J. Phytopathology **155**, 342–349 (2007)
doi: 10.1111/j.1439-0434.2007.01237.x

Department of Plant Biology, Faculty of Biology, University of Murcia, Espinardo, Spain

Biological Control of *Phytophthora* Root Rot of Pepper Using *Trichoderma harzianum* and *Streptomyces rochei* in Combination

M. Ezziyyani[1], M. E. Requena[1], C. Egea-Gilabert[2] and M. E. Candela[1]

Authors' addresses: [1]Department of Plant Biology, Faculty of Biology, University of Murcia, Campus de Espinardo, 30100 Espinardo (Murcia); [2]Department of Science and Agrarian Technology, ETS Ingeniería Agronómica, University Politecnic of Cartagena, Paseo Alfonso XIII, 48, 30203 Cartagena, Spain (correspondence to M. E. Candela. E-mail: mcandela@um.es)

Received July 19, 2006; accepted December 6, 2006

Keywords: biocontrol, pepper, *Phytophthora capsici*, root rot, *Streptomyces rochei*, *Trichoderma harzianum*, *Capsicum annuum*

Abstract

A combination of two compatible micro-organisms, *Trichoderma harzianum* and *Streptomyces rochei*, both antagonistic to the pathogen *Phytophthora capsici*, was used to control root rot in pepper. The population of the pathogen in soil was reduced by 75% as a result. Vegetative growth of the mycelium of *P. capsici* was inhibited *in vitro* on the second day after *P. capsici* and *T. harzianum* were placed on the opposite sides of the same Petri plate. *Trichoderma harzianum* was capable of not only arresting the spread of the pathogen from a distance, but also after invading the whole surface of the pathogen colony, sporulating over it. Scanning electron microscopy showed the hyphae of *P. capsici* surrounded by those of *T. harzianum*, their subsequent disintegration, and the eventual suppression of the pathogen's growth. *Streptomyces rochei* produced a zone of inhibition, from which was obtained a compound with antioomycete property secreted by the bacteria. When purified by high-pressure liquid chromatography, this compound was identified as 1-propanone, 1-(4-chlorophenyl), which seems to be one of the principal compounds involved in the antagonism. A formulation was prepared that maintained the compound's capacity to inhibit growth of the pathogen for up to 2 years when stored at room temperature in the laboratory on a mixture of plantation soil and vermiculite. The two antagonists, added as a compound formulation, were effective at pH from 3.5 to 5.6 at 23–30°C. The optimal dose of the antagonists in the compound formulation was 3.5×10^8 spores/ml of *T. harzianum* and 1.0×10^9 FCU/ml of *S. rochei*. This is the first report of a compound biocontrol formulation of these two antagonists with a potential to control root rot caused by *P. capsici*.

Introduction

Root rot in pepper (*Capsicum annuum*) caused by the oomycete *Phytophthora capsici* results in substantial losses worldwide. The disease is fatal; by the time the first symptoms appear, the tissues are already totally invaded and the plant dies within a few days. The problem is especially serious when the pathogen becomes established in greenhouse soils on which bell pepper cultivars, which are extremely sensitive to *P. capsici* and of high commercial value, are grown. As no bell pepper cultivars resistant to this pathogen have been reported so far, fumigating the soil with methyl bromide has been the only means of controlling the pathogen. However, methyl bromide was banned in 2005. Our research group has been greatly successful in controlling the root rot caused by *P. capsici* in pepper through biological means (Sid Ahmed et al., 1999, 2003; Ezziyyani et al., 2004). However, despite several trials to identify the most suitable dose, no single biocontrol agent has proved capable of inhibiting the infection totally, which reflects the findings of other groups (Bora et al., 2004; Akköprü and Demir, 2005). The solution may be to use combinations of biocontrol agents, especially when they exhibit complementary modes of action, a supposition which has proved correct in several combinations, each involving a bacterial species and a fungal species (Elad et al., 1994; Guetsky et al., 2002; Szczech and Shoda, 2004, 2006; Li et al., 2005). The present study describes one such attempt, which involved *T. harzianum*, a fungus, and a bacterial isolate, *S. rochei*, obtained from the rhizosphere of pepper plants that exhibited antagonistic activity towards *P. capsici*.

Materials and Methods

Plant materials and pathogen

Disinfected seeds of *C. annuum* L., cv. California Wonder, were sown in $4 \times 4 \times 14$ cm pots filled with a potting mixture consisting of one part vermiculite, two parts sand and six parts autoclaved peat (by volume). The pots were placed in a growth chamber and maintained at 21 ± 3°C, 78% relative humidity and a 12-h

photoperiod. After 3 months, seedlings were transferred to larger pots and/or soil where they were treated. The causal agent of pepper root rot, *P. capsici* isolate 15, obtained from our culture collection, was maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25 ± 2°C (Candela et al., 1995). Roots of pepper seedlings were inoculated with the stock cultures of *P. capsici*, the pathogen was re-isolated from surface-sterilized root tissues, and a monoconidial isolate used to produce fresh inoculum for the experiment.

Biocontrol agents

Trichoderma harzianum was used as the fungal partner in the biocontrol agent. The isolate, no. 2413, came from the Colección Española de Cultivos Tipo (CECT), Valencia, Spain and was maintained in the dark on potato dextrose broth (PDB; Merck, Darmstadt, Germany) at 4°C. *Streptomyces rochei* was the bacterial partner, isolated by our group from the rhizosphere of a healthy plant of pepper grown in Murcia, Spain and selected from 467 isolates of bacteria associated with roots of pepper and tested for both *in vitro* antagonistic activity towards *P. capsici* and synergism with *T. harzianum*. This particular isolate was named *S. rochei* 'Ziyani', and its cultures were maintained on nutrient agar (NA; Merck).

Production and storage of inoculum

The inoculum of *P. capsici* was grown in vermiculite–PDB medium obtained by adding 300 ml of PDB to a 1-l Erlenmeyer flask containing 150 g sterilized vermiculite. This mixture was autoclaved and then seeded with five discs (5 mm diameter) cut from the edge of an actively growing colony of *P. capsici*. The initial oomycete population in the vermiculite–PDB medium was counted as described by Sid Ahmed et al. (1999) and expressed as propagules per gram (ppg). The inoculum of *T. harzianum* was prepared in vermiculite–Avy3 medium (Sid Ahmed et al., 1999) in the same way as that of *P. capsici*: discs 3 mm in diameter cut from the edge of a colony of *S. rochei* actively growing on NA were added to 250-ml Erlenmeyer flasks containing 150 ml of sterilized nutrient broth (NB; Merck). The bacterial colonies thus obtained were separated from the medium and transferred to 1000-ml Erlenmeyer flasks containing 150 g of vermiculite and 200 ml of NB to help conserve them. All flasks were incubated at 25°C for 2 weeks with the cultures being shaken at 120 rpm.

***In vitro* experiments**

To check the feasibility of using the two micro-organisms jointly, they were grown together and also along with *P. capsici*. To measure the degree of antagonism, two discs, each 5 mm in diameter, one covered with actively growing mycelium of *P. capsici* and one with either of the two organisms antagonistic to it, were placed 6 cm apart on either side of a Petri dish. *Trichoderma harzianum* was grown on PDA enriched with laminarine and glucose (3 : 1, v/v) and *S. rochei* on NA. The Petri dishes (three in each set) were incubated at 23, 25, 27 and 30°C for 72 h. Antagonism was verified in two ways: (i) by directly observing the Petri dishes and measuring the diameters of the colonies and the presence (+) or absence (−) of an inhibition zone between the antagonists and the pathogen and (ii) by scanning electron microscopy (SEM) of the zone of interaction.

Scanning electron microscopy

To observe the interaction between the antagonists themselves and between them and the pathogen, 0.5 cm pieces of agar were cut from the *in vitro* interaction zones. The pieces were fixed first in glutaraldehyde and then in 1% osmium tetroxide, dehydrated with ethanol, mounted on a pedestal with graphite-conductive paint, and the splutter covered with gold. A Jeol T-6.100 microscope (Freehold, NJ, USA) was used for observing the samples.

Isolation and detection of the antioomycete substance produced by *S. rochei*

Five discs taken from 7-day-old cultures of *S. rochei* growing on NA were placed in 1 l of NB. The broth was incubated at 28°C on a rotary shaker at 130 rpm for 20 days and then centrifuged at $1300 \times g$ for 30 min. The supernatant was collected by filtering through a Whatman no. 2 filter paper and mixed with 2 : 1 n-buthanol : ethyl acetate mixture (50 : 50, v/v). The organic phase was evaporated to dryness and the residue suspended in 5.0 ml of methanol. These crude extracts were filtered through 0.2-μm syringe Millipore filters (MILLEX-HV 0. Bedford, MA, USA) and kept in sterile Eppendorf tubes at 4°C until use.

The extraction products were separated by thin layer chromatography (TLC) on silica gel 60 plates (20 × 20 cm, layer thickness 0.25 mm; Merck) and allowed to develop for 35 min in a rectangular TLC tank with chloroform : methanol (70 : 30) elution solvent. The antagonistic activity towards *P. capsici* was examined by the following inhibition test made on a 2.5-cm-wide lane at the edge of the TLC plate. For this, the TLC strip was impregnated with 10 ml of molten PDA and, while it was still liquid (when it reached a temperature of 45°C), the strip was completely covered with 1 ml of a 10^9 FCU/ml spore suspension of *P. capsici*. When solidified, the TLC strip was placed in a sterile glass moisture chamber and incubated at 25 ± 2°C for 3 days before calculating the R_f (retention factor) of the pathogen inhibition zone. In the rest of the chromoplate, the medium that made up the inhibition zone was scraped off with a spatula and eluted with 5 ml ethanol. It was then filtered through 0.45-μm syringe Millipore filters, concentrated to 1 ml with N_2, and kept at 4°C in a sterile Eppendorf tube until use. To purify the compounds of the inhibition zone, a C_{18} reverse-phase high-performance liquid chromatograph (HPLC; Hewlett-Packard, 1100, Waldrom, Germany) equipped with G1315 diode-array detector was used. The major band

was registered at 248 nm. The analytical column was a LiChroCART 250 × 4 mm I.D. packed with LiChrospher 100, RP-18 e (spherical particles 5 μm in diameter). The precolumn was an ODS-Hypersil (C_{18}), 5 μm, 20 × 4.0 mm (Hewlett-Packard). The antioomycete substances were eluted at room temperature using a linear-gradient solvent system from 10% acetonitrile in water Milli-Q (Millipore, Billerica, MA, USA) to 100% acetonitrile at a flow rate of 1.0 ml/min. The injection loop size was 20 μl. The elution protocol lasted 10 min. The compounds were collected at the outlet of the detector, the collection coinciding with the beginning of the peak's descent. Each eluate was concentrated with N_2, re-dissolved in methanol, and evaluated for its antagonistic properties towards *P. capsici*. For this, a disc of mycelium cut from the edge of a growing colony of *P. capsici* was placed in the centre of a Petri dish containing PDA. Two holes 5 mm in diameter were made equidistant from the centre and each was filled with 20 μl of the metabolic eluates of the purified extract. The dish was incubated at 25°C in dark and the diameter of the colony was measured after 24, 48, 72 and 96 h. The control consisted of methanol, which was the solvent used for dissolving the purified compound during HPLC. The compound showing antioomycete activity was identified by mass spectrometry in an Agilent, 5973 (G2577A, mass selective detector) chromatograph (Agilent Technologies, Ramsey, MN, USA) and then in nuclear magnetic resonance spectrometer (Bruker Avance 300, NMR-^{13}C; Bruker, Ettlingen, Germany).

In vivo experiments

When the pepper seedlings in the nursery were 3 months old, they were transplanted into pots and also to the field. The potting mix comprised one part vermiculite, two parts sand and six parts peat (v/v), to which was added the inoculum. The experiment consisted of three treatments (inocula): *T. harzianum*, *S. rochei* and *T. harzianum* + *S. rochei* (*T. harzianum* at a concentration of 5.0×10^5 ppg and *S. rochei* at 7.0×10^4 CFU/g). The vermiculite–antagonist mixture was added to the pots in different doses: 5, 10, 15 and 20 g per pot. The effect of pH on plant growth was assessed by adding different proportions of 1 M KCl to the soil to give pH values of 5.61, 5.44, 4.95, 4.58, 4.20 and 3.48. Seven days after transplantation, a shallow trench, as deep as the rhizosphere, was dug around each plant; 9 g of vermiculite (in which the pathogen *P. capsici* had been grown so as to get a concentration of 1.2×10^6 ppg of substrate) was placed in the trench and covered with soil. The plants were watered with tap water every 2 or 3 days using self-compensating emitters at 4 l/h, enriched every 2 weeks with potassium, nitrogen, phosphorous (KNP) solution by a fertilizer injection system (ATF0040, Novo Ris, Zaragoza, Spain) connected to the irrigation network. The temperature ranged from 23 to 30°C and relative humidity was 65–85%. The resulting plants represented four groups: those inoculated with the pathogen as well as treated with the antagonists (I + T), those not inoculated but treated with the antagonists but (NI + T), those inoculated but not treated (I + NT) and those neither treated nor inoculated (NT + NI). There were 10 plants per treatment and each treatment was replicated three times. All the plants were examined daily for symptoms of root rot for 2 months, after which the plants were dug up and their roots washed before evaluating the effect of the different treatments.

In the case of seedlings transplanted to the field, the antagonists and then the pathogen were placed in holes dug in the soil with a spacing of 70 × 40 at a similar manner as in the pots. As far as possible, the same external conditions were maintained for both the potted plants and the plants grown in the field. Fifty plants were grown and two experiments were carried out between April and September. All plants were examined daily and then evaluated 2 months after transplanting, in the same way as the potted plants.

To add the antagonists to the plant we used a mixture of plantation soil and the vermiculite–culture medium in which the antagonists were grown, vermiculite–NB for *S. rochei* and vermiculite–Avy3 for *T. harzianum*. The exact formula is the subject of a patent application. To confirm the stability of the antagonists in the vermiculite–soil mixture, they were kept at room temperature in the laboratory and their viability checked every month for 2 years. For this, 5 g of the culture of each antagonist was cultured on Petri dishes. In the case of *T. harzianum*, this was performed on PDA treated with Chlortetracycline Sigma (C-4881) and Penicillin G Sigma (P-8306) at 50 mg/l (Sigma, Steinheim, Germany). In the case of *S. rochei*, the NA was enriched with Captan and Benomyl at 5 mg/l. The dishes were incubated in dark in an oven at 25 ± 2°C. The survival of the antagonists was checked visually after 72 h by assessing the vegetative growth of the mycelium of *T. harzianum* and of the colonies of *S. rochei*. Subsequently, the capacity of the antagonists to inhibit growth of the pathogen was checked *in vitro* and then every 6 months *in vivo* for 2 years.

Statistical analysis

The effects of the treatments on disease severity was analysed by one-way ANOVA using STATGRAPHICS PLUS for Windows, V.2.1 (Statistical Graphics Corp., Baltimore MD, USA) and the mean values were compared using the least significant difference (LSD) test at $P = 0.05$.

Results

Figure 1 shows the growth of the micro-organisms: *P. capsici* and *T. harzianum* on PDA and *S. rochei* on NA. *Trichoderma harzianum* showed a clear antagonism towards *P. capsici*. Initially, *T. harzianum* kept the pathogen at bay and kept growing, sharing the same substrate until it had invaded the whole surface of the pathogen colony and even sporulated over it. This microparasitism could be observed under the SEM

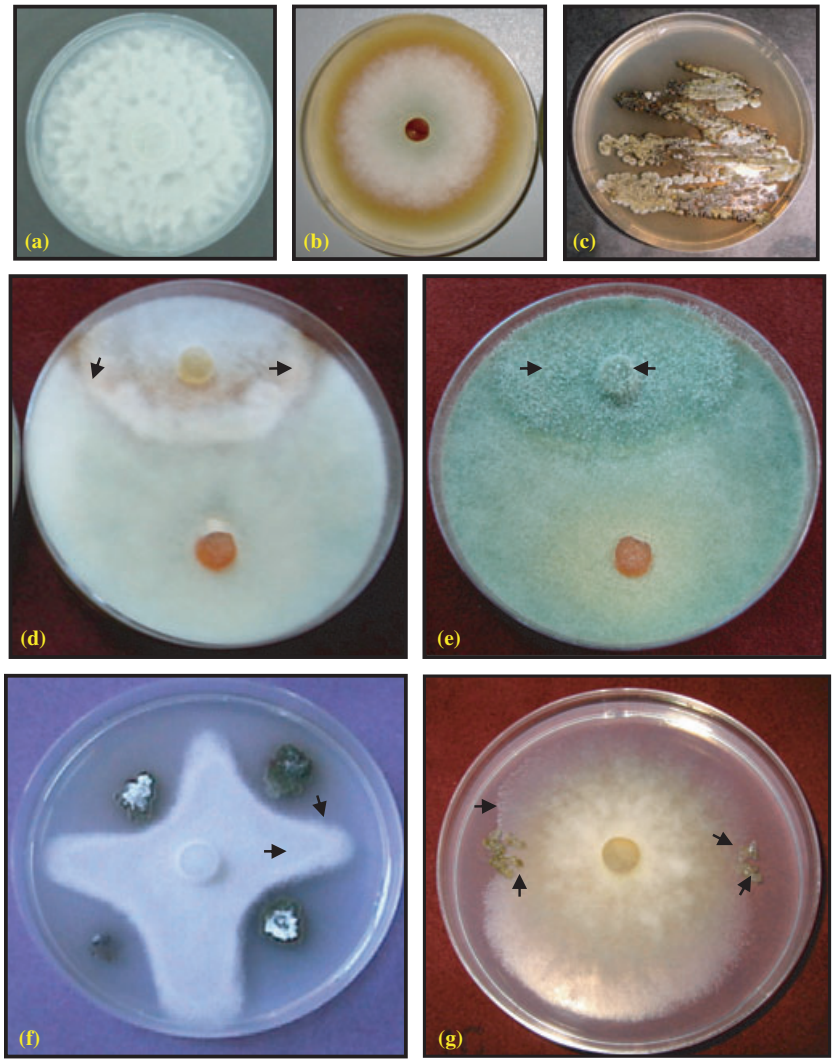

Fig. 1 (a–c) *In vitro* growth at 48 h of the pathogen *Phytophthora capsici* in potato dextrose agar (PDA; a) and the antagonists, *Trichoderma harzianum* in PDA (b) and *Streptomyces rochei* in nutrient agar medium (c). (d–g) Dual confrontations of the antagonists against the pathogen and against each other in PDA medium. *Trichoderma harzianum* inhibited *P. capsici* growth by hyperparasitism at 72 h (d) and sporulating over the pathogen colony after 5 days (e). Inhibition of growth of *P. capsici* caused by the antioomycete discharged by *S. rochei* deposited in the culture medium, after 72 h of interaction (f). Synergic compatibility in the confrontation between the antagonists *T. harzianum* and *S. rochei* in 48 h cultures (g). The arrows indicate the interaction zones of the microorganisms

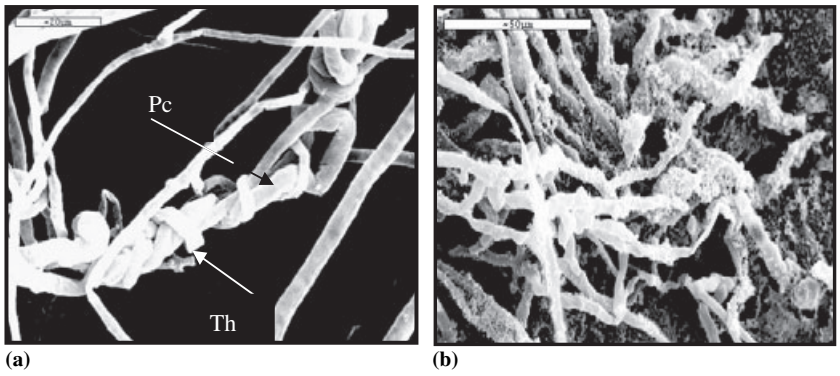

Fig. 2 Scanning electron micrographs of the interaction between the pathogen *Phytophthora capsici* (Pc) and the antagonists *Trichoderma harzianum* (Th) and *Streptomyces rochei* (S) obtained in potato dextrose agar cultures after 48 h. (a) Microparasitism by massive envelopment of Pc hyphae (arrows). (b) Disintegration of the Pc mycelium by sporulation of the bacterium over the oomycete hyphae; note the presence of bacterial spores and formation of spherical hypertrophy in the Pc hyphae

(Fig. 2a), where hyphae of *T. harzianum* were seen enveloping, severing and disintegrating *P. capsici* hyphae, thereby preventing their growth. In PDA plates seeded simultaneously with the *S. rochei* isolate and *P. capsici* at either ends, the pathogen was inhibited. The inhibition zone clearly showed that antimicrobial compounds with antioomycete capacity were being produced by the bacteria (Fig. 1f). Microscopic observation of the zone of interaction between *S. rochei* and *P. capsici* with the SEM clearly showed the microparasitic effect of the filamentous bacteria that led to curling, deformation and vacuolization of

P. capsici hyphae (Fig. 2), ending with their lysis and the eventual destruction of the fungal colony. In plates seeded simultaneously with *T. harzianum* and *S. rochei*, both micro-organisms grew normally: the radial growth of the fungus was not inhibited by the bacteria; in fact, after 2 days, the fungal mycelium surrounded the bacterial colony, demonstrating their compatibility (Fig. 1g). Microscopic examination of the interaction zone showed that *T. harzianum* hyphae to be growing uniformly with no sign of deformation or disintegration (results not shown). Temperature had no effect on the symbiotic action between the two antagonists. For these reasons, interaction between the two antagonists, namely *T. harzianum*, and *S. rochei*, can be described as antagonistic synergism as they are compatible and can be used together against the pathogen.

Isolation and detection of an antioomycete substance produced by *S. rochei*

The inhibition zone arising from the action of the antagonist *S. rochei* led to an attempt to identify the antioomycete substance produced by the bacteria. The extract obtained was separated by TLC, which provided a visible band at 360 nm, 12 mm wide and with an R_f of 0.8. A bioassay was carried out on this extract to identify any antimicrobial compounds responsible for the inhibition zone. After purification by HPLC, two main peaks were obtained, one with a retention time (RT) of 2.795 min and 55.8% intensity and the other with an RT of 3.819 and 34.4% intensity. Figure 3 shows the inhibition (antioomycete activity) by the first compound (RT: 2.795) compared with the methanol control. The same test carried out with the other compound (RT: 3.819) showed no inhibition of pathogen growth and an image identical to that of the methanol control (results not shown). This confirmed that only the compound with an RT of 2.795 is antagonistic towards *P. capsici*.

Figure 4 shows the kinetics of oomycete growth tested at 24, 48, 72 and 96 h following exposure to the active compound RT of 2.795; the progressive reduction in the diameter is clearly visible. While the antioomycete compound was active from 24 h onwards, decreasing the growth of the pathogen and inhibiting the expansion of its colony approximately a concentration of 10 μg/ml, methanol had no such effect. The compound isolated from the antagonist bacteria *S. rochei* was identified by mass spectrometry as C_9H_9ClO), 1-propanone, 1 (4-chlorophenyl) (Fig. 5) with a molecular weight of 168.034, while NMR-^{13}C spectra showed the following chemical displacement of the carbon atoms expressed in C1: 135.50, C2: 128.80, C3: 129.40, C4: 139.10, C5: 129.40, C6: 1288, C7: 198.8, C8: 31.8, C9: 8.20.

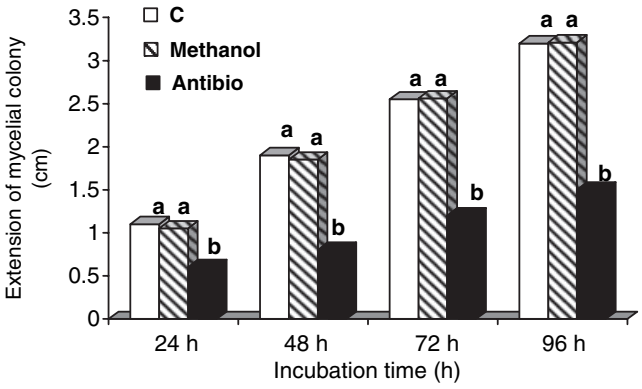

Fig. 4 Dynamic growth evaluation of the *Phytophthora capsici* colony during a 4-day period. C: control with the oomycete growing in isolation, without adding any compound; methanol: control of the oomycete against two methanol discs; antibio: against two discs of the antioomycete sample dissolved in methanol. Values are mean of three experiments with three replicates each. Mean values followed by the same letter are not significantly different according to Fisher's LSD at P = 0.05

Effect of the different treatments on root rot caused by *P. capsici in vivo*

Samples of 100 g each were collected from the inoculated mixture added to the pots containing vermiculite + soil to count the initial population of each antagonist and, by a series of dilutions, the following concentrations were obtained for each dose used (in g/pot): 5, 1.3×10^4; 10, 2.0×10^5; 15, 8.0×10^6 and 20,

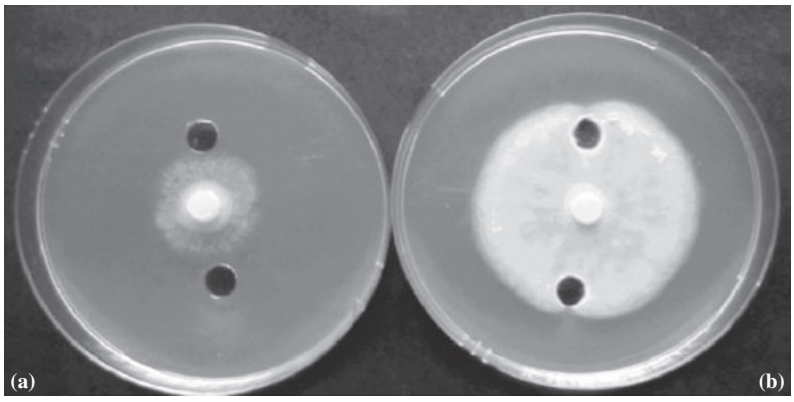

Fig. 3 Bioassay of antioomycete activity against *Phytophthora capsici* in potato dextrose agar. (a) Inhibition of pathogen growth caused by the compound produced by the antagonist bacterium *Streptomyces rochei* which had a retention time (RT) of 2.795 after purification in high-performance liquid chromatography (HPLC). (b) Control with methanol, which did not inhibit pathogen growth. Growth of similar diameter was obtained when the fungus was cultivated alone and in the presence of the compound RT of 3.819 in HPLC

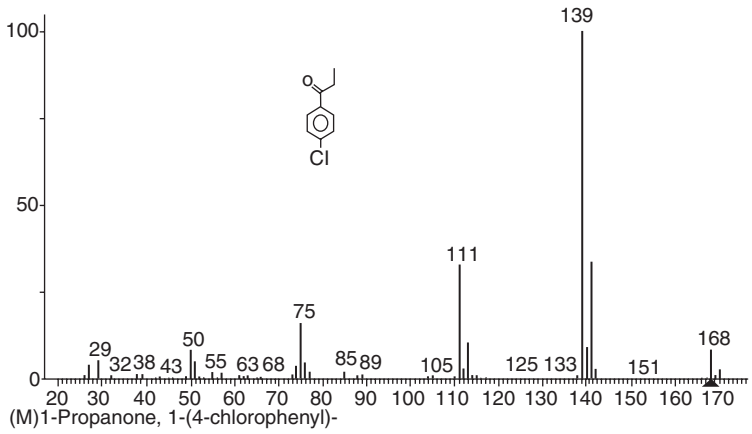

Fig. 5 Mass spectrum of the *Streptomyces rochei* antioomycete produced

Table 1
Root rot caused by *Pc* (*Phytophthora capsici*) in pepper plants and percentage of disease reduction when the soil was mixed with antagonist micro-organisms *Th* (*Trichoderma harzianum*) and *Sr* (*Streptomyces rochei*)

	Pepper plants grown in:	Root rot caused by *P. capsici*	Decrease of disease (%)
Control: (NT and NI)	pots or soil	0.00 a	
Pc: (NT and I)	pots or soil	5.00 c	
Th: (T and NI)	pots or soil	0.00 a	
Sr Ziyani: (T and NI)	pots or soil	0.00 a	
Th + *Sr*: (T and NI)	pots	0.00 a	100
Th (1) + *Pc*: (T and I)	pots	3.56 a	28.9
Th (2) + *Pc*: (T and I)	pots	3.48 a	30.4
Th (3) + *Pc*: (T and I)	pots	3.40 a	32.0
Th (4) + *Pc*: (T and I)	pots	3.11 a	37.8
Sr (1) + *Pc*: (T and I)	pots	3.73 a	25.5
Sr (2) + *Pc*: (T and I)	pots	3.26 a	34.9
Sr (3) + *Pc*: (T and I)	pots	2.29 a	40.3
Sr (4) + *Pc*: (T and I)	pots	2.22 a	55.6
Th (4) + *Sr* (4) + *Pc*: (T and I)	pots	1.01 b	79.8
Th (4) + *Sr* (4) + *Pc*: (T and I)	soil	1.26 b	74.8

In each dose used, the initial population of each antagonist was [1] = 1.3×10^4, [2] = 2.0×10^5, [3] = 8.0×10^6 and [4] = 35.0×10^7 spores/ml of *T. harzianum* and [1] = 9×10^2, [2] = 1.9×10^4, [3] = 2.8×10^6 and [4] = 1.0×10^9 CFU/ml of *S. rochei*. NT and NI, plants neither treated nor inoculated; NT and I, plants not treated but inoculated; T and NI, plants treated but not inoculated; T and I, plants both treated and inoculated. The severity of *P. capsici* root rot was evaluated applying the following scale: 0 = <1% of root rotted, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–90%, 5 = >91% or dead plants. Values are mean of three experiments with five replicates each. Mean values followed by the same letter are not significantly different according to Fisher's LSD at P = 0.05.

35.0×10^7 spores/ml of *T. harzianum* and, respectively, 9×10^2, 1.9×10^4, 2.8×10^6, and 1.0×10^9 CFU/ml of *S. rochei*.

Table 1 shows the incident of root rot in pots and in soil 2 months after the different treatments. As all the plants infected but not treated (I + NT) died and all those treated but not infected (T + NI) survived, it is clear that not only was the pathogen sufficiently virulent to kill the plants if they were not treated with the biocontrol agents, but also that the biocontrol agents are harmless to the plants. In the plants that were infected as well as treated (I + T), the dose of antagonistic bacteria affected the time at which the symptoms of root rot appeared: the lowest doses of 9×10^2 [1] and 1.9×10^4 [2] CFU /ml led to the highest number of plants dying, while the highest doses delayed the appearance of symptoms (and hence the disease); some plants even flowered before they died (the kinetics of individual plant deaths is not reflected in the table). The greatest reduction in mortality was obtained with the isolate *S. rochei* at a concentration of 1×10^9 CFU/ml. The use of different concentrations of the fungus *T. harzianum*, on the other hand, did not significantly influence the appearance of the disease. When the antagonists were applied jointly to the pot-grown plants, the incidence of the disease was reduced by 79.8%.

For seedlings transplanted to the field, the individual and joint addition of the antagonists was tested using dose no. 4 of each. Table 1 shows the reduction in the severity of root rot in the 50 plants treated with the combination of both antagonists. The severity was reduced to 1.26, compared with the maximum of 5, representing a decrease of 74.8%. This reduction was less than that obtained in pots, perhaps because the medium supporting the plants was autoclaved. Consequently, the antagonist micro-organisms do not have to compete for nutrients and space with other micro-organisms making up the basal flora and therefore find it easier to colonize the rhizosphere of the pepper plants and so offer greater protection against the pathogen.

The antagonists were kept in sealed flasks with vermiculite + culture medium + plantation soil (inoculum) at room temperature for 2 years. During this period, the preparations of both antagonists were

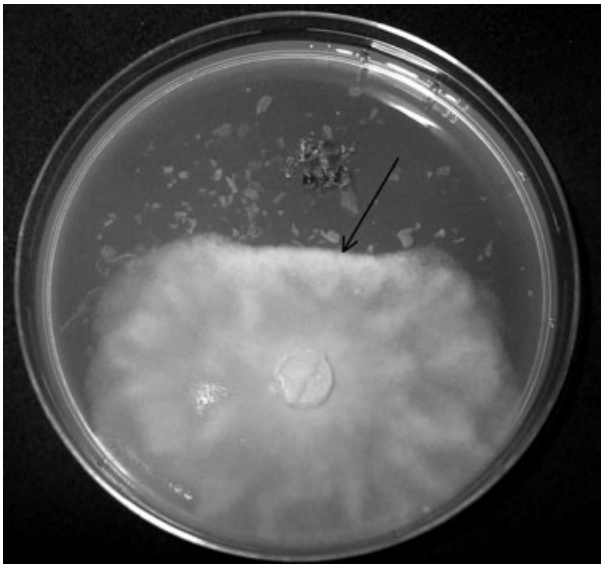

Fig. 6 48-h cultures of the confrontation of *Phytophthora capsici* and 0.1 g of the formulate formed by vermiculite + *Streptomyces rochei* in nutrient agar + soil that had been stored for 2 years. The pathogen growth inhibition zone (arrow) is similar in extent to that obtained with the recently prepared formulate

extremely effective both *in vitro* and *in vivo*. Figure 6 shows the *in vitro* confrontation of *P. capsici* and 0.1 g of the inoculum *S. rochei* kept at room temperature for 2 years. Similar results were obtained with the antagonist *T. harzianum*, confirming that the capacity of both antagonists to inhibit growth of the pathogen had been maintained during storage and was similar to that of recently prepared inocula (results not shown, but similar to Fig. 6). *In vivo*, too, the mixture of both antagonists inhibited the pathogen to the same degree as freshly prepared inocula did. The results agree with those of other authors (Ongena et al., 2000; Innes and Allan, 2001; Clarkson et al., 2004; De la Fuente et al., 2004; McLean et al., 2005) who studied the colonizing capacity of strains of *Pseudomonas*, *Streptomyces* and/or *Trichoderma* in soils and roots for biocontrol experiments against different pathogens.

Discussion

Previous studies have demonstrated that the extent to which *P. capsici* is inhibited by *T. harzianum in vitro* varies according to the culture medium, temperature and pH, being maximum on PDA enriched with laminarin–glucose (3 : 1, v/v; Ezziyyani et al., 2005). The present study shows that the inhibition of pathogen growth is gradual due to the fact that *T. harzianum* grows rapidly at the outset and then invades the colony of *P. capsici* by a marked process of hyperparasitism. Other authors have attributed this effect to the fact that *T. harzianum* contributes numerous antibiotics and enzymes (β-1,3-glucanase, chitinase, protease and cellulase) to the medium, where they degrade cell walls and play a key role in microparasitism (Papavizas and Lumsden, 1980; Elad et al., 1982; Lorito et al., 1993; Lahsen et al., 2001).

Streptomyces rochei belongs to the order Actinomycetales, whose importance is due to its members being a good source of bioactive secondary metabolites of industrial and commercial value (El-Tarabily et al., 2000; Bressan and Figueiredo, 2005). To date, several *Streptomyces* antibiotics, which are known to be active against fungal pathogens, have been isolated (Kim et al., 1999; Hwang et al., 2001; Rodríguez et al., 2002; Remsing et al., 2003). In this study, we identify an antioomycete compound produced by one isolate *S. rochei* as being partly responsible for the *in vitro* inhibition of pathogen growth and which seems to play an important role in the biocontrol mechanism that reduces root rot caused by *P. capsici* in pepper *in vivo*. To the best of our knowledge, there are no data in the literature that point to an antifungal/antibiotic compound produced by actinomycetes and related to 1-propanone, 1(-4-chlorophenyl). Given the clear interest that such a compound will generate, more experiments are being carried out to assess its contribution to the biocontrol of *P. capsici in vivo*, measuring such parameters as its concentration in the rhizosphere, its recovery at different times after addition to the soil, and its antioomycete ability when applied directly to the rhizosphere of different plants.

The main conclusion is that the antagonists, *T. harzianum* and *S. rochei*, despite belonging to different genera, are synergic and can be added together to produce a greater effect on the pathogen. Another advantage of the biopreparation is that it is made of already germinated micro-organisms with abundant mycelial mass, which enables them to adapt readily to a growth medium containing vermiculite and plantation soil. This facilitates their adherence and adaptation to the soil and makes them more effective than ungerminated spores, which would have to overcome the competition from other micro-organisms of the basal flora of the plant rhizosphere. The *in vivo* assay confirmed that the antagonists are harmless to the plants and that they are more effective in combination than when acting individually in reducing root rot caused by *P. capsici* (reduction up to 75%). This result supports the use of a combination of compatible antagonists selected for their different biological properties (including antibiosis, microparasitism, enzymatic lysis and, probably, the ability to compete for space and nutrients), lending weight to the suggestion of other authors (Guetsky et al., 2001; Schmidt et al., 2004; Li et al., 2005) that the use of more than one biocontrol agent is necessary for reducing the vagaries of biological control. Using the characteristics of a combination of antagonists, as developed in this study, opens up the possibility of designing future strategies for transferring the knowledge gained to the control of other pathogens.

Acknowledgements

This research was funded by the Project 57/PCMC/02 de la Fundación Séneca. Comunidad Autónoma de la Región de Murcia (Spain).

References

Akköprü A, Demir S. (2005) Biological control of Fusarium wilt in tomato caused by *Fusarium oxysporum* f. sp. *licopersici* by AMF *Glomus intraradices* and some rhizobacteria. *J Phytopathol* **153**:544–550.

Bora T, Özaktan H, Göre E, Aslan E. (2004) Biological control of *Fusarium oxysporum* f. sp. *melonis* by wettable powder formulations of the two strains of *Pseudomonas putida*. *J Phytopathol* **152**:471–475.

Bressan W, Figueiredo JEF. (2005) Biological control of *Stenocarpella maydis* in maize seed with antagonistic *Streptomyces* sp. isolates. *J Phytopathol* **163**:623–626.

Candela ME, Alcázar MD, Espin A, Egea-Gilabert C, Almela L. (1995) Soluble phenolic acids in *Capsicum annuum* stems infected with *Phytophthora capsici*. *Plant Pathol* **44**:116–123.

Clarkson JP, Mead A, Payne T, Whipps JM. (2004) Effect of environmental factors and *Sclerotium cepivorum* isolate on sclerotial degradation and biological control of white rot by *Trichoderma*. *Plant Pathol* **53**:353–362.

De la Fuente L, Thomashow L, Wéller D, Bajsa N, Quagliotto L, Chernin L, Arias A. (2004) *Pseudomonas fluorescens* UP61 isolated from birdsfoot trefoil rhizosphere produces multiple antibiotics and exerts a broad spectrum of biocontrol activity. *Eur J Plant Pathol* **110**:671–681.

El-Tarabily KA, Soliman MH, Nassar AH, Al-Hassani HA, Sivasithmparam K, Mckenna F, Hardy G. E. St. J. (2000) Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. *Plant Pathol* **49**:573–583.

Elad Y, Chet I, Henis Y. (1982) Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can J Microbiol* **28**:719–725.

Elad Y, Köhl J, Fokkema NJ. (1994) Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic bacteria and fungi. *Eur J Plant Pathol* **100**:315–336.

Ezziyyani M, Requena ME, Pérez-Sánchez C, Candela ME. (2005) Efecto del sustrato y la temperatura en el control biológico de *Phytophthora capsici* en pimiento (*Capsicum annuum* L.). *Anal Biol* **27**:119–126.

Ezziyyani M, Pérez-Sánchez C, Requena ME, Sid Ahmed A, Candela ME. (2004) Evaluación del biocontrol de *Phytophthora capsici* en pimiento (*Capsicum annuum* L.) por tratamiento con *Burkholderia cepacia*. *Anal Biol* **26**:61–68.

Guetsky R, Shtienberg D, Elad Y, Dinoor A. (2001) Combining biocontrol agents to reduce the variability of biological control. *Phytopathology* **91**:621–627.

Guetsky R, Shtienberg D, Elad Y, Fischer E, Dinoor A. (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**:976–985.

Hwang BK, Lee JY, Kim BS, Moon SS. (2001) Isolation and *in vivo* and *in vitro* antifungal activity of phenylacetic acid and sodium phenylacetate from *Streptomyces humidus*. *Appl Environ Microbiol* **67**:3739–3745.

Innes CM, Allan EJ. (2001) Induction, growth and antibiotic production of *Streptomyces viridifaciens* L-form bacteria. *J Appl Microbiol* **90**:301–308.

Kim BS, Moon SS, Hwang BK. (1999) Isolation, antifungal activity, and structure elucidation of the glutarimide, streptimidone, produced by *Micromonospora coerulea*. *J Agric Food Chem* **47**:3372–3380.

Lahsen HA, Soler A, Rey M, De la Cruz J, Monte E, Llobell A. (2001) An antifungal exo-a-glucanase (AGN1) from the biocontrol fungus *Trichoderma harzianum*. *Appl Environ Microbiol* **67**:5833–5839.

Li GQ, Huang HC, Acharya SN, Erickson RS. (2005) Effectiveness of *Coniothyrium minitans* and *Trichoderma atroviride* in suppression of sclerotinia blossom blight of alfalfa. *Plant Pathol* **54**:204–211.

Lorito M, Harman GE, Hayes CK, Broadway RM, Transmo A, Woo SL, Di Pietro A. (1993) Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* **83**:302–307.

McLean KL, Swaminathan J, Frampton CM, Hunt JS, Ridgway HJ, Stewart A. (2005) Effect of formulation on the rhizosphere competence and biocontrol ability of *Trichoderma atroviride* C52. *Plant Pathol* **54**:212–218.

Ongena M, Daayf F, Jaques P, Thonart P, Benhamou N, Paulitz TC, Bélanger RR. (2000) Systemic induction of phytoalexins in cucumber in response to treatments with fluorescent peudomonads. *Plant Pathol* **49**:523–530.

Papavizas GC, Lumsden RD. (1980) Biological control of soil borne fungal propagules. *Annu Rev Phytopathol* **18**:389–413.

Remsing L, González AM, Nur-e-Alam M, Fernández-Lozano MJ, Braña AF, Rix U, Oliveira MA, Méndez C, Salas JA, Rohr J. (2003) Mithramycin SK, a novel antitumor drug with improved therapeutic index generated by combinatorial biosynthesis in the mithramycin producer *Streptomyces argillaceus*. *J Am Chem Soc* **125**:5745–5753.

Rodríguez L, Aguirrezabalaga I, Allende N, Braña AF, Méndez C, Salas JA. (2002) Engineering deoxysugar biosynthetic pathways from antibiotic-producing microorganisms: a tool to produce novel glycosylated bioactive compounds. *Chem Biol* **9**:721–729.

Schmidt CS, Agostini F, Simon A-M, Whyte J, Townend J, Leifert C, Killham K, Mullins C. (2004) Influence of soil type and pH on the colonisation of sugar beet seedlings by antagonistic *Pseudomonas* and *Bacillus* strains, and on their control of *Pythium* damping-off. *Eur J Plant Pathol* **110**:1025–1046.

Sid Ahmed A, Pérez-Sanchez C, Egea C, Candela ME. (1999) Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathol* **48**:58–65.

Sid Ahmed A, Ezziyyani M, Pérez-Sánchez C, Candela ME. (2003) Effect of chitin on biological control activity of*Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *Eur J Plant Pathol* **109**:418–426.

Szczech M, Shoda M. (2004) Biocontrol de *Rhizoctonia* damping-off of tomato by *Bacillus subtilis* combined with *Burkholderia cepacia*. *J Plant Pathol* **152**:549–556.

Szczech M, Shoda M. (2006) The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*. *J Phytopathol* **154**:370–377.