Root-colonization ability of antagonistic *Streptomyces griseoviridis*. *Plant and Soil* 163, 77–83.
Kortemaa H., T. Pennanen, A, Smolander and K. Haahtela, 1997. Distribution of antagonistic *Streptomyces griseoviridis* in rhizosphere and non-rhizosphere sand. *Journal of Phytopathology* 145, 137–143.
Linser A., L. Cazzara and G. Barbieri, 2006. Cedomon® (*P. chlororaphis* MA342) the first natural seed dressing fungicide for wheat and barley. In: *Atti Giornate Fitopatologiche* (A. Brunelli, A. Canova, ed.), vol. 2, Clueb Bologna, Italia, 27–34.
Lorito M., G.E. Harmann, C.K. Hayes, R.M. Brodway, S.L. Woo and A. Di Pietro, 1993. Chitinolitic enzymes produced by *T. harzianum*. Antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* 83, 302–307.
Lorito M., C.K. Hayes, A. Di Pietro, S.L. Woo and G.E. Harman, 1994. Purification, characterization and synergistic activity of a glucan 1,3-β-glucosidase and an N-acetylglucosaminidase from *T. harzianum*. *Phytopathology* 84, 398–405.
Manandhar J.B., R.N. Thapliyal, K.J. Cavanaugh and J.B. Sinclair, 1987. Interaction between pathogenic and saprobic fungi isolated from soybean roots and seeds. *Mycopathologia* 98, 69–75.
Martyn R.D., 1996. Fusarium Crown and Foot Rot of Squash. In: *Compendium of Cucurbits Diseases* (T.A. Zitter, D.L. Hopkins, C.E. Thomas, ed.), The American Phytopathological Society, St. Paul, MN, USA, 16–17.
Montesinos E., A. Bonaterra, E. Badosa, J. Frances, J. Alemany, I. Llorente and C. Moragrega, 2002. Plant-microbe interactions and the new biotechnological methods of plant disease control. *International Microbiology* 5, 169–175.
Nagao H., K. Sato and S. Ogiwara, 1994. Susceptibility of *Cucurbita* spp. to the cucurbit root-rot fungus *Fusarium solani* f. sp. *cucurbitae* race 1. *Agronomie* 2, 95–102.
Naseby D.C., J.A. Pascual and J.M. Lynch, 2000. Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* populations, soil microbial communities and soil enzyme activities. *Journal of Applied Microbiology* 88, 161–169.
Nelson P.E., T.A. Toussoun and W.F.O. Marasas (ed.), 1983. *Fusarium Species. An illustrated manual for Identification*. The Pennsylvania State University Press, University Park PA, USA, 193 pp.
O'Donnell K., 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia* 92, 919–938.
O'Donnell K., H.C. Kistler, E. Cigelnik and R.C. Ploetz, 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. In: *Proceedings of the National Academy of Sciences of the United States of America*, USA, 95, 2044-2049.
Ohtakara A., 1988. Chitinase activity. In: *Methods in Enzymology* (W.A. Wood, S.T. Kellogg, ed.), Academic Press, San Diego, 462–470.
Okon Y., I. Chet and Y. Henis, 1973. Effect of lactose, ethanol

and cycloheximide on the translocation pattern of radioactive compound and on sclerotium formation in *Sclerotium rolfsii*. *Journal of General Microbioogy* 74, 251–258.

Petrini O., P.J. Fisher and L.E. Petrini, 1992. Fungal endophytes of bracken (*Pteridium aquilinum*) with some reflections on their use in biological control. *Sydowia* 44, 282–293.

Pisi A., R. Roberti, E. Zakrisson, G. Filippini, W. Mantovani and A. Cesari, 2001. SEM investigation about hyphal relationships between some antagonistic fungi against *Fusarium* spp. foot rot pathogen of wheat. *Phytopathologia Mediterranea* 40, 37–44.

Raatikainen O.J., T.H. Päivinen and R.T. Tahvonen, 1994. HPLC separation and subsequent detection of aromatic heptaene polyenes in peat after treatment with *Streptomyces griseoviridis*. *Pesticide Science* 41, 149–154.

Roberti R., E. Zakrisson, F. Flamigni, L. De Vero and A. Cesari, 2002. Antagonistic fungi producing hydrolytic enzymes, active in degrading the cell wall of some foot rot pathogens (*Fusarium* spp.) of wheat. *Journal of Plant Diseases and Protection* 109, 101–108.

Roberti R, A. Veronesi and C. Coltelli, 2008a. Marciume del piede in espansione su zucchino. *L'Informatore Agrario* 30, 57–59.

Roberti R., A. Veronesi, A. Cesari, A, Cascone, I. Di Berardino, L. Bertini and C. Caruso, 2008b. Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. *Plant Science* 175, 339–347.

Roberts W.K. and C.P. Selitrennikoff, 1988. Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology* 134, 169–176.

Rojo F.G., M.M. Reynoso, M. Fererz, S.N. Chulze and A.M. Torres, 2007. Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Protection* 26, 549–555.

Schirmböck M., M. Lorito, Y.L, Wang, C.K. Hayes, I. Arisan-Atac, F. Scala, G.E. Harman and C.P. Kubicek, 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology* 60, 4364–4370.

Sherf A.F. and A.A. MacNab (ed.), 1986. *Vegetable diseases and their control*. 2th edition, John Wiley and Sons, New York, NY, USA, 728 pp.

Siddiqui I.A. and S.S. Shaukat, 2003. Combination of *Pseudomonas aeruginosa* and *Pochonia chlamydosporia* for control of root-infecting fungi in tomato. *Journal of Phytopathology* 151, 215–222.

Sivan A. and I. Chet, 1993. Integrated control of *Fusarium* crown and root rot of tomato with *Trichoderma harzianum* in combination with methyl bromide or soil solarization. *Crop Protection* 12, 380–386.

Snyder W.C., S.G. Georgopoulos, R.K. Webster and S.N. Smith, 1975. Sexuality and genetic behavior in the fungus *Hypomyces (Fusarium) solani* f. sp. *cucurbitae*. *Hilgardia* 43, 161–185.

Srinivasan K., G. Gilardi, A. Garibaldi and M.L. Gullino, 2009. Efficacy of bacterial antagonists and different commercial products against *Fusarium* wilt on rocket. *Phytoparasitica* 37, 179–188.

Stravato V.M. and C. Cappelli, 1996. Marciume pedale dello zucchino in provincia di Latina. *Colture Protette* 7/8, 75–77.

Tousson T.A. and W.C. Snyder, 1961. The pathogenicity, distribution, and control of two races of *Fusarium (Hypomyces) solani* f. sp. *cucurbitae*. *Phytopathology* 51, 17–22.

Tronsmo A. and G.E. Harman, 1993. Detection and quantification of N-acetyl-J-D-glucosaminidase, chitobiosidase and endochitinase in solutions and on gels. *Analytical Biochemistry* 208, 74–79.

Valois D., K. Fayad, T. Barasubiye, M. Garin, C. Dery, R. Brzezinski and C. Beaulieu, 1996. Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. *Applied and Environmental Microbiology* 62, 1630–1635.

Van Loon L.C., P.A.H.M. Bakker and C.M.J. Pieterse, 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36, 453–483.

Vannacci G. and P. Gambogi, 1980. *Fusarim solani* f. sp. *cucurbitae* razza 1 su semi di *Cucurbita pepo* L.: reperimento del patogeno e influenza di condizioni colturali sull'andamento della malattia. *Phytopathologia Mediterranea* 19, 103–114.

Veronesi A., C. Sigala and R. Roberti, 2009. Identification and characterization of *Fusarium solani* f. sp. *cucurbitae* race 1 on zucchini in the growing area of Bologna. *Micologia Italiana* 38, 24–33.

Vitale S., M. Maccaroni and A. Belisario, 2006. First report of zucchini collapse by *Fusarium solani* f. sp. *cucurbitae* race 1 and *Plectosporium tabacinum* in Italy. *Plant Disease* 91, 325.

Viterbo A., O. Ramot, O. Chernin and I. Chet, 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie van Leeuwenhoek* 81, 549–556.

Von Rad U., M.J. Mueller and J. Durner, 2005. Evaluation of natural and synthetic stimulants of plant immunity by microarray technology. *New Phytologist* 165, 191–202.

Weller D.M., 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* 26, 379–407.

White T.J., T.D. Bruns, S.B. Lee and J.W. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, ed), New York Academic Press, New York, NY, USA, 315–322.

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