Phytopathol. Mediterr. (2006) 45, 231–237

Improving control of Fusarium wilt of leguminous plants by combined application of biocontrol agents

Omar A. ABDUL WAHID

Department of Botany, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt

Summary. In dual culture tests, *Trichoderma pseudokoningii* and *Bacillus subtilis* parasitized and inhibited the growth of *Fusarium oxysporum* f. sp. *fabae* and of *F. oxysporum* f. sp. *lupini*, which cause wilt on broad bean and lupine respectively. When applied to the seeds of these crops in field experiments, both antagonists controlled wilt of broad bean and lupine (75.2% healthy plants). A mixture of both biocontrol agents was more effective than either agent used alone. Moreover, the biocontrol agents provided a higher percentage of healthy plants than a fungicide tested for comparison. Fresh filtrate of both antagonists was more effective in suppressing pathogen growth than stored filtrate.

Key words: *Vicia faba*, *Lupinus termis*, combined control, broad bean, lupine.

Introduction

Leguminous plants are widely consumed all over the world. They are used to compensate for a lack of animal protein in the diet, especially by vegetarians. Broad bean and lupine are among the most important sources of non-animal proteins. These two crops are intensively utilized in Egypt as dry and green seeds. Both plants are subject to fungal diseases wherever they are grown. Fusarium wilt is a limiting factor that reduces yield. This disease is conventionally controlled by chemical fungicides, but as these crops are used for human and animal consumption, chemical fungicides represent hazardous choice for disease control. Instead, biological control offers a safer alternative way for disease control and the production of blemish-free plants. Many microorganisms have been tested for their potential as biocontrol agents. *Trichoderma* and *Bacillus* are commonly used to control many plant diseases.

The aim of this study was to evaluate the ability of *T. pseudokoningii* and *B. subtilis* to control Fusarium wilt of broad bean and lupine. Another aim was to test the interaction between the two antagonists in the field, and to discuss their role.

Materials and methods

Host, pathogen and biocontrol agents

The hosts used in this investigation were *Vicia faba* L. cultivar Giza 2 (broad bean) and *Lupinus termis* Folk. cultivar Giza 2 (lupine). The two wilt pathogens, *Fusarium oxysporum* f. sp. *fabae* Yu & Fang (Fof) and *F. oxysporum* f. sp. *lupini* Snyder & Hansen (Fol), were isolated previously from infected plants. They were identified according to Booth (1977) and were evaluated for their pathogenicity to their respective crop. The biocontrol candidates *Trichoderma pseudokoningii* Rifai and *Bacillus*

To correspond with the author:
E-mail: oabdulwahid@gawab.com

subtilis (Ehrenberg) Cohn were isolated from the soil of the Botanical Garden of the Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt, where the field experiments were conducted. All the organisms were maintained on potato dextrose agar (PDA) slopes until use.

Delivery of biocontrol agents

An experiment was designed to select the most suitable means of applying the candidate biocontrol organisms. This experiment was carried out in 30-cm diameter pots, each containing 5 kg naturally infested soil. The pots were divided into 3 groups. The first group of pots was planted with seeds coated with either *T. pseudokoningii* or *B. subtilis*. In the second group the top 5 cm of the soil was thoroughly mixed with wheat bran inoculated with either *T. pseudokoningii* or *B. subtilis*. The third group served as a control and did not receive any treatment. The last two groups were seeded with uncoated broad bean seeds.

Field experiment

The field experiment was carried out in the Botanical Garden of the Suez Canal University, in a plot that had a long history of vegetable and cereal cropping since it was regularly cultivated with various leguminous and cereal crops necessary for running the educational courses, such as morphology, pathology, economic botany, etc. The previous crop had been peanuts preceded by kidney beans and tomato. Some physical and chemical properties of the soil are given in Table 1. Though this field may harbour several pathogens, in this investigation we focused only on *F. oxysporum*. To evaluate the biocontrol capability of the candidate organisms, the seed coating technique (Windles and Kommedahl, 1982; Taylor *et al*., 1994; Zhang *et* al., 1996) was adopted since this technique was found to be the most suitable means of application. The candidate organisms were grown on PDA medium for an appropriate period of time, then the spores of *T. pseudokoningii* and the cells of *B. subtilis* were harvested by flooding the culture with sterile distilled water (SDW). Each candidate was separately mixed with sterilized carboxymethyl cellulose (CMC). The mixtures were intermingled with either broad bean or lupine seeds, and left to dry under aseptic conditions. The seeds thus coated were planted in naturally infested plots in the Botanical Garden of the Botany Department. Two controls were used, one of seeds coated with the fungicide Vitavax (2,3-dihydro-5-carboxanilido-6-methyl-1,4-oxathiin) (3 g kg^{-1} seed), and one of seeds coated with CMC only. The experimental design was a randomized complete block with four replicates.

Efficacy of antagonists *in vitro*

The interaction between the pathogen and the candidate antagonists was examined in dual culture. Both competitors were grown 4 cm apart on PDA dishes. The dishes were inoculated with 2 discs (5-mm diam) taken from the edge of 10-day old culture, except for *Bacillus* where the discs were taken after 24 h. The dishes were incubated at 28°C in the dark for a sufficient length of time to clarify the type of interaction. Depending on the growth rate of the opponents, the one growing more slowly was plated earlier than the one with faster growth.

The candidate organisms were also grown, singly or in combination with each other, in Czapek's yeast extract broth (CYEB). It should be noted that *B. subtilis* was grown in nutrient broth (NB) as well. Aliquots of 100 ml were dispensed in 250-ml Erlenmeyer flasks; each flask received a 1 ml cfu suspension of either candidate (10^5 cfu ml^{-1}). After a period of incubation, the broth was sterilized through a 0.22 mm Millipore filter. The effect of the filtrate was tested with the bore–diffusion

Table 1. Physical and chemical properties of the soil used in the experiment.

Parameter	Value
Sand (%)	94.5
Silt (%)	2.5
Clay (%)	3.0
Texture	Sandy
pH[a]	8.04
CaCO (%)	0.55
Ece (dsm^{-1})[b]	1.40
Organic (g kg^{-1} soil)	1.05
Total N (g kg^{-1} soil)	0.09
Available N (mg kg^{-1} soil)	6.45
Available (mg kg^{-1} soil)	4.08

[a] In soil-water suspension 1:2.5.
[b] In soil saturation extract.

method. It was tested either immediately, as fresh filtrate, or after being stored for four weeks in a refrigerator. Non-inoculated CYEB and SDW were used as controls.

Statistical analysis

Results were subjected to ANOVA and treatments were separated using the least significant differences (LSD) test (SAS, 1988).

Results

Delivery of biocontrol agents

Seed coating was superior to soil amendment for wilt control, giving a higher percentage of healthy plants (HP) with both biocontrol candidates. Nevertheless, even soil amendment achieved a highly significant improvement compared with the control (14% HP). *B. subtilis* was more effective than *T. pseudokoningii*. With *B. subtilis* 81% of plants were healthy, while with *T. pseudokoningii* the percentage of HP was only 77% (Fig. 1).

Field experiment

Both *T. pseudokoningii* and *B. subtilis* significantly reduced percent disease incidence in the field. They allowed the development of about 75.2% of HP. Each biocontrol agent was effective when applied alone. *Trichoderma* was highly effective in the control of *Fusarium* wilt of leguminous plants, causing about 66.1% HP compared with 33.3% HP in the control. *B. subtilis* was also effective against *F. oxysporum*, leading to about 61.4% HP. However, a mixture of these agents was most effective in wilt control. Although the two pathogens differed in their response, the difference between them was not significant. The biocontrol candidates were more effective than the chemical fungicide Vitavax, which gave 57.2% of HP, while the biocontrol mixture gave 75.2% (Table 2).

In vitro interaction of pathogens and candidates

Dual culture

When pathogens and candidates were paired *in vitro*, they showed two types of interactions: 1. mycoparasitism, in which *Trichoderma* parasitized *F. oxysporum* and overgrew the pathogen colony, restricting its growth; and 2. inhibition of *Fusarium* through the production of antifungal compounds. Both biocontrol candidates stopped the *Fusarium* some distance away from their own colonies.

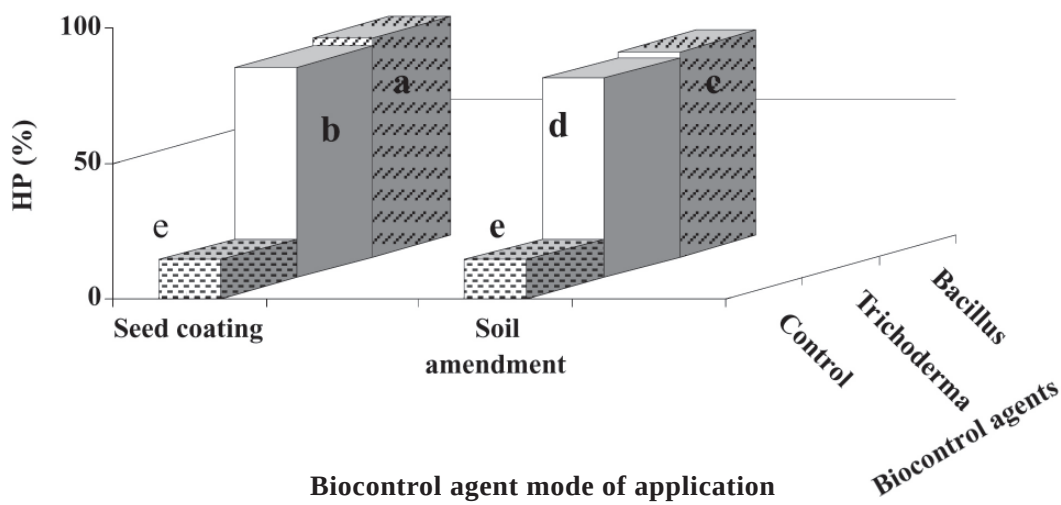

Fig. 1. Pot evaluation of the effect of the means of application (seed coating; soil amendment) of two biocontrol agents (*Bacillus subtilis*, *Trichoderma pseudokoningii*) on the percentage of healthy plants (% HP) of broad bean grown in Fusarium infested soil. In the control the seeds did not receive any treatment. LSD=1.1658. Means with the same letter are not significantly different (P=0.05).

Table 2. Field assessment of the effectiveness of biocontrol agents in controlling *Fusarium oxysporum* causing wilt of broad bean and lupine, expressed as a percentage of healthy plants.

Treatment	Healthy plants (%)		
	Broad bean	Lupine	Overall[a]
F. oxysporum	28.6	37.9	33.3 d
T. pseudokoningii	67.9	64.3	67.0 b
B. subtilis	62.4	60.3	61.4 bc
Mixture of the two biocontrol agents	73.6	76.8	75.2 a
Vitavax	56.6	57.71	57.2 c

[a] LSD=7.5559. Means with the same letter are not significantly different (P=0.05).

Effectiveness of the culture filtrates

To ensure the inhibition effectiveness of the biocontrol candidates, their filtrates were tested against both pathogens with the bore diffusion method. *Trichoderma* filtrate was more effective against both pathogens than *Bacillus* filtrate (Fig. 2). It was noticed moreover that a mixture of the filtrates of the two candidates was less effective than each one applied alone. Filtrate effectiveness, particularly that of *Bacillus*, decreased significantly after storage.

On the other hand the nutrient media had a strong influence on the effectiveness of the candidates. Filtrate of *B. subtilis* grown in NB more strongly inhibited both pathogens than *B. subtilis* filtrate derived from CYEB (Fig. 3).

Discussion

When using biocontrol agents to suppress pathogens, it is vital to introduce the agents into the soil in such a way as most likely to disturb the

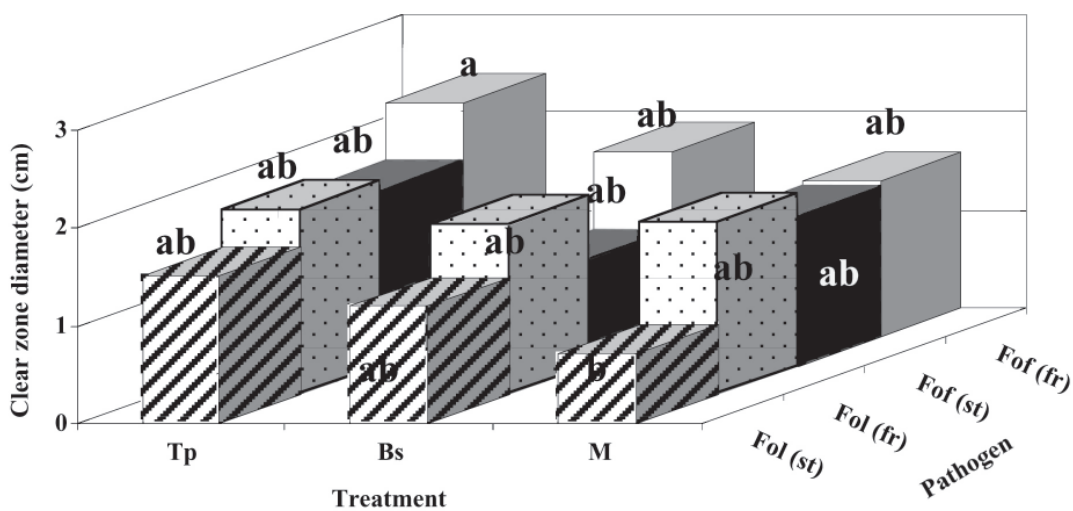

Fig. 2. Effect of filtrate of the biocontrol agents on the growth of the pathogens *in vitro* (expressed as clear zone diameter). LSD=1.2902. Means with the same letters are not significantly different (P=0.05). Tp, *Trichoderma pseudokoningii*; Bs, *Bacillus subtilis*; M, Mixture of these two organisms; Fof (fr), *Fusarium oxysporum* f. sp. *fabae*, fresh filtrate; Fol (fr), *F. oxysporum* f. sp. *lupini*, fresh filtrate; Fof (st), *F. oxysporum* f. sp. *fabae*, stored filtrate; Fol (st), *F. oxysporum* f. sp. *lupini*, stored filtrate.

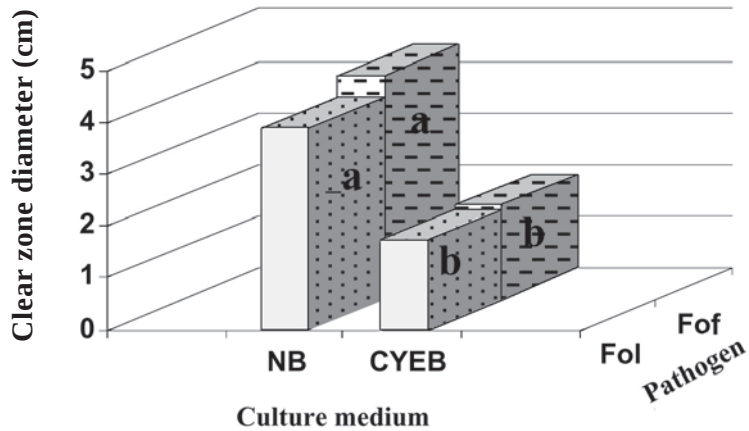

Fig. 3. Effect of the culture medium on the activity of the biocontrol agent (expressed as clear zone diameter). LSD=0.4442. Means with the same letters are not significantly different (P=0.05). NB, Nutrient broth, CYEB, Czapek's yeast extract broth; Fof, *Fusarium oxysporum* f. sp. *fabae*. Fol, *Fusarium oxysporum* f. sp. *lupini*.

microbial status in the spermosphere and rhizosphere of heavily infested soils. Although soil treatment with antagonist-inoculated wheat bran produced a high percentage of healthy plants, it was less effective than coating the seeds with the biocontrol agents. A wheat bran formulation may provide a nutrient source for resident mycobiota and other microorganisms to antagonize the biocontrol agents in their turn (Ristaino *et al*., 1994; Nagtzoam and Bollen, 1997). Coating seeds of broad bean and lupine with *T. pseudokoningii* and *B. subtilis* effectively controlled the wilt caused by *Fusarium oxysporum*. Seed coating is the most suitable method for biocontrol in both the spermosphere and the rhizosphere, where colonization of seeds and roots is very fast and extensive in a few hours, and is complete in a few days (Callan *et al*., 1990; Lewis, 1991) since the seed-coating agents will be the first colonizers (Windels, 1981; Nagtzoam and Bollen, 1997) and will move along the growing roots (Nagtzoam and Bollen, 1997).

The complexity of the soil ecosystem is a constraint that makes the biological control of root pathogens by means of antagonists a challenge (Pierson and Weller, 1994). The biocontrol organisms may become subject to the inimical behavior of other resident microorganisms (Paulitz *et al*, 1987; Seifert, 1988). To ensure good establishment of introduced candidates, they must be able to resist any metabolites secreted by other resident organisms. For this reason it is preferable to use native candidates rather than introduce foreign agents; native candidates have a better chance to be the first colonizer of the seeds (Cook, 1993).

Xifildou *et al*. (1998) found that *Trichoderma* spp. only lowered disease incidence when they were inoculated simultaneously with *F. oxysporum*, but that they effectively suppressed *F. oxysporum* when inoculated either before or after the pathogen (Michereff *et al*., 1995). The success of *Trichoderma* as a biocontrol agent is partly due to its production of a large variety of metabolites with diverse functions, to its aggressive growth, and to its hyperparasitism. The data of the present study indicate that mycoparasitism and antibiosis are two possible ways by which *Trichoderma* acts as a biocontrol agent.

Many *Bacillus* species have been used either alone or in combination to control fungal pathogens (Guetsky *et al*., 2002; Yan *et al*., 2002; Falconi *et al*., 2004). This can be attributed to a variety of mechanisms: the induction of systemic resistance, the promotion of host growth (Jacobsen *et al*., 2004; Kloepper *et al*., 2004) and/or antibiosis. The present study suggests that antibiosis was the control mechanism displayed by *B. subtilis*.

Given that the establishment of a threshold density for an antagonist is a key factor for biolog-

ical control, the use of mixtures of antagonists is a logical approach. Mixtures mimic a community more closely and exhibit multiple mechanisms of disease suppression (Sneh *et al*., 1984; Raaijmakers *et al*., 1995; Duffy *et al*., 1996; Schisler *et al*., 1997). In the present study, a combination of the two candidates reduced disease incidence (with 75.2% HP) more than either candidate used alone. This could be attributed to a synergistic interaction between the component antagonist organisms. According to Pierson and Weller (1994), the greater activity of a mixture is due to the biodiversity of the component organisms used. Subsequently mixtures are also more effective colonizers of the root and display a wider variety of traits inhibiting disease. The antagonists studied here may exhibit two mechanisms: mycoparasitism and antibiosis in a synergistic way. The occurrence of multiple mechanisms has also been suggested for other biocontrol agents (Dennis and Webster, 1971; Chang *et al*., 1986; Windham *et al*., 1986; Duffy and Weller, 1999; Yedidia *et al*., 2000; Tsahouridou and Thanassoulopoulos, 2001 a, b; Guetsky *et al*., 2002; Yan *et al*., 2002). Therefore using different antagonists in a mixture is a reliable means to reduce fluctuation and increase the reliability of biological control, and to control a wider range of pathogens (Larkin and Fravel, 1998; Guetsky *et al*., 2001), especially when the antagonists have different ecological and nutritional requirements as well as different mechanisms of action. In the present study, a mixture of antagonists was also superior to fungicides, including the fungicide Captan (Mao *et al*., 1997).

The durability of biocontrol agents may limit their application. In this study, the storage of metabolites impaired their effectiveness against *F. oxysporum*. Howell (1991) reported that the potency of *Trichoderma* species may be impaired by storage at ambient temperature. The duration of storage is a factor determining how long a biocontrol agent will remain commercially viable (Becker and Schwinn, 1993).

On the other hand, the starting media also have a strong influence on the biocontrol effectiveness of potential antagonists. In this study, *Bacillus* grown on NB was more active than *Bacillus* grown on Czapek's yeast extract broth. Larena *et al*. (2002) likewise found that the method of producing biocontrol agents strongly affected its effectiveness and shelf life.

Due to their biological properties the biocontrol agents studied offered good prospects for integrated disease management. However, human safety, effectiveness under commercial conditions and stability of action are important points that should be clarified before these antagonists can be considered to be powerful plant protectants.

Literature cited

Becker J.O. and F.J. Schwinn, 1993. Control of soil-borne pathogens with living bacteria and fungi: status and outlook. *Pesticide Science* 37, 355–363

Booth C., 1977. Fusarium: *Laboratory Guide to the Identification of the Major Species*. Commonwealth Mycological Institute, Kew, UK, 85 pp.

Chang Y.C., Y. Chang, R. Baker, O. Kleifeld and I. Chet, 1986. Increased growth of plants induced by the biological control agent *Trichoderma harzianum*. *Plant Disease* 70, 145–148.

Cook R.J., 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annual Review Phytopathol*ogy 31, 53–80.

Dennis L. and J. Webster, 1971. Antagonistic properties of *Trichoderma*. I, Production of non-volatile antibiotics. *Transactions of the British Mycological Society* 57, 25–39.

Duffy B. and D. Weller, 1999. *Trichoderma* used alone and combined with *Pseudomonas* for biocontrol of wheat take-all: inoculum dosage and placement effects. *Phytopathology* 89(6), S22 (abstract).

Duffy B.K., A. Simon and D.M. Weller, 1996. Combination of *Trichoderma koningii* with fluorescent *Pseudomonads* for control of take-all on wheat. *Phytopathology* 86, 188–194.

Falconi C.E, A.R. Oleas and V.R Yanez, 2004. Biological control of monilia pod rot (*Moniliophthora roreri*) on "high flavor" cocoa's field using biopesticides based on *Bacillus subtilis* and *Pseudomonas cepacia*. *Phytopathology* 94, S28 (abstract).

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

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

Howell C.R, 1991. Biocontrol of *Pythium* damping off of cotton with seed-coating preparations of *Gliocladium virens*. *Phytopathology* 81, 738–741.

Jacobsen B.J., N.K. Zidaek and B.J. Larson, 2004. The role of *Bacillus* based biological control agents in integrated pest management system: Plant diseases. *Phytopathology* 94, 1272–1275.

Kloeppor J.W., C-M. Ryu and S. Zhang, 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259–1266.

Larena I, P. Melgarigo and A. De Col, 2002. Production, survival, and evaluation of solid substrate inocula of *Penicillium oxalicum*, a biocontrol agent against *Fusarium* wilt of tomato. *Phytopathology* 92, 863–869.

Larkin R.P. and D.R. Fravel, 1998. Efficacy of various fungal and bacterial biocontrol organisms for control of *Fusarium* wilt of tomato. *Plant Disease* 82, 1022–1028.

Lewis J.A., R.D. Lumsden and J.C. Locke, 1996. Biocontrol of damping-off diseases caused by *Rhizoctonia solani* and *Pythium ultimum* with alginate pills of *Gliocladium virens*, *Trichoderma hamatum*, and various food bases. *Biocontrol Science and Technol*ogy 6, 163–173.

Mao W., J.A. Lewis, P.K. Hebbar and R.D. Lumsden, 1997. Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*. *Plant Disease* 81, 450–454.

Michcreff S.J., N.S.S. da Silveira, A. Reis and R. Mariano, 1995. Greenhouse screening of *Trichoderma* isolates for control of *Curvularia* leaf spot of yam. *Mycopathologia* 130, 103–108

Nagtzoam M.P.M. and G.J. Bollen, 1997. Colonization of roots of eggplant and potato by *Talaromyces flavus* from coated seed. *Soil Biology and Biochemistry* 299, 1499–1507.

Paulitz T.C., C.S. Park and R. Baker, 1987. Biological control of *Fusarium* wilt of cucumber with nonpathogenic isolates of *Fusarium oxysporum*. *Canadian Journal of Microbiology* 33, 349–353.

Pierson E.A. and D.M. Weller, 1999. Use of mixtures of fluorescent *Pseudomonads* to suppress take-all and improve the growth of wheat. *Phytopathology* 84, 940–947.

Raaijmakers J.M., M. Leeman, M.M.P. van Oorschot, I. Vander Sluis, B. Schippers and P.A.H.M. Bakker, 1995. Dose-response relationships in biological control of *Fusarium* wilt of radish by *Pseudomonas* spp. *Phytopathology* 85, 1075–1081

Ristaino J.B., J.A. Lewis and R.D. Lumsden, 1994. Influence of isolate *Gliocladium virens* and delivery system on biological control of southern blight on carrot and tomato in field. *Plant Disease* 78, 153–156.

Schisler D.A, P.J. Slininger and R.J. Bathast, 1997. Effects of antagonist cell concentration and two-strain mixtures on biological control of *Fusarium* dry rot of potatoes. *Phytopathology* 87, 177–183.

Seifert K.A, 1988. Biological control and wood protection. Canadian wood preservers Association. *Proceedings of the 9th Annual Meeting*, 124–137.

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 Protiction*, 12, 380–386.

Sneh B., M. Dupler, Y. Elad and R. Baker, 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from *Fusarium* suppressive soil. *Phytopathology* 74(9), 1115–1124.

Taylor A.G., G.E. Harman and P.A. Nielson, 1994. Biological seed treatments using *Trichoderma harzianum* for horticultural crops. *Horticulture Technology* 4,105–108.

Tsahouridou P.C. and C.C. Thanassoulopoulos, 2001a. Parasitism of *Sclerotium rolfsii* Sacc. sclerotia by the fungus *Trichoderma koningii* Rifai. *Phytopathologia Mediterranea* 40, 95 (abstract).

Tsahouridou P.C. and C.C. Thanassoulopoulos, 2001b. Biological control of *Sclerotium rolfsii* Sacc. with *Trichoderma* spp. *Phytopathologia Mediterranea* 40, 95 (abstract).

Windham M., Y. Elad and R. Baker, 1986. A mechanism of increased plant growth induced by *Trichoderma* spp. *Phytopathology* 76, 518–521.

Windels C.E., 1981. Growth of *Penicillium oxalicum* as a biological seed treatment on pea seed in soil. *Phytopathology* 71, 929–933.

Windels C.E. and T. Kommedahl, 1982. Rhizosphere effects of pea seed treatment with *Penicillium oxalicum* in the field. *Phytopathology* 72, 190–194.

Xifildou S., F.T. Gravanis and A.C. Brugger, 1998. Wilt disease caused by isolates of *Fusarium oxysporum* and *F. oxysporum* var. *redolens* on tomatoes and biological effect of *Trichoderma viride* and *T. hamatum* on disease severity. Ninth Hellenic Phytopathological congress, Athens, Greece, 20–22 October 1998 (abstract).

Yan Z., M.S. Reddy, C.M. Ryu, J.A. Mc Inroy, M. Wilson and J.W. Klopper, 2002. Induced systemic protection against tomato late blight, elicited by plant growth promoting rhizobacteria, *Phytopathology* 92, 1329–1333.

Yedidia I., N. Benhamou, Y. Kapulnik and I. Chet, 2000. Activation of plant defense responses following the colonization and penetration of cucumber roots by *Trichoderma harzianum*. *Plant Physiology and Biochemistry* 38, 863–873.

Zhang J., C.R. Howell and J.L. Starr, 1996. Suppression of *Fusarium* colonization of cotton roots and *Fusarium* wilt by seed treatments with *Gliocladium virens* and *Bacillus subtilis*. *Biocontrol Science and Technol*ogy 6, 175–187.

Accepted for publication: October 8, 2006