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Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants

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The ability of *Trichoderma harzianum* to control the rotting of pepper (*Capsicum annuum*) plant roots caused by *Phytophthora capsici* was studied. Interactions between the fungi were assessed *in vitro* on three culture media (V8c, Czapek and 2% water agar) and *in vivo* in plants grown in a substrate inoculated with *P. capsici* and *T. harzianum*. Studies on mutual antagonism *in vitro* showed that *P. capsici* was inhibited by *T. harzianum*; however, the intensity of inhibition differed according to the medium used, being greatest on Czapek. Analysis of the fungal populations in the plant growth substrate showed that *T. harzianum* consistently reduced that of *P. capsici* over time. This reduction in the pathogen population was associated with a reduction in root rot of between 24 and 76%, although plant growth (dry weight) was still reduced by 21·2–24·7%, compared with the uninoculated control. In the absence of *T. harzianum* with the same pathogen inoculum levels, the reduction in dry weight was 59·8–68·6%, suggesting that *T. harzianum* reduced the damage.

Keywords: antagonism, *Capsicum annuum*, biocontrol, mycoparasitism, *Phytophthora* blight, *Trichoderma harzianum*

Introduction

Phytophthora capsici causes rot in both the aerial and subterranean parts of many plants (Leonian, 1922; Barnett & Binder, 1973) including pepper (*Capsicum annuum*), in which it causes serious economic losses. Attempts to control *Phytophthora* spp. by, for example, pre-plant soil fumigation, and by the use of resistant plants, have shown little success (Umaerus *et al*., 1983). Furthermore, the use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens (Bruin & Edgington, 1980). It is therefore necessary to develop alternative ways of control. One such alternative is biological control, in which micro-organisms are selected for their ability to antagonize pathogens. *Trichoderma* spp. have been widely used in biological control studies against *Rhizoctonia solani* (Beagle-Ristaino & Papavizas, 1984) and *Pythium* spp. (Hadar *et al*., 1984) among others. However, little attention has been paid to their ability to control *Phytophthora* spp. in general, and *P. capsici* in particular, although their inhibitory effect on some pathogenic fungi that form zoospores suggests that they may have a role to play (Smith *et al*., 1990).

The present study evaluates the biological control potential of *T. harzianum* against *P. capsici* by examining the *in vitro* antagonism between the fungi and the *in vivo* effect on both populations in a plant growth substrate, with the concomitant effect on the incidence of root rot caused by *P. capsici* and the effects of infection on the growth of pepper plants grown in pots in a glasshouse.

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Materials and methods

Plant and fungal material

Pepper plants (*Capsicum annuum* cv. Yolo Wonder) were grown from seed previously disinfected with 7% sodium hypochlorite for 5 min and sown in alveolar trays containing a 2:1 mixture (v/v) of peat and sand that had been sterilized twice at 121°C for one hour on each of two consecutive days. The trays were placed in a Fisons® Growth chamber with a 16 h photoperiod at 25°C and a relative humidity of 75–80%. They were watered with running water every three days until the plants had five leaves, at which stage they were used for experiments.

The fungi used were *T. harzianum*, isolate 2413 from the Colección Española de Cultivos Tipos de Valencia, Spain, and *Phytophthora capsici*, isolate 17 (Candela

et al., 1995). Both were maintained in the dark on V8c agar and potato dextrose agar (PDA), at 4°C.

Antagonism of *T. harzianum* against *P. capsici in vitro*

The interactions were studied in Petri dishes (85 mm diameter) containing three different media: V8c, Czapek and water agar (WA), all adjusted to pH 5·6. A disc (5 mm diameter) from the edge of an actively growing *P. capsici* colony was transferred to each dish and a similar sized disc of *T. harzianum*, cut in the same manner, was placed at a distance of 4 cm. The dishes were incubated at 25°C in darkness. As controls, discs of agar were added to similar plates inoculated with *P. capsici*. Any interactions were observed daily for six days using an optical microscope, noting any morphological changes and recording the inhibition according to the following formula:

$$I = 100 - (100R_2/R_1)$$

where I = inhibition of vegetative growth of the fungi, R_1 = radius of the control colony in mm, and R_2 = the distance in mm travelled by the *P. capsici* colony over the *T. harzianum* colony. Such confrontations were prepared in triplicate and the experiment was repeated three times.

Preparation of the inoculum used to infest the potting mix

The *T. harzianum* inoculum was prepared by the method of Smith *et al*. (1990), with slight modifications. Four or five discs (5 mm diameter) were cut from the edge of the actively growing *T. harzianum* colony and added to a glass Petri dish (90 mm diameter) containing a mixture of peat and wheat bran (3:1, v/v) that had been moistened with 15 mL of distilled water and sterilized for 15 min at 121°C on each of two consecutive days. The same procedure was followed for the *P. capsici* inoculum. The dishes were incubated at 25°C in darkness for 15 days.

Preparation and inoculation of the potting mix used for the plants, and determination of soil populations

The effect of *T. harzianum* on the population dynamics of *P. capsici* and associated root rot caused by *P. capsici* was studied in a potting medium prepared by mixing peat and sand (2:1, v/v) and sterilizing in autoclave bags for one hour at 121°C on each of two consecutive days. The sterilized mix was then inoculated with both fungi prepared previously at a rate of 2% (v/v) of each, or with 4% *P. capsici* and 2% *T. harzianum*. Control treatments were inoculated independently with *P. capsici* alone at 2% or 4%, or *T. harzianum* at 2%, or left uninoculated. Mixing was accomplished by rotation in inflated plastic bags.

Each combination was placed in 11 × 11 cm pots, which were kept at room temperature (18–25°C) for 6–8 h while initial counts of the two fungal populations were made.

For this initial count, 2 g of substrate were taken from each of 5 pots of the appropriate treatment and mixed. This mixture was then divided into two subsamples of 5 g each, one of which was brought to 45 mL in 0·1% (w/v) sterile peptone water. From this suspension (10^{-1}) a series of decimal dilutions was made to arrive at 10^{-6}. Populations of *P. capsici* and *T. harzianum* were determined by plating 1 mL of each of serial dilution in triplicate onto P5VPP-BH (Papavizas *et al*., 1981) and TSM (Askew & Lang, 1993) plates, respectively. P5VPP-BH contained Difco cornmeal agar (17 g L^{-1}) acidified to pH 4 with 1·0 N HCl. After autoclaving, antimicrobial agents were added: 5 μg mL^{-1} pimaricin, 200 μg mL^{-1} vancomycin, 100 μg mL^{-1} pentachloronitrobenzene, 100 μg mL^{-1} penicillin G, 2·5 μg mL^{-1} benomyl and 20 μg mL^{-1} hymexazol. TSM contained 0·2 g $MgSO_4$ ($7H_20$), 0·9 g K_2HPO_4, 0·15 g KCl, 1·0 g NH_4NO_3, 3·0 g anhydrous glucose, 0·15 g rose bengal, 20 g agar and 950 mL distilled water. After autoclaving, 50 mL of antimicrobial agents were added (g/50 mL): 0·25 g chloramphenicol, 0·2 g quintozene, 0·2 g captan and 1·6 g matalaxyl. The plates were incubated at 25°C for 24–36 h and counts were expressed as propagules per gram (ppg) of substrate from plates that developed between 20 and 100 colonies.

Growth of plants in the potting medium and evaluation of the treatments

Pepper plants of approximately the same height and with five true leaves were transferred to previously prepared pots, which were kept permanently moist throughout the experiment. The pots were arranged on greenhouse benches (15–30°C) in a randomized complete block design. Plant growth was measured periodically during eight weeks by reference to the apices at the time of planting, marked by indelible marker at the beginning of the experiment. To determine whether the addition of nutrient had any effect on the populations of the fungi and on suppression of root rot, the potting mixes were enriched with wheat bran (1%, v/v) at the end of the second week. To facilitate the introduction of the wheat bran, it was first ground to a powder, which was then added around the base of the stem and the substrate surface was then prodded with a wire to mix the bran with the substrate before watering. After a further eight weeks the plants were extracted and the roots were washed clean of adhering substrate. The roots were examined for possible lesions following the method of Smith *et al*. (1990). This is based on the visual observation of the extent of rotting, which is expressed as follows: 0 = < 1%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–89%, 5 = > 90% root rot.

The roots and shoots of the plant were separated and dried at 80°C for 24 h before being weighed. The experiment was repeated three times, with five replicates of each treatment and experiment.

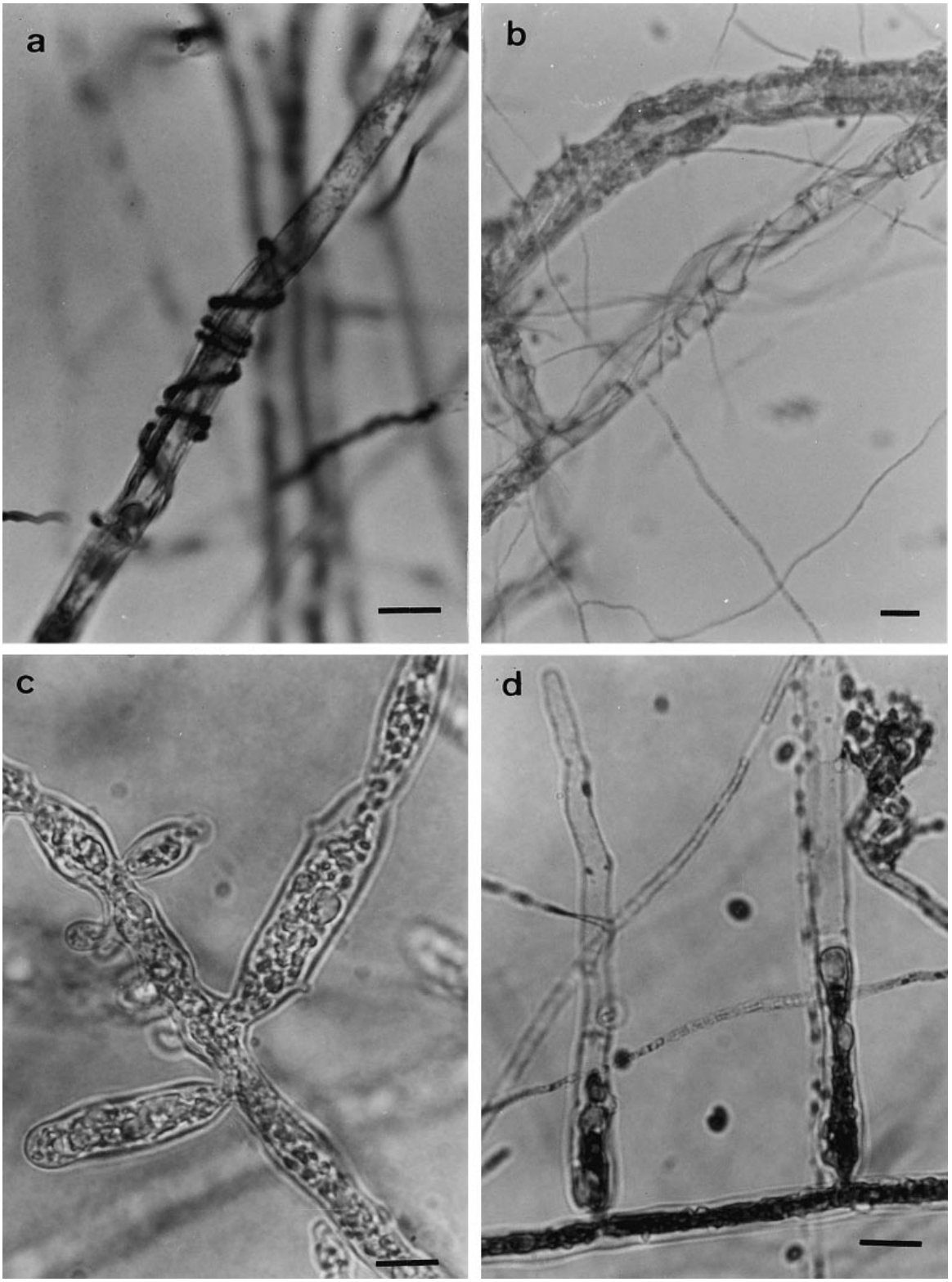

Figure 1 Photomicrographs of hyphal interactions between *P. capsici* and *T. harzianum*. (a) Coiling of *T. harzianum* hyphae around those of *P. capsici*. (b) Disintegration of *P. capsici* hyphae. (c) Vacuolization of *P. capsici* hyphae. (d) Vacuolization of *P. capsici* cells in the presence of *T. harzianum* in WA medium. (Bars, 10 μm).

Growth of the fungal populations in the plant substrate

The population density of both fungi was monitored every two weeks using the method described above: dilution plating from decimal dilutions in selective media, P5PP-BH for *P. capsici* and TSM for *T. harzianum*. The fungi were distinguished by their macroscopic and microscopic differences.

Statistical analysis

Data referring to the number of propagules per gram were subjected to log transformation and all data were then subjected to analysis of variance. Effects of the different combinations of fungus on weight, growth, root rot and fungal populations were analysed statistically using Fisher's LSD (Least Significant Difference) test.

Results

Antagonistic interaction between *T. harzianum* and *P. capsici in vitro*

The following *in vitro* interactions were observed: (a) rapid colonization of the medium by *T. harzianum*, which grew over the *P. capsici* mycelium; (b) inhibition of *P. capsici* growth; (c) *T. harzianum* hyphae coiled around those of *P. capsici*; (d) vacuolization and disintegration of *P. capsici* hyphae and (e) production of sporangia by *P. capsici*.

The intensity of these reactions differed according to the medium on which the confrontation took place. On Czapek medium, *T. harzianum* growth, although less dense, resulted in almost all the plate being rapidly colonized while *P. capsici* was inhibited by 53% (SE = 0·25). *T. harzianum* hyphae were observed coiled around those of *P. capsici* (Fig. 1a), resulting in the disintegration of the host hyphae (Fig. 1b). On V8 agar, colonies of both fungi were very dense and rapid growth of T. *harzianum* resulted in most of the culture medium being invaded: *P. capsici* growth was inhibited by 38% (SE = 0·18). Microscopic observation of the interactive zone showed vacuolization of the *P. capsici* hyphae (Fig. 1c) followed by cell disintegration. The growth and the density of both fungi was less on WA medium, where *T. harzianum* inhibited the growth of *P. capsici* by 44% (SE = 0·18) and produced vacuolization of the hyphae followed by disintegration (Fig. 1d). No hyphal coiling was observed. Production of sporangia was stimulated in all combinations and on all the culture media.

Changes in the *T. harzianum* population in the potting medium

When acting as the sole inoculum, the population of *T. harzianum* fell from 3×10^5 to $1{\cdot}5\times10^5$ ppg during the first two weeks (Fig. 2). When applied jointly with *P.*

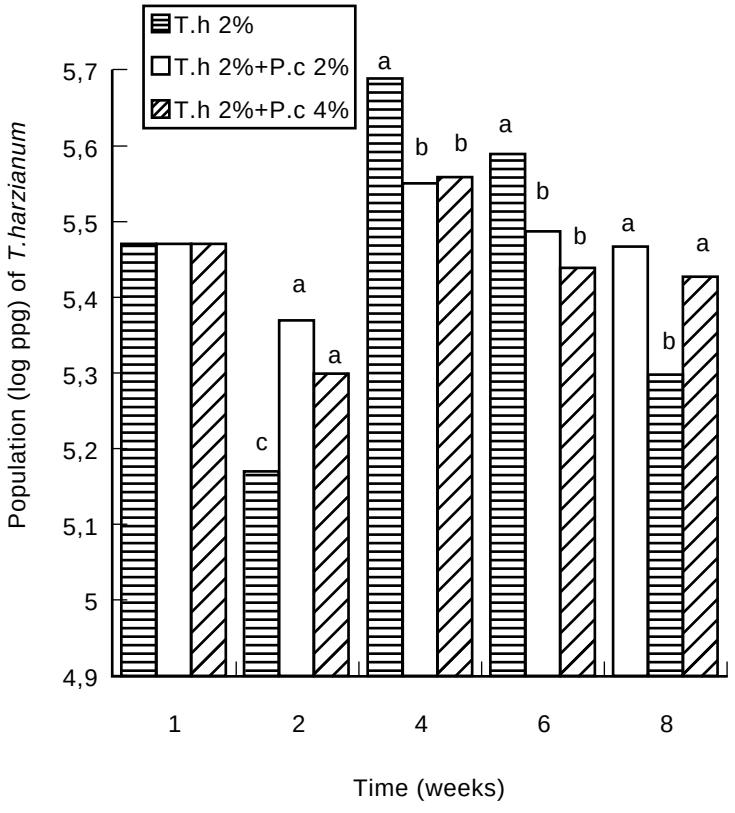

Figure 2 Variation in the population density (log propagules per gram) of *T. harzianum* with or without *P. capsici* in the substrate used for growing pepper (*Capsicum annuum*) plants. *T. harzianum* was used at 2% (v/v) inoculum concentration and *P. capsici* at 2% and 4%. Wheat bran was added at the end of the second week. The values of each count assigned the same letter are not significantly different according to Fisher's LSD-test ($P = 0{\cdot}05$).

capsici at 2% (v/v) inoculum concentration, the population density fell from 3×10^5 to $2{\cdot}35\times10^5$ ppg, while in the presence of 4% *P. capsici*, it fell from 3×10^5 to 2×10^5 ppg. According to Fisher's test, the decrease of the population of *T. harzianum* in both combinations (*T. harzianum* 2% + *P. capsici* 2% or 4%) was significantly less than when the fungus was used alone ($P=0{\cdot}05$).

In the fourth week, two weeks after adding 1% wheat bran, the population density of *T. harzianum* when alone rose to 5×10^5 ppg (Fig. 2), and in the presence of *P. capsici* at 2 and 4% to $3{\cdot}6\times10^5$ and to $3{\cdot}7\times10^5$ ppg, respectively ($P=0{\cdot}05$). From the fourth week onwards, the density of *T. harzianum* alone, and in both combinations, fell gradually until week eight.

The density of the *P. capsici* population, when added at 2 and 4% in combination with 2% *T. harzianum*, fell from 7×10^4 to 1×10^2 ppg and from 1×10^5 to $2{\cdot}5\times10^2$ ppg, respectively, by the end of the second week (Fig. 3). This fall was significantly greater than when *P. capsici* was inoculated alone ($P=0{\cdot}05$). When wheat bran was added at the end of the second week, the densities of *P. capsici* at 2 and 4% inoculum concentration rose to 3×10^3 and 5×10^3 ppg, respectively. At the same time, in the combinations with *T. harzianum*, the density of *P. capsici* rose to $8{\cdot}5\times10^2$ and $7{\cdot}8\times10^2$ ppg, respectively. From the fourth to the eighth week, the population density of *P. capsici* at 2 and 4% inoculum combined with *T. harzianum* decreased to 80 and 40 ppg,

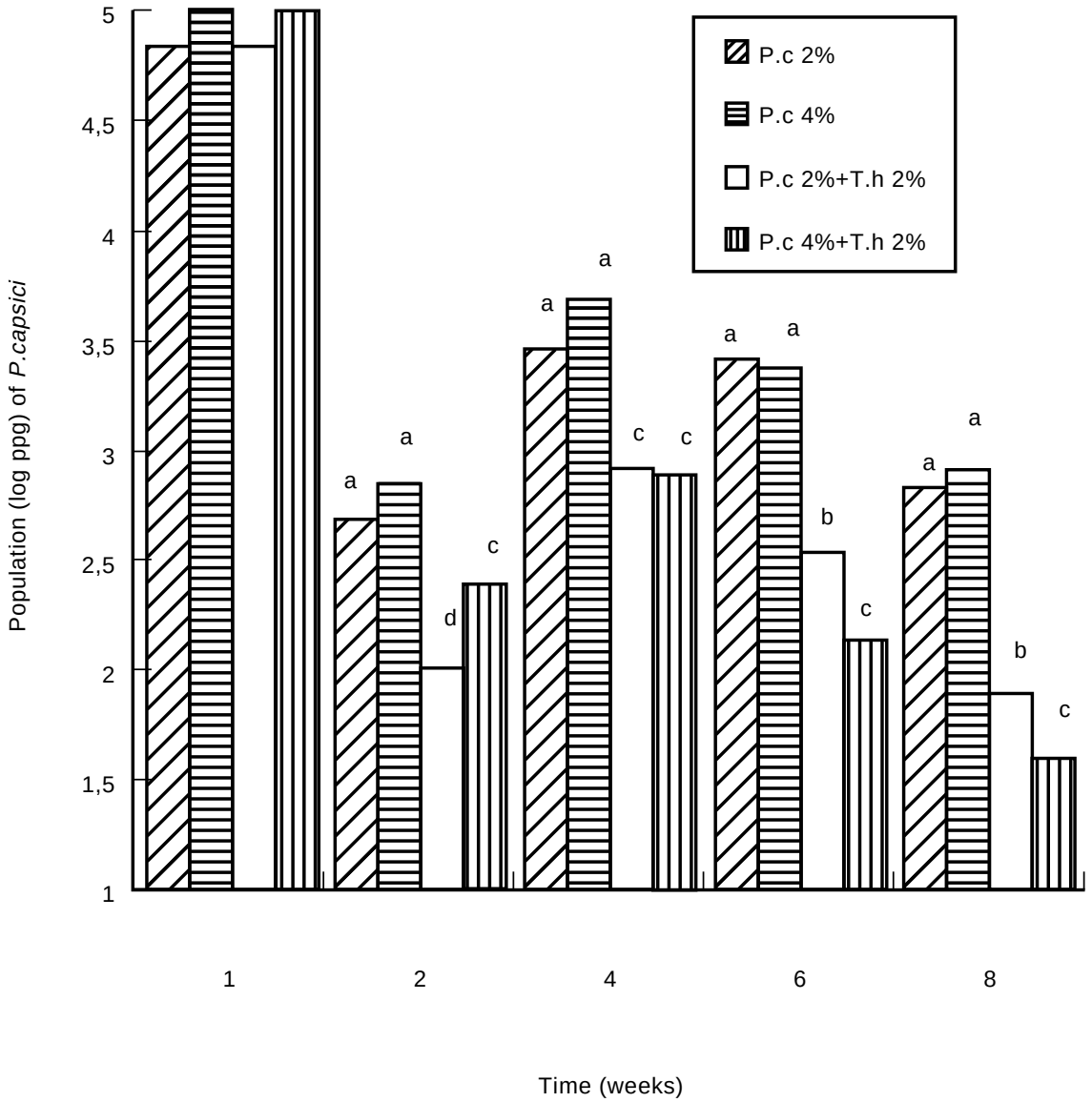

Figure 3 Variation in the population density (log propagules per gram) of *P. capsici* with or without *T. harzianum* in the substrate used for growing pepper plants. *P. capsici* was used at 2% and 4% (v/v) inoculum concentration and *T. harzianum* at 2%. Wheat bran was added at the end of the second week. The values of each count assigned the same letter are not significantly different according to Fisher's LSD-test ($P=0{\cdot}05$).

respectively, a significantly greater decrease than when *P. capsici* was used alone ($P = 0{\cdot}05$).

Efficacy of *T. harzianum* in suppressing the disease caused by *P. capsici* in pepper plants

When *P. capsici* was used at 4%, plant apical growth was reduced by 65%, from 160 mm to 55 mm and plant dry weight by 68·6%, from 1940 mg to 610 mg (Fig. 4). Rot affected 96% of the root (Fig. 5). In the presence of *T. harzianum* (2%) apical growth was reduced by 21·3%, to 126 mm, dry weight by 21·2%, to 1530 mg, and rot affected 20% of the roots.

At a 2% inoculum concentration *P. capsici* reduced apical growth by 50·6%, from 160 mm to 79 mm, and plant weight by 59·8% (Fig. 4), while 36% of the roots were rotted (Fig. 5). In the presence of *T. harzianum* the reduction in apical growth was 26·9% and in dry weight was 24·7%, and rot affected 12% of the roots.

Compared with the plants inoculated with 2 and 4% *P. capsici*, treatment with *T. harzianum* reduced to root rot by 24% and 76%, respectively.

Plant weight was also reduced ($P = 0{\cdot}05$) in the presence of *T. harzianum* but no symptoms of disease were observed.

Discussion

Although the interactions between many fungi have been studied, those involving *Phytophthora* spp. and other fungal groups have received less attention.

In vitro and *in vivo* studies of the behaviour of *Phytophthora* spp. in the presence of antagonistic fungi such as *T. harzianum*, *Gliocladium* spp. (Smith *et al.*, 1990) or *Pythium nunn* and *Penicillium funiculosum* (Fang & Tsao, 1995a, b) have shown the biocontrol capacity of these fungi on *Phytophthora* spp. under controlled conditions.

The results reported here suggest that the presence of *T. harzianum* in the substrate significantly reduces the root rot caused by *P. capsici* in pepper plants. The reduction seems to be related to reduced population density of *P. capsici* in the substrate and perhaps to alterations caused by *T. harzianum* in the *P. capsici* hyphae, as observed *in vitro*.

The establishment of an antagonist in a soil or substrate and its subsequent proliferation may be an important factor in biological control of pathogens (Lewis & Papavizas, 1984). The presence of *T. harzianum* in the substrate alongside *P. capsici* generally resulted in a significant decrease in the population density of the pathogen. This antagonistic effect occurred despite the fluctuations in the *T. harzianum* population observed during the experimental period. The decrease in its density was probably the result of exhaustion of the nutrient supply because, when wheat bran was added, the population density rose.

These results support the observations of other authors (Malajczuk, 1983; Lewis & Papavizas, 1985; Lewis & Papavizas, 1984; Smith *et al.*, 1990), who emphasized the importance of the organic matter content in stimulating the density of fungal populations and proposed manipulation of organic matter levels to promote biological control of *P. cinnamomi*. However, the proliferation of *T. harzianum* may also depend on age of the inoculum used. Lewis & Papavizas (1984) observed that the population of *T. viride* did not increase

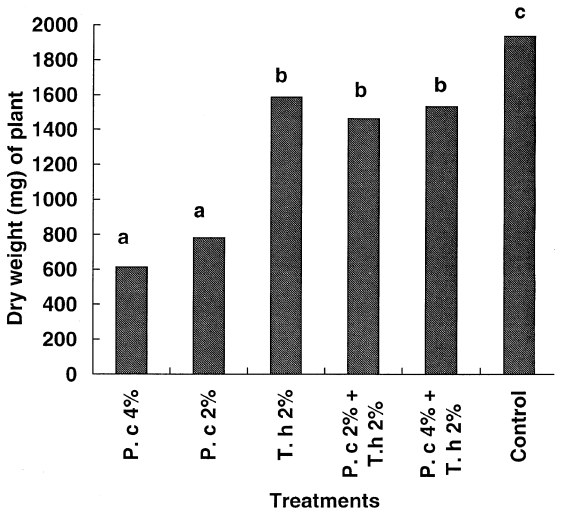

Figure 4 Dry weight of pepper plant (*Capsicum annuum*, var. Yolo Wonder) in the presence of *T. harzianum* (T. h) and *P. capsici* (P. c) after eight weeks. *T. harzianum* was used at 2% (v/v) inoculum concentration and *P. capsici* at 2% and 4%. Each value is the mean of 15 observations (three experiments with five replicate plants each). The columns headed by the same letter are not significantly different according to Fisher's LSD-test ($P = 0{\cdot}05$).

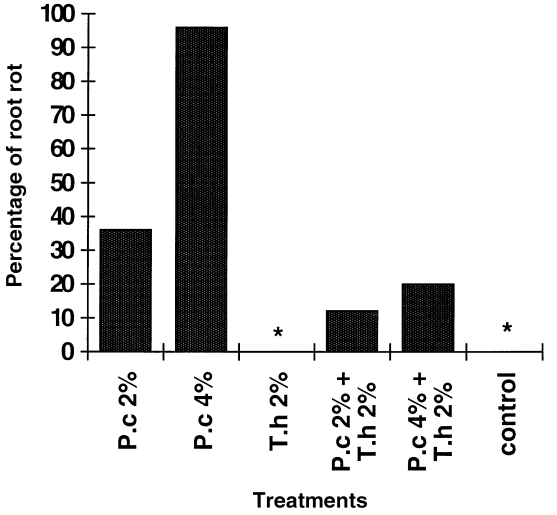

Figure 5 Percentage of root rot in pepper plants grown in substrate infected with *P.capsici* (P. c) at 2% or 4% (v/v) inoculum concentration and *T. harzianum* (T. h) at 2%. The values represent the mean of 15 observations (three experiments with five replicate plants each); * denotes no sign of lesions.

when 15–40-day-old inocula on wheat bran were used, although an increase was observed with 1–3-day-old inocula.

Although the *T. harzianum* population fell during the first two weeks, its presence still led to a diminution of the *P. capsici* population. However, the decrease in the *T. harzianum* population was less pronounced than when it was alone in the substrate.

Malajczuk (1983) described *Phytophthora* spp. as weak competitors and *Trichoderma* spp. as active parasites of *Phytophthora* spp., contributing to their breakdown and decay in soil.

The gradual drop in the *T. harzianum* population during the last four weeks might explain the percentages of root rot observed. According to Papavizas & Lumsden (1980), this can be remedied by adding an energy source and other organic materials. To alleviate a fall in antagonist density, Fang & Tsao (1995b) suggested a periodical amendment of the substrate by adding fresh inoculum of the antagonist.

The addition of wheat bran led to increases in the populations of both fungi when they were applied to the substrate together, suggesting that this nutrient source stimulated not only *T. harzianum* but also *P. capsici*, although to a lesser degree. This behaviour may result from the ability of the pathogens to use the added nutrient.

The reduction of root rot was not related to stimulation in plant growth. In the mix inoculated with *P. capsici* and *T. harzianum*, growth was less than that of the uninoculated plants. This suggests that the *T. harzianum,* while reducing the *P. capsici* root rot, could not promote plant growth. In fact, *T. harzianum* alone reduced apical growth and dry weight of plants by 15·7% and 18·6%, respectively. Ghisalberti *et al*. (1990) observed that *T. harzianum* isolates reduced take-all even when they did not seem to promote plant growth. Fang & Tsao (1995a) observed that *Pythium nunn* at 1000 ppg reduced the incidence of the disease caused by *Phytophthora* spp. but did not favour plant growth. According to Harman & Hadar (1983), the increased growth of plants in the presence of *Trichoderma* sp. may result from the elimination of minor pathogens in the rhizosphere. In the present experiments, the planting mix was sterilized before use, which should result in the absence of pathogens other than *P. capsici*. According to Windham *et al*. (1986) the effect on growth might be the result of the production of growth regulators by *Trichoderma* spp. It appears that the reduction of root rot and the concomitant reduction in plant growth in the present study may be due to the reduction in the *P. capsici* population and/or induction of host plant resistance by *T. harzianum* without the production of plant growth promoters. This is currently under investigation and results will be published in a future paper.

The correlation of the *in vivo* and *in vitro* results on the pathogen populations suggests that the mechanisms responsible for the decrease in the *P. capsici* population and the reduction in root rot are probably the same under both conditions. According to Papavizas & Lumsden (1980) the mechanisms involved in the control of pathogens by *Trichoderma* spp. are probably: antibiosis, lysis, competition and mycoparasitism. However, Ayers & Adams (1981) indicated that interactions observed *in vitro* do not necessarily confirm their operation for the decrease in pathogen populations and reduction in diseases observed in natural conditions.

The *in vitro* culture of *P. capsici* and *T. harzianum* together in three culture media led to a variety of interactions. *P. capsici* growth was generally inhibited, the host cell contents disorganized and the hyphae lysed on all the media and hyphae were intensely parasitized by *T. harzianum* on Czapek medium. Similar reactions were reported previously by Barnett & Binder, (1973) and Elad *et al*. (1983) who noticed inhibition of growth, lysis and parasitism by *Trichoderma* spp. of some species of *Phytophthora*, but not of *P. capsici*. *T. harzianum* produces various toxic and antibiotic metabolites (Dennis & Webster, 1971a, b; Claydon *et al*., 1987; Lorito *et al*., 1994) and enzymes (Lorito *et al*., 1993) which are involved in the inhibition and lysis of pathogenic fungi. The stimulation of sporangium production by *P. capsici* in the presence of *T. harzianum* observed in all the culture media is associated with the inhibition of *P. capsici* vegetative growth. This phenomenon has been observed by several investigators, who have suggested that it is a result of an antagonistic action (Brasier, 1975).

Acknowledgements

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