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Biological control of *Phytophthora capsici* root rot of pepper (*Capsicum annuum*) using *Burkholderia cepacia* and *Trichoderma harzianum*

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ABSTRACT

Objective: Study the antagonistic capacity of a combination of two compatible microorganisms, the bacterium *Burkholderia cepacia* and the fungus *Trichoderma harzianum* against the pathogen *Phytophthora capsici,* the causal agent of rot in pepper.

Methodology and results: Evaluation of the two antagonists against the pathogen and between themselves was carried out by dual *in vitro* interactions in several cultural media, at different pH and temperature conditions. Both antagonists affected the survival and development of the pathogen *P. capsici*, through a variety of mechanisms. *B. cepacia* showed a high degree of antibiosis while *T harzianum* showed greater competition for space and nutrients, and a tendency to mycoparasitism and enzyme lysis. Biomass production of the antagonists was optimised in an Oat-Vermiculite medium, which proved to be efficient, cheap and rapid. The optimal doses of the antagonists were 3.5×10^8 spores/ml for *T. harzianum* and 10^9 CFU/ml for *B. cepacia* in a pH range of 3.5 - 5.6 and temperature range of 23 - 30 °C. *In vivo* treatment with the combination of *B. cepacia* + *T. harzianum* reduced the incidence of wilt caused by the pathogen *P. capsici* on pepper by up to 71%.

Conclusion and application of findings: One of the reasons for the success obtained is that we have obtained total compatibility between the two antagonists used, which belong to different genera with a wide antifungal spectrum. The use of such antagonistic microorganisms in biological control of plant pathogens provides an alternative to chemical products such as methyl bromide (MB) whose use is prohibited by the Montreal protocol, due to its environmentally harmful effects, especially thinning of the ozone layer.

Key words: Biocontrol, *Capsicum annuum*, *Phytophthora capsici*, *Burkholderia cepacia*, *Trichoderma harzianum.*

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INTRODUCTION

Numerous bacteria and fungi are known to possess an antagonistic capacity against other microorganisms. Such antagonism is manifested in many different ways, including inhibiting the development of the pathogens responsible for causing disease. The antagonists can be exploited for biocontrol as long as their lack of pathogenicity towards plants or even animals and man can be demonstrated. Among the genera classified as antagonists are the bacteria *Pseudomonas* and *Bacillus* (Elad & Baker, 1985; Leeman *et al*., 1995; Kimer *et al*., 1998; Loper & Henkels, 1997; Sid Ahmed *et al*., 2003a, 2003b; Ezziyyani *et al*., 2004; Szczech & Shoda, 2006; Romero *et al*., 2007).

The genus *Trichoderma* possesses good qualities for controlling diseases in plants caused by pathogenic soil fungi, especially those of the genera *Phytophthora, Rhizoctonia, Sclerotium*, *Pythium* and *Fusarium,* among others. Species of Trichoderma act as competitive hyperparasites, producing antifungal metabolites and hydrolytic enzymes that cause structural changes at cell level, such as vacuolisation, granulation, cytoplasm disintegration and cell lysis, which have been observed in organisms with which they interact.

Species of the genus *Trichoderma* are the most widely used antagonists for controlling plant diseases caused by fungi due to their ubiquitous nature, ease with which they can be isolated and cultured, their rapid growth on a variety of substrates and the fact that they do not attack higher plants (Papavizas *et al*., 1982). The mechanisms by which *Trichoderma* suppress phytopathogens are basically three, i.e. direct competition for space or nutrients (Elad & Baker, 1985; Elad & Chet, 1987; Chet & Ibar, 1994; Belanger *et al*., 1995; Sid Ahmed *et al*., 1999), the production of antibiotic metabolites, whether volatile or not (Chet *et al*., 1997, Sid Ahmed *et al*., 2000) and direct parasitism on phytopathogenic fungi (Yedidia *et al*., 1999).

The selection of the antagonists used in this study was based on numerous recent contributions of many authors (including Hoitink & Fahy, 1986; Martín & Hancock, 1986; Martín & Loper, 1999, Li *et al*., 2005; Gupta *et al*., 2006; Huang & Erickson, 2007), who have shown that bacteria such as *Pseudomonas* spp. and fungi such as *Trichoderma* spp., grow and colonise organic matter and, in so doing, inhibit its colonisation by pathogens.

To establish a successful biological control programme, it is necessary to first establish the optimal requirements of the pathogen-antagonist and antagonist-antagonist systems *in vitro* in terms of temperature, moisture, nutrients, pH, pathogen and antagonist to be used, etc, before moving on to greenhouse experiments. The results obtained through this strategy would constitute the basis for using a given combination of antagonists selected for their different biological possibilities. In this particular study, the result will facilitate the design of future strategies to biologically control the soil pathogenic fungus *P. capsici* and enable possible transfer of this technology to the control of other pathogens.

MATERIALS AND METHODS

Plant material: The pepper (*Capsicum annuum* L.) variety California Wonder was selected for the study since it is highly susceptible to the oomycete *Phytophthora capsici*. Seeds were disinfected in a commercial solution of 2% sodium hypochlorite for 5 minutes, washed three times with sterile distilled water and sown in 48-alveolar trays (4x4x14 cm) containing a sterilised mixture of peat, sand and vermiculite (3:1:0.5). The trays were placed in a greenhouse at the Experimental Field Station of the University of Murcia, Spain. When the fifth or sixth true leaf had developed, plants were transplanted and inoculated with *P. capsici* (Candela *et al*., 1995).

Pathogen culture: As pathogen, we used the oomycete *Phytophthora capsici*, isolate 15, which is an extremely aggressive strain. The fungus was kept on potato dextrose agar (PDA, Difco). For the biocontrol experiments, the fungus was also cultured in «vermiculite-PDB (potato-dextrose broth)» medium, obtained by adding 200 ml of PDB to a 1000 ml Erlenmeyer flask containing 150 g vermiculite previously sterilised twice at 121 °C for 30 min on two consecutive days. The mixture was autoclaved and inoculated aseptically using four, 5 mm diameter discs cut from the edge of a mycelial colony of *P. capsici* grown on PDA. These were then incubated in an oven at 25°C for three weeks, which was sufficient time to produce abundant inoculum. To obtain a large quantity of zoospores, *P. capsici* was grown in PDB in darkness while shaking in 250 ml flasks at 25 °C for 15 days. The mycelia were then separated and washed, put into

distilled water again and shaken for another 5-6 days during which spores were formed and released.

Culturing antagonists: As antagonist, we used the fungus *Trichoderma harzianum*, isolate 2413 from the Spanish type collection (CECT) in Valencia (Spain), which we have previously used (Sid Ahmed *et al*., 2001; Ezziyyani *et al*. 2005, 2007). The fungus was kept on PDA and Avy-3 media (Ezziyyani *et al*., 2004). The bacterial strain *Burkholderia cepacia*, isolate 322 was also obtained from the Spanish type collection (CECT), Valencia (Spain) and kept on NA medium (Nutritive Agar, standard II, Merck). Depending on the type of assay to be carried out, the inocula and bacterial suspensions were prepared in solid or liquid medium to treat the seeds and plants by three different procedures described previously by Ezziyyani *et al*., (2004).

In vitro interaction between antagonists and *P. capsici*: The activity of the antagonists *B. cepacia* and *T. harzianum* was evaluated by interactions with the pathogen *P. capsici* on four different media: PDA, Czapek Dox-Agar, 2% Water-Agar (W-A) and PDA enriched with Laminarine and glucose (3:1, v/v). The inhibition test was used in each medium. A 5 mm disc was cut from the border of the colony of the pathogen *P. capsici* growing in PDA and sowed 2 cm from the edge of each dish containing one of the media and inoculum of one of the antagonists. In the case of *T. harzianum* a 5 mm disc was taken from the edge of its growing dish and placed 5 cm from the disc of the pathogen.

In the case of the bacterium, 50µl of a pure culture of *B. cepacia* was used. The dishes were incubated at 25 ± 2ºC. Three, four and five days later, visual observations were made of the interaction by optical microscopy and/or SEM. Inoculated media were then incubated at 23, 25, 27 and 30°C, and the interaction was observed after three days. Inoculated media were then incubated at 23, 25, 27 and 30°C, and the interaction was observed after three days.

In vitro interaction between antagonists *B. cepacia* and *T. harzinum*: The interactions between the antagonists themselves were carried out as described for the interactions between them and the pathogen. The different culture media served to see the degree of compatibility between them. The PDA medium facilitated the optimal growth of de *P. capsici* and *T. harzianum*. In this medium, too, the bacteria developed very well and so we were able to confirm the inhibition of the pathogen by *B. cepacia* and *T. harzinum* and the compatibility between them. The symbiotic action of *B. cepacia* was confirmed after two days' incubation at 23, 25, 27, and 30°C, when the compatibility with the fungus *T. harzinum* was observed. The symbiotic action of *B. cepacia* was confirmed after two days' incubation at 23, 25, 27, and 30°C, when the compatibility with the fungus *T. harzinum* was observed.

Microscopic observations: Samples from the interaction zone between the pathogen and *B. cepacia* on PDA were analysed by scanning electron microscope (Jeol T- 6.100), processing the samples as follows: 2x2 mm sections of agar were taken from the interaction zone between the antagonist and *P. capsici.* The samples were immersed in a 1% aqueous solution of agar and prefixed at 4°C by immersion for 2 h in a solution of 3% glutaraldehyde (25%) in 0.1M sodium cacodylate (R 23/25, Merck) at pH 7.2, followed by three rinses with the same buffer for 30 minutes in the dark. They were then fixed by immersion in 1% osmium tetraoxide for 2 hours at 4°C in the dark. The samples were dehydrated in three 15 minute steps with increasing concentrations of ethanol (30, 50, 70, 90 and 100%) before drying at critical point (ethanol/liquid CO_2). The sections were mounted on a pedestal with conductive graphite paint and covered in gold sputter. The samples analysed by optical microscopy were taken from the interaction zone between *P. capsici* and *T. harzianum* on PDA medium on a glass support.

The inoculum of *T. harzianum* was prepared: a) in 1000 ml Erlenmeyer flasks containing a mixture of ground oat (8 g), 150 g of fine vermiculite and 250 ml distilled water (Avy-3 medium), b) in flasks containing 300 g vermiculite and 200 ml PDB medium and c) in flasks containing 300 g of sand, 150 ml of liquid Czapek medium (Difco). The flasks were sterilised twice at 121°C for 30 minutes on two consecutive days. Four 5 mm diameter discs cut from the edge of the mycelial colony grown on PDA were transferred aseptically to the flasks containing the prepared mixture. The flasks with oat, PDB and Czapek-Dox were incubated in a store at 25°C for 7 and 15 days, and more than a month.

The inoculum of *B. cepacia* was prepared in 500 ml Erlenmeyer flasks containing 150 ml of NB medium (Nutrient Broth, Difco) sterilised in an autoclave at 121°C for 30 minutes. This medium was

then inoculated with *B. cepacia* transferred from NA using a sterile rod. The flasks were incubated while being shaken at room temperature in the laboratory for three days. The bacteria were collected in sterile tubes and centrifuged at 15,000 rpm for 20 minutes. The precipitate was resuspended in 100 ml sterile distilled water and transferred to a 1000 ml Erlenmeyer flask containing 150 g fine vermiculite buffered in 150 ml sterilized NB. Finally, the flasks were incubated in an oven at 28° C for 5 days.

In vivo treatment of the pathogen with each antagonist and with a combination of *T. harzianum* and *B. cepacia*: Experiments were carried out in soils of the University of Murcia Experimental Field Station to evaluate the potential of each antagonist, separately and in combination, to control *P. capsici*. In all the assays 15 g of *P. capsici* grown in vermiculite-PDB was added directly to the pots in order to ensure infection. The initial population determined by the most probable number (MPN) (Collins *et al.,* 1991) varied between 1.2 x 10^6 and 8.1 x 10^7 propagules/g. The plants were cultivated as described above. The inoculum of the antagonists used was that prepared in vermiculite, oat and water at a rate of 5, 10, 15 and 20 g/pot. Samples (100 g) of the inoculated mixture were taken to calculate the initial population of antagonist by means of serial dilutions using a non-ionic detergent. Holes were made in the soil in a 70 x 40 cm design, into which 25 grams of vermiculite mixed with each antagonist or a combination of both were introduced along with one plantlet. After seven days the soil at the level of the rhizosphere was mixed to form a circle around the plants, which were then infected with *P. capsici*. The drip irrigation system used four self-compensating emitters per plant, providing 4 litres per hour, while fertilisation (NPK) was provided every three weeks by an injection system (ATF0040, Novo Ris, Zaragoza, Spain) connected to the irrigation line. Observations were taken at the end of the second month following transplantation both in control and the treated plants. The temperature during the experimental period varied between 25 and 30°C. One hundred plants per treatment were analysed and the treatments were repeated three times.

RESULTS AND DISCUSSION

Effect of antagonists against *P. capsici* in vitro

The extent of inhibition of *P. capsici* by *B. cepacia* varied according to the culture medium used, and appeared optimal on PDA (Fig. 2a). The interaction was also to an extent influenced by the incubating temperature and medium pH. On PDA, the inhibition zone produced by *B. cepacia* in its action against the pathogen increased with time, and it was accompanied by destruction of oomycete mycelium (Fig. 2d). The viability or death of the pathogen was periodically checked, and it was observed that the growth of the pathogen had been effectively suppressed.

Several authors have attributed the inhibition or destruction of pathogen mycelia to one or several antibiotics produced by bacteria (Elander *et al*., 1968; Rosales *et al*., 1995, Kraus & Loper, 1995; De La Fuente *et al*., 2001). Working with different strains of *Pseudomonas cepacia,* these authors detected antibiotics such as pirrolnitrine and pioluteorine, which have a wide antibiotic spectrum. The influence of incubation temperature and media pH on the antagonistic activity also needs to be taken into account. In Czapek-Dox and PDA media, the inhibition of *P. capsici* by *B. cepacia* at temperatures ≥ 25 °C was greater than that observed at 23°C. At pH 4.5 a notable reduction in the rate of growth and colony density of *P. capsici* was observed.

In the interactions between *B. cepacia* and *P. capsici* observed by Scanning Electron Microscopy (SEM) the alteration in the vegetative growth of the pathogen was noticed from the second day of interaction (Fig. 3d). The interactions in the inhibition zone were manifested in the form of morphological deformation, disorganisation and formation of spherical hypertrophy on the hyphae of *P. capsici,* while the disintegration of the mycelial walls (Fig. 3e) leads to the total destruction of the colony. These results demonstrate the particular parasitic ability of the antagonists, which, contributes to inhibition of pathogen growth.

T. harzianum showed a clearly antagonistic effect against *P. capsici* (Fig. 2b), especially on PDA medium enriched with laminarine-glucose (3:1, v/v), which is reported to increase the antifungal activity through secretion of the hydrolytic enzyme, β-1,3-glucanase (Lahsen *et al*., 2001; Ezziyyani, 2004). *T. harzianum* grew over and totally reduced the *P. capsici* colony. The intensity of *P. capsici* inhibition by *T. harzianum in vitro* varied with the culture medium, temperature and pH. Inhibition was intense on PDA,

and least on Water Agar. The inhibition zone produced by *T. harzianum* against the pathogen increased with time, an increase that was accompanied by the destruction of the fungal mycelium that had developed so far (Fig. 2e). The first thing to be noticed was suppressed growth of *P. capsici* with the suppression zone increasing as the growth of *T. harzianum* increased. This was followed by a marked hyperparasitic effect that was manifested by reduced pathogen mycelial growth, because the some *T. harzianum* isolate tested produces antibiotics (Wilson *et al.,* 2008) as 6-pentyl-alpha-pyrone (El-Hasan *et al.,* 2008) and enzymes (β-1,3- glucanase, chitinase, protease and cellulase), all of which degrade the cell wall and play an important role in mycoparasitism (Lorito *et al*. 1993, Papavizas & Lumsden, 1980; Herrera *et al.,* 1999, Sid Ahmed *et al*., 2000). After seven days, *T. harzianum* had totally invaded the *P. capsici* colony's surface and even sporulated on it. Under transmission microscope, the hyphae of *T. harzianum* could be seen coiling over and around those of the pathogen, further preventing its growth (Fig. 3 b, c).

Our results agree with those published by other (Elad *et al*., 1982, Bara *et al*., 2003) using laminarine as substrate in the culture media for strains of *Trichoderma*, who observed an increase in β-1,3-glucanase activity of up to 67% and greater lytic action against pathogens, e.g. *Sclerotinia rolfsii* and *Rhizoctonia solani*. Carpenter *et al.* (2005) using substractive hybridisation have found novel genes involved in the mycoparasitic interaction of *Trichoderma harzianum* with the phytopathogen *Sclerotinia sclerotiorum*. The proteins encoded by the novel genes included three monooxygenases, a metallo-endopeptidase, a gluconate dehydrogenase, an endonuclease and a proton ATPase. The strain transformed with genes for hydrolases showed an antifungal capacity against *Rhizoctonia solani* that was greater than that obtained with the wild strain. Other authors (Lahsen *et al*., 2001, Sousa *et al*., 2002, Sáez & Cipriano, 2003) reported to have cloned genes in strains of *T. harzianum* with a high degree of antifungal expression against *Phytophthora cinnamomi* and *Rosellinia necatrix*.

Interactions between *B. cepacia* and *T. harzianum*, assessed on various media resulted in zero inhibition of *T. harzianum* growth. The growth of *T. harzianum* continued until the fourth day, totally surrounding the colonies of *B. cepacia* (Fig. 2c, f), which provided macroscopic and microscopic evidence of their total compatibility. Optical microscopic examination showed that the hyphae of *T. harzianum* grew uniformly and showed no deformations or disorganisation. The temperature did not affect the symbiotic action between the two antagonists, at least between ambient temperature and 30°C. The pH only affected the rate of vegetative growth and pigmentation of the fungus, which changed from green to white. For this reason, the relationship established between the two selected antagonists, *T. harzianum* and *B. cepacia*, could be defined as an antagonistic symbiosis, and they can be used jointly against the pathogen.

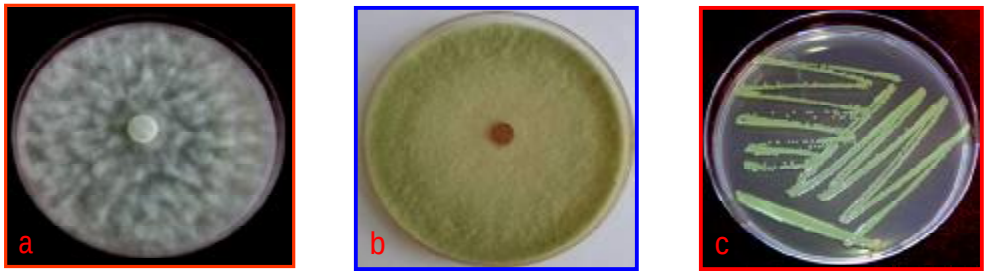

Figure 1: Growth of pathogen and the antagonists [a]: *Phytophthora capsici* on Potato Dextrose Agar (PDA) medium; [b]: *Trichoderma harzianum* on PDA; [c]: *Burkholderia cepacia* in Nutrient Agar.

Effect of antagonists against *P. capsici* in vivo.
From the results obtained in greenhouse comparing two pathogen inoculation methods, using infected vermiculite was determined to be better than using pathogen zoospores. In vermiculite with PDB, the pathogen is present in mycelial form which more virulent, while the zoospores of *P. capsici* even when added to the rhizosphere in optimal physiological condition for growth in the laboratory, lose their aggressive character, probably due to environmental reasons or due to the stress they undergo when transferred from a reduced substrate to the soil (Ezziyyani *et al*., 2007). Preparation of antagonist inoculum for treating the plants was best in AVy3 medium, which was cheapest and easiest to prepare, and resulting in fast growing, viable and abundant inoculum, with a high degree of inhibition of the rot caused by *P. capsici*. This medium was much better vermiculite-PDB and sand- liquid Czapek mixtures.

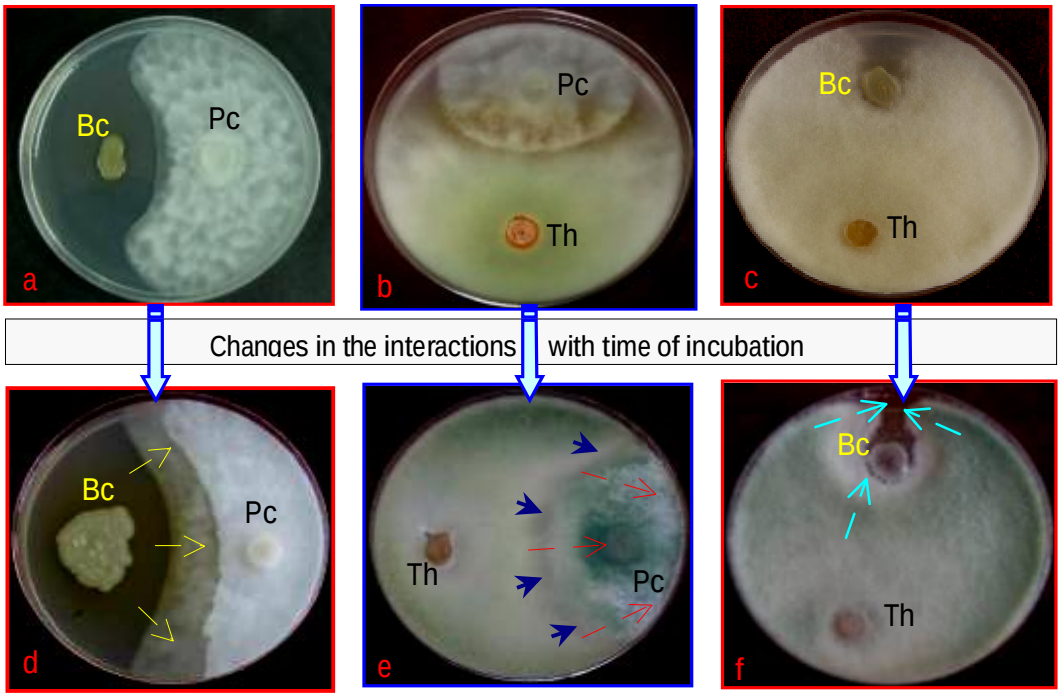

Figure 2: Interaction between *Phytophthora capsici* and the antagonists.
Fig. (a and d): Inhibition of the vegetative grow of *P. capsici (Pc)* by *B. cepacia (Bc)* on PDA. Fig. (b and e): Inhibition of the vegetative growth of *P. capsici* by *T. harzianum (Th)* on PDA. Fig. (c and f): Interaction between the antagonists *B. cepacia* and *T. harzianum* indicating good compatibility between them. The arrows indicate the interaction zones of the microorganisms.

The best result was obtained using *B. cepacia* at $1x10^9$ UFC.ml-1 and *T. harzianum* at $3.5×10^8$ spores/ml. In the plants: Non-treated and Inoculated (NT&I) with *P. capsici* root rot reached 5.0 on the scale used. Treatment of the plants with *T. harzianum* and *B. cepacia*: Treated and Inoculated (T&I) reduced rot severity to 3.15 and 2.22, respectively, equivalent to a reduction of 37.8 and 56%, respectively. Evaluation on scale of 0-5: 0, no symptom; 5, plant death. Maximum reduction of rot severity (1.26 on scale) was obtained when the roots were treated with a combination of the two antagonists (*T. harzianum* + *B. cepacia*), being equivalent to a 71% reduction in disease severity. Analysis of variance (ANOVA) showed significant differences for the interaction of two factors (time of death and antagonistic strain). Of greatest note was the delay in the appearance of rot symptoms in the plants treated and inoculated with the pathogen: Treated and inoculated (T&I), since some plants even reached flowering.

According to Ezziyyani *et al* (2004, 2007), the best treatment and/or inoculation method for a bio-

preparation is one in solid medium. Sztejnberg *et al*. (1987) observed that the effectiveness of *T. harzianum* is influenced by the application method. For example, vermiculite with PDB or NB is a good medium in which the antagonist is in mycelial or colonial form and fully virulent. In comparison, the cells of *B. cepacia* or *T. harzianum* spores, although added to the rhizosphere of the plant in optimal physiological conditions for growth in the laboratory, lose their effectiveness as a result of biotic and/or abiotic factors due to stress through transfer from microbial suspensions with reduced substrate to soil.

Based on our findings, we do not agree with Herrera López *et al*. (1999), who argued that the reduced establishment in soil of *T. harzianum* may be due to the antagonist using up its energy to produce secondary metabolites rather than for its own reproduction. However, our results confirm the observations of others (Smith *et al*., 1990; McLeod *et al*., 1995; Sid Ahmed *et al*., 2000) that the pre-selection of antagonist by means of dual cultures in vitro is useful, but does not guarantee its effectiveness in vivo under greenhouse or field conditions. The antagonistic effect was reduced in our experiment from practically 100% *in vitro* to between 37.8 - 71% *in vivo*. In view of these findings, we suggest that it might be a good strategy to study the *in vitro* and *in vivo* characteristics of each antagonist in the search for compatible combinations and to improve the production of solid-based bio preparations based on the abiotic and biotic factors.

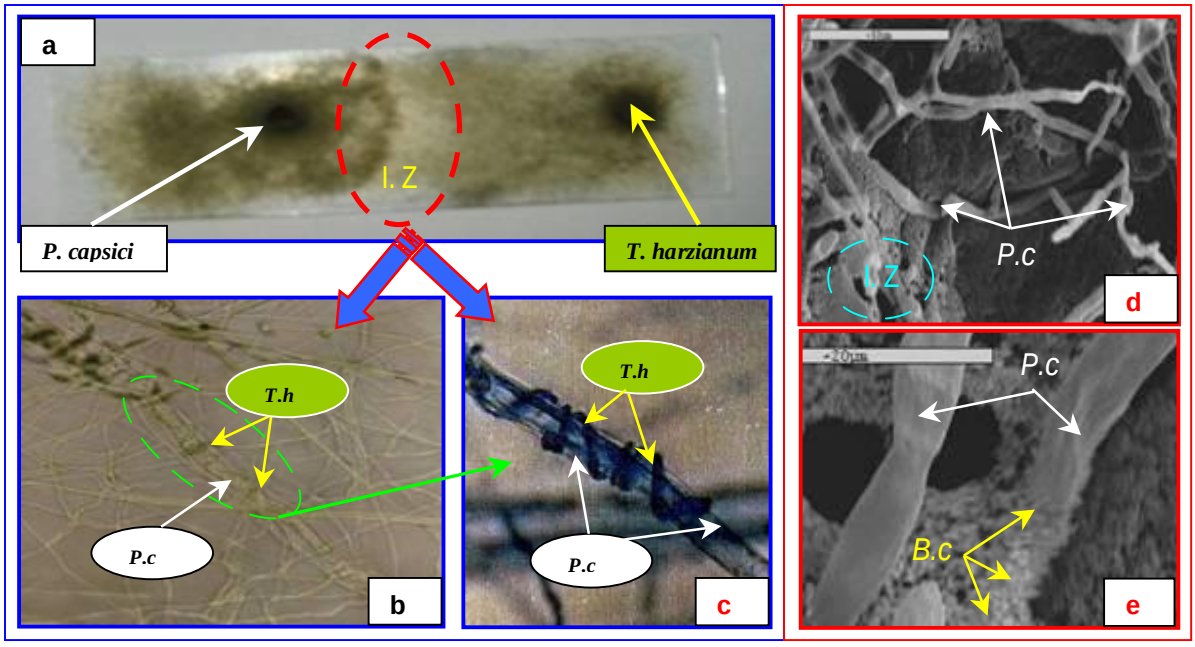

Figure 3: Optical microscopy (b and c) and Scanning electron microscope images (d and e) showing inhibition of the vegetative grow of *Phytohthora capsici* by *Trichoderma harzianum* on PDA medium:
(a) Interaction between *P. capsici* (*P.c.*) and *T. harzianum* (*T.h.*) on microscope glass slide. Interaction zone (I. Z) between *T. harzianum* and *P. capsici. T. harzianum* hyperparasitises and invades the entire *P. capsici* colony surface, sporulating on it. (b) *T. harzianum* parasitizes *P. capsici* inducing lysis and disintegration of *P. capsici* hyphae. (c) Microparasitism by massive envelopment of *P. capsici* hyphae around *T. harzianum*. (d) Disorganization on *P. capsici* hyphae by *B. cepacia* on PDA medium. Interaction zone (I. Z) between *B. cepacia* and *P. capsici* on PDA medium. (e) Details under SEM of adhesion, proliferation and hypertrophy on the *P. capsici* hyphae caused by *B. cepacia.* Disintegration of the *P. capsici* mycelium by sporulation of the bacterium over the oomycete hyphae.

Table 1: Effect of *in vivo* treatment with the antagonists on root rot in pepper plants caused by *P.capsici.* Evaluation on scale of 0-5: 0, no symptom; 5, plant death. The root rot values are the means per plant and treatment. Mean values followed by the same letters are not significantly different according to LSD at P = 0.05. Plants (T = treated; NT = non-treated; I = inoculated; NI = non-inoculated).

Treatments	*Phytophthora capsici rot severity*	Disease reduction
Control: (NT&NI)	0.00 a	---------
P. capsici: (NT&I)	5.00 c	---------
B. cepacia: (T&NI)	0.00 a	---------
T. harzianum : (T&NI)	0.00 a	---------
T. harzianum + B. cepacia: (T&NI)	0.00 a	---------
T. harzianum + P. capsici: (T&I)	3.15 b	37.80%
B. cepacia + *P. capsici*: (T&I)	2.22 a	65.00%
T. harzianum + *B. cepacia*+ *P. capsici*: (T&I)	1.26 a	71.00%

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