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Effects of Commercial and Indigenous Microorganisms on Fusarium Wilt Development in Chickpea[1]

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The purpose of this research was to determine whether *Bacillus subtilis*, nonpathogenic *Fusarium oxysporum*, and/or *Trichoderma harzianum*, applied alone or in combination to chickpea (*Cicer arietinum* L.) cultivars 'ICCV 4' and 'PV 61' differing in their levels of resistance to Fusarium wilt, could effectively suppress disease caused by the highly virulent race 5 of *Fusarium oxysporum* f. sp. *ciceris*. Seeds of both cultivars were sown in soil amended with the three microbial antagonists, alone or in combination, and 7 days later seedlings were transplanted into soil infested with the pathogen. All three antagonistic microorganisms effectively colonized the roots of both chickpea cultivars, whether alone or in combination, and significantly suppressed Fusarium wilt development. In comparison with the control, the incubation period for the disease was delayed on average about 3 days and the final disease severity index and standardized area under the disease progress curve were reduced significantly between 14 and 33% and 16 and 42%, respectively, by all three microbial antagonists. Final disease incidence only was reduced by *B. subtilis* (18–25%) or nonpathogenic *F. oxysporum* (18%). The extent of disease suppression was higher and more consistent in 'PV 61' than in 'ICCV 4' whether colonized by *B. subtilis*, nonpathogenic *F. oxysporum*, or *T. harzianum*. The combination of *B. subtilis* + *T. harzianum* was effective in suppressing Fusarium wilt development but it did not differ significantly from treatments with either of these antagonists alone. In contrast, the combination of *B. subtilis* + nonpathogenic *F. oxysporum* treatment was not effective but either antagonist alone significantly reduced disease development. © 1998 Academic Press

***Key Words:* *Bacillus* spp., *Cicer arietinum*, nonpathogenic *Fusarium oxysporum*, *Fusarium oxysporum* f. sp. *ciceris*, *Trichoderma harzianum*, biological control, Fusarium wilt.**

INTRODUCTION

The chickpea (*Cicer arietinum* L.) is one of the most important food legumes grown worldwide (Saxena, 1990). In the European Union, chickpea production is concentrated in the Mediterranean Basin, with Spain being the principal producer. One of the major constraints to chickpea production in this area and many other parts of the world is Fusarium wilt caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato. This disease can completely destroy the crop (Halila and Strange, 1996) or cause significant annual yield losses (Trapero-Casas and Jiménez-Díaz, 1985).

Crop rotation, pathogen-free seed, removal of plant debris, and fungicide seed treatment are several of the disease management strategies that have been employed for control of Fusarium wilt (Nene and Reddy, 1987), but have met with limited success. Host plant resistance appears to offer the best practical and economical strategy for control of this disease. Good progress has been made in the identification of sources of resistance to Fusarium wilt (Haware *et al.*, 1990; Jiménez-Díaz *et al.*, 1991) in both "desi" (small, angular, colored seeds) and "kabuli" (large, ramhead-shaped, beige seeds) germplasm, and "kabuli" cultivars resistant to Fusarium wilt have been developed. However, the effectiveness of these resistant cultivars may be curtailed because of the occurrence of races in *F. oxysporum* f. sp. *ciceris* pathogenic to them. To date, seven races of this pathogen, designated 0 to 6, have been identified in California, India, Morocco, Spain, and Tunisia (Halila and Strange, 1996; Haware and Nene, 1982; Jiménez-Díaz *et al.*, 1993).

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Another strategy attempted in recent years for the management of Fusarium wilt and other diseases of chickpea caused by soilborne pathogens is biological control using bacterial or fungal antagonists (Hervás *et al.,* 1995, 1997; Kaiser *et al.,* 1989; Kaur and Mukhopadhyay, 1992; Kumar and Dube, 1992; Landa *et al.,* 1997a,b; Vidhyasekaran and Muthamilan, 1995). For example, Kaur and Mukhopadhyay (1992) demonstrated that the "chickpea-wilt complex" caused by several soilborne fungi, including *F. oxysporum* f. sp. *ciceris,* was effectively controlled by *Trichoderma harzianum* Rifai. Hervás *et al.* (1995) showed that treatment of germinated chickpea seeds with either nonpathogenic races of *F. oxysporum* f. sp. *ciceris* or nonpathogenic isolates of *F. oxysporum* could suppress Fusarium wilt. Application of *Pseudomonas fluorescens* Migula to chickpea seeds also significantly reduced Fusarium wilt incidence while increasing the yields over the control by more than 100% (Vidhyasekaran and Muthamilan, 1995). Similar results were obtained when seed bacterization with a fluorescent pseudomonad RBT13 suppressed Fusarium wilt by 52% while increasing seed germination, growth, and yield of chickpea (Kumar and Dube, 1992). More recently, species of *Bacillus* were isolated from the rhizosphere of chickpea and demonstrated to inhibit conidial germination and hyphal growth of *F. oxysporum* f. sp. *ciceris* (Landa *et al.,* 1997b) and suppress Fusarium wilt development (Landa *et al.*, 1997a).

Selection of biocontrol agents for controlling diseases such as Fusarium wilt of chickpea has emphasized the use of individual agents. However, it would seem logical that increasing the number of biological control agents as a mixture may result in treatments that could persist longer in the rhizosphere, provide a wider array of biocontrol mechanisms, and/or function under a broader range of environmental conditions, especially if these mixtures were of different species (Pierson and Weller, 1994). Several attempts already have been made using nonpathogenic *F. oxysporum* and different *Pseudomonas* species (Lemanceau and Alabouvette, 1993; Lemanceau *et al.,* 1993; Park *et al.,* 1988). In these studies, the combinations of the pseudomonads and nonpathogenic *F. oxysporum* were more effective in controlling Fusarium wilt than when these antagonists were used individually. Duffy and Weller (1995) demonstrated that combinations of *Gaeumannomyces graminis* Arx & Olivier var. *graminis* with strains of fluorescent *Pseudomonas* spp. were significantly more suppressive of take-all of wheat than either treatment alone but only under greenhouse conditions.

These aforementioned findings suggest a potential for reducing plant stress and suppressing root diseases such as Fusarium wilt of chickpea. Moreover, the combination of selected species of microorganisms may be synergistic. Based on this hypothesis, using a combination of two or more biocontrol agents for controlling a root disease has the possibility of reducing soil populations of the pathogen, as well as limiting root necrosis and/or wilting to severity levels below those experienced with either agent alone. In addition, the effect of the microbial combination may further enhance limited or partial resistance while improving overall plant health, and host genotypes differing in resistance to the pathogen can differ in their ability to support biological control (i.e., Hervás *et al.,* 1997; Smith *et al.,* 1997; Van Peer *et al.,* 1991). Therefore, the purpose of this research was to determine whether three antagonistic microorganisms, namely *Bacillus subtilis* (Ehrenberg) Cohn, nonpathogenic *F. oxysporum* isolate Fo 90105, and *T. harzianum,* applied either alone or in combination to two chickpea genotypes with different levels of resistance to Fusarium wilt, could effectively suppress disease development caused by the highly virulent *F. oxysporum* f. sp. *ciceris* race 5.

MATERIALS AND METHODS

Microorganisms and Inoculum Production

Bacillus subtilis isolate GB03 from the commercial formulation Kodiak HB (Gustafson, Inc., Dallas, TX), which contained 1.2×10^{10} endospores per gram of the formulation, was used. *Trichoderma harzianum* strain KRL-AG2 from the commercial formulation T-22G (BioWorks Inc., Geneva, NY), containing 1×10^{7} cfu per gram dry weight, was used. *Fusarium oxysporum* f. sp. *ciceris* race 5 isolate Foc 8012 and nonpathogenic *F. oxysporum* isolate Fo 90105 were also used in this study. Isolate Foc 8012 was obtained from an infected chickpea in southern Spain and has been used in previous studies (Hervás *et al.,* 1995, 1997; Jiménez-Díaz *et al.,* 1991). Isolate Fo 90105 was originally isolated from the root of a healthy chickpea grown in a naturally infested field at Santaella, Córdoba, southern Spain. This isolate has been shown to be effective against Fusarium wilt of chickpea either when seedlings were inoculated with a conidial suspension of Fo 90105 prior to being challenged with *F. oxysporum* f. sp. *ciceris* race 5 (Hervás *et al.,* 1995) or treated seeds were sown in soil artificially infested with the pathogen (Hervás *et al.,* 1997). Monoconidial fungal isolates were stored in sterile soil in tubes at 4°C. Active cultures were obtained from small aliquots of soil plated on potato–dextrose agar (PDA) and incubated at 25°C with a 12-h photoperiod of fluorescent and near UV light at 36 $\mu E.m^{-2}.s^{-1}$.

Inoculum of nonpathogenic *F. oxysporum* isolate Fo 90105 was increased in a cornmeal–sand (CMS) mixture (Trapero-Casas and Jiménez-Díaz, 1985) and incubated under the same conditions as the stock cultures for 2 weeks. The inoculum concentration was determined by dilution plating the CMS inoculum on V8

juice–oxgall–PCNB agar (VOPA), a *Fusarium*-selective medium (Bouhot and Rouxel, 1971), and incubating the plates as above for 7 days. The inoculum concentration was about 8.3×10^7 colony forming units (cfu) per gram of CMS. Inoculum of *F. oxysporum* f. sp. *ciceris* consisted of chlamydospores used as previously reported (Hervás *et al.*, 1997). Chlamydospore suspensions were mixed with sterile soil and stored at 4°C.

Growth Chamber Experiments

Three experiments (I, II, III) were conducted to determine the ability of *T. harzianum*, nonpathogenic *F. oxysporum*, and *B. subtilis*, either alone or in combination, to suppress the development of Fusarium wilt caused by *F. oxysporum* f. sp. *ciceris* race 5 in 'kabuli' chickpea cvs. 'ICCV 4' and 'PV 61.' These cultivars are susceptible to race 5, but completely resistant to races 0 and 1 and moderately resistant to race 0 of *F. oxysporum* f. sp. *ciceris*, respectively (Jiménez-Díaz *et al.*, 1993; and unpublished; Kumar *et al.*, 1985). Seeds were surface-disinfested in 2% NaOCl for 3 min, washed three times in sterile distilled water, and dried for several hours under a stream of filtered air.

For Experiment I, disinfested seeds were sown in plastic trays ($60 \times 40 \times 10$ cm; 120 seeds per tray) filled with a sterile soil mixture (clay loam/peat, 2:1, vol/vol) amended with *T. harzianum* or *B. subtilis*, either alone or in combination. Experiment II was conducted using nonpathogenic *F. oxysporum* isolate Fo 90105 and *B. subtilis* as the biological control agents applied to sterile soil, either alone or in combination. For Experiment III, five treatments were used: *B. subtilis* (Bs), *F. oxysporum* (Fo), *T. harzianum* (Th), Bs + Fo, and Bs + Th. Seeds sown in sterile soil served as the controls for the three experiments. The inoculum concentrations of the biocontrol agents were the following: *T. harzianum* and nonpathogenic *F. oxysporum* were amended at 1×10^5 cfu per gram of soil, whereas *B. subtilis* was amended at 1×10^6 cfu per gram of soil. Seedlings were grown in a growth chamber adjusted to 25°C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 $\mu E.m^{-2}.s^{-1}$ for 7 days. Seedlings were then removed from the trays, selected for uniformity, and transplanted into 15-cm-diameter clay pots (four plants per pot) filled with a soil mixture not infested (control) or infested with chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5. The inoculum concentration of *F. oxysporum* f. sp. *ciceris* race 5 in the infested soil was determined before use by dilution plating on VOPA medium. In Experiment I there were two inoculum concentrations of the pathogen (1.3×10^3 and 6.7×10^3 cfu per gram of soil), whereas in Experiments II and III the pathogen was used at 3.7×10^3 and 1.2×10^3 cfu per gram of soil, respectively. Plants were grown in a growth chamber for at least 40 days as described previously. Plants were watered as needed and fertilized weekly with 100 ml of Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Rhizosphere Colonization by Biological Control Agents

In all experiments, colonization of 'ICCV 4' and 'PV 61' by *B. subtilis*, nonpathogenic *F. oxysporum*, and *T. harzianum* was determined on 10 seedlings selected at random per treatment just before transplanting. Seedlings were uprooted delicately from the trays and shaken gently to remove all but the most tightly adhering rhizosphere soil. The roots were cut into 1-cm segments, and 1 g of these segments was placed into 10 ml of sterile water and sonicated for 15 min to remove bacteria and/or fungi from the roots. Serial dilutions of the washings were plated onto salt V8 agar *Bacillus*-selective medium (Turner and Backman, 1991), VOPA, and *Trichoderma* medium E (Papavizas and Lumsden, 1982) to determine population of *B. subtilis*, nonpathogenic *F. oxysporum*, and *T. harzianum*, respectively, expressed as cfu/g of fresh root tissue.

Dual Cultures

A dual culture experiment was conducted *in vitro* to assay the ability of *B. subtilis* to inhibit hyphal growth of *F. oxysporum* f. sp. *ciceris* race 5, nonpathogenic *F. oxysporum* Fo 90105, and *T. harzianum*, as well as to observe interactions between colonies of *F. oxysporum* f. sp. *ciceris* race 5 and the two fungal antagonists. Assays were performed in petri plates containing PDA. *Bacillus subtilis* was first incubated in the dark for 24 h at 30°C. Mycelial disks, cut from actively growing colonies of fungal isolates, were placed 3 cm from the bacterial colonies. Dual cultures of the respective fungal antagonist and *F. oxysporum* f. sp. *ciceris* race 5 were prepared by placing PDA disks from the growing margins of fresh fungal cultures onto PDA. Each combination of microorganisms was replicated five times. The bacterial and fungal isolates were plated separately as controls. Plates were incubated at 25°C and a 12-h photoperiod of fluorescent and near UV light at 36 $\mu E.m^{-2}.s^{-1}$.

Disease Assessment and Data Analyses

Disease reactions were assessed by the incidence (percentage) and severity of symptoms at 2- to 4-day intervals using a 0 to 4 rating scale of the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Percentage values were arcsine transformed $(Y/100)^{1/2}$ before analysis of variance. Data on disease severity were used to calculate the following: (i) a disease intensity index (DII) determined as $DII = (\Sigma S_i \times N_i) \div (4 \times N_t)$, where S_i is the symptoms severity, N_i is the number of plants with S_i symptoms severity, and N_t is the total number of plants; (ii) incubation period (IP) established as the number of

days from transplanting until DII > 0; (iii) final disease severity index (final DII); and (iv) standardized area under the disease progress curve of DII plotted over time (days) (SAUDPC) calculated according to Campbell and Madden (1990). All experiments were conducted following a factorial design with four replications in a randomized complete block design (four pots per replication and four plants per pot). The factors were two cultivars, three antagonistic microorganisms and, in Experiment I, two inoculum concentrations of the pathogen. Data were analyzed by analysis of variance using Statistix (NH Analytical Software, Roseville, MN). There was variance heterogeneity between the two chickpea cultivars according to the Bartlett's test of equal variances. Therefore, separate analyses were performed for each cultivar. Treatment means for the IP, the final DII, the final disease incidence (DI), and SAUDPC were compared using Fischer's protected least significant difference (LSD) test at $P = 0.05$.

RESULTS

Dual Cultures

Bacillus subtilis inhibited the *in vitro* hyphal growth of *F. oxysporum* f. sp. *ciceris* race 5, nonpathogenic *F. oxysporum* Fo 90105, and *T. harzianum*. Growth of *F. oxysporum* f. sp. *ciceris* race 5 was suppressed also by *T. harzianum*. Four days after placing both cultures onto PDA, the colony of *F. oxysporum* f. sp. *ciceris* was completely overgrown by *T. harzianum*, and *T. harzianum* had begun to sporulate profusely. *Trichoderma harzianum* killed the mycelia of *F. oxysporum* f. sp. *ciceris* because the pathogen failed to grow when transferred to fresh PDA. Antagonism was not observed between the pathogenic isolate and nonpathogenic *F. oxysporum* Fo 90105 in dual cultures on PDA.

Rhizosphere Colonization by Biological Control Agents

The roots of chickpea cvs. 'ICCV 4' and 'PV 61' were colonized by *B. subtilis*, nonpathogenic *F. oxysporum* Fo 90105, and *T. harzianum* whether alone or in combination in all experiments (Table 1). Since there was no variance heterogeneity among experiments according to Bartlett's test for equal variances, results from all experiments were pooled for data analysis. Mean population sizes of *B. subtilis* and *T. harzianum*, whether alone or in combination, were not significantly different ($P = 0.05$) between the two chickpea cultivars (Table 1). Mean population size of *B. subtilis* alone was significantly ($P < 0.05$) higher than that for the Bs + Fo combination on 'ICCV 4,' whereas population size for the *B. subtilis* treatment alone was significantly higher than that for both combination treatments on 'PV 61.'

Significant differences in population sizes of *T. harzianum* were not observed between *T. harzianum* alone and the combination of Bs + Th (Table 1). However, there was a 10-fold increase in the population of *T. harzianum* on the roots in all treatments from the initial amended population of 1×10^5 cfu/g soil. Also, significant differences were not observed between the population sizes of nonpathogenic *F. oxysporum* Fo 90105 alone and that in the combination treatment of Bs + Fo for either chickpea cultivar. However, mean population sizes of the nonpathogenic *F. oxysporum* on the roots of the Bs + Fo treatment were significantly ($P < 0.05$) higher on 'ICCV 4' than on 'PV 61.'

TABLE 1

Populations of *Bacillus subtilis* Isolate GB03, *Trichoderma harzianum* Isolate KRL-AG2, and Nonpathogenic *Fusarium oxysporum* Isolate Fo 90105 Recovered from Rhizosphere Soil of 7-Day-Old Chickpeas 'ICCV 4' and 'PV 61' after Soil Amendment with the Microorganisms

	Mean population[a] (Log cfu/g fresh root)					
	B. subtilis[b]		*T. harzianum*[c]		*F. oxysporum*[d]	
Treatment	ICCV4	PV61	ICCV4	PV61	ICCV4	PV61
B. subtilis (Bs)	6.01[e] a	6.21 a	—	—	—	—
F. oxysporum (Fo)	—	—	—	—	4.99	5.02
T. harzianum (Th)	—	—	6.25	6.24	—	—
Bs + Fo	5.78 b	5.42 c	—	—	5.10 A	4.79 B
Bs + Th	5.79 ab	5.87 b	6.13	6.24	—	—

[a] Populations of the microorganisms were determined as described under Materials and Methods.
[b] *Bacillus subtilis* isolate GB03 was amended to the soil at 1×10^6 cfu/g from a commercial formulation (Kodiak, HB).
[c] *Trichoderma harzianum* strain KRL-AG2 was amended to the soil at 1×10^5 cfu/g from a commercial formulation (T-22G).
[d] Nonpathogenic *Fusarium oxysporum* isolate Fo 90105 was increased in a CMS mixture and amended to the soil at 1×10^5 cfu/g.
[e] Data are the average of three experients for *B. subtilis* and two experiments for *T. harzianum* and nonpathogenic *F. oxysporum* from four 1-g fresh root samples per treatment. Means followed by the same lowercase letter or no letter are not significantly different according to Fisher's protected LSD ($P = 0.05$). Comparisons between treatments within a cultivar are indicated with lowercase letters. Comparisons between chickpea cultivars within a treatment are indicated with capital letters.

Growth Chamber Experiments

Experiment I: Effect of B. subtilis and T. harzianum alone and in combination on Fusarium wilt development in chickpea cvs. 'ICCV 4' and 'PV 61'. Prior colonization of chickpea seedling roots by either *B. subtilis* alone, *T. harzianum* alone or in combination, and inoculum concentration of *F. oxysporum* f. sp. *ciceris* race 5 influenced the development of Fusarium wilt in cvs. 'ICCV 4' and 'PV 61' (Fig. 1, Table 2). Symptoms were not observed in control plants nor in plants treated with the biocontrol agents grown in noninfested soil (data not shown). Control plants grown in infested soil showed wilt symptoms characteristic of those caused by *F. oxysporum* f. sp. *ciceris* race 5 in the two cultivars. Disease developed severely reaching a final DI of 93–100% (Fig. 1, Table 2). Disease development was not significantly ($P = 0.05$) influenced by the inoculum concentration of the pathogen, except for the final DII. Thus, the final DII for both 'ICCV 4' and 'PV 61' was higher with an inoculum concentration of *F. oxysporum* f. sp. *ciceris* race 5 at 6.7×10^3 cfu/g soil than with 1.3×10^3 cfu/g of soil (Table 2).

Colonization of 'PV 61' roots by *B. subtilis,* whether alone or in combination with *T. harzianum* (but not *T. harzianum* alone), resulted in significantly ($P < 0.05$) less disease only at the lowest inoculum concentration (1.3×10^3 cfu/g of soil) of *F. oxysporum* f. sp. *ciceris* race 5 (Table 2). Thus, this bacterial treatment significantly ($P < 0.05$) delayed the IP by 4 to 5 days and reduced the final DII by 25.3 to 32.5%, SAUDPC by 32.6 to 40.8%, and the final DI by 18.8 to 21.8%, compared with the control at this same inoculum concentration of the pathogen. These effects were not apparent at the higher inoculum concentration (6.7×10^3 cfu/g soil) of *F. oxys-*

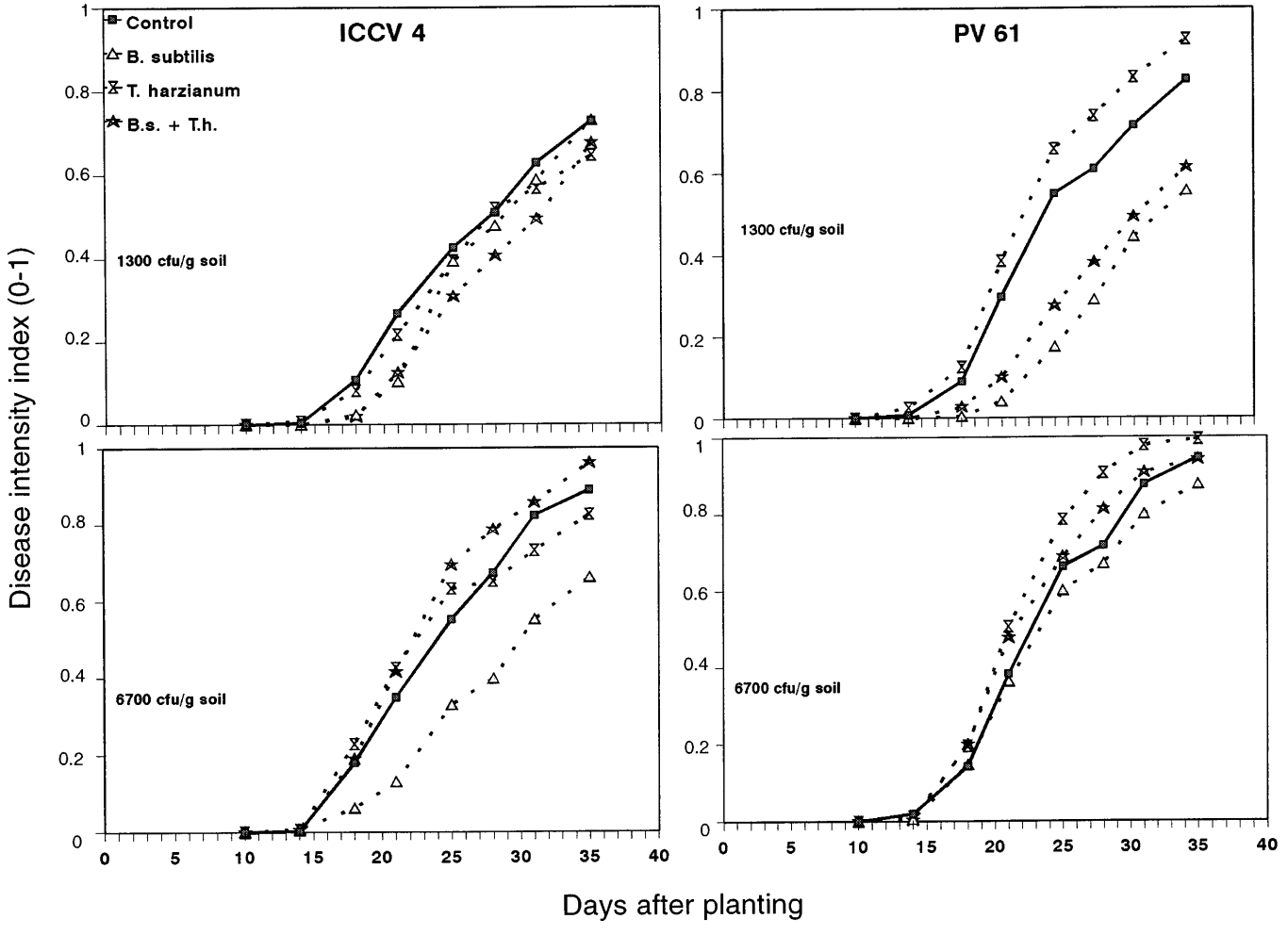

FIG. 1. Effect of *Bacillus subtilis* isolate GB03 and *Trichoderma harzianum* strain KRL-AG2, alone and in combination, on the development of Fusarium wilt in susceptible chickpea cvs. 'ICCV 4' and 'PV 61.' Seeds were sown in sterile soil amended with a commercial formulation (Kodiak, HB) of *B. subtilis,* with a commercial formulation (T-22G) of *T. harzianum,* or with the two microorganisms. Seven-day-old seedlings were transplanted into soil artificially infested with 1.3×10^3 or 6.7×10^3 cfu/g of *Fusarium oxysporum* f. sp. *ciceris* race 5. Disease was assessed by means of a disease intensity index calculated from data on incidence and severity of symptoms. Data are the average of four replications with four pots per replication and four plants per pot.

TABLE 2

Effect of *Bacillus subtilis* (Bs) and *Trichoderma harzianum* (Th), Alone and in Combination, on Fusarium Wilt Development in Susceptible Chickpeas 'ICCV 4' and 'PV 61' Transplanted into Soil Infested with *Fusarium oxysporum* f. sp. *ciceris* Race 5

Cultivar	Race 5 (cfu/g soil)[a]	Treatment[b]	Disease assessment[c] (35 days)			
			IP (days)	Final DII	SAUDPC	Final DI (%)
ICCV 4	1300	Control	20.2 b	0.73 *	0.40	93.7
		Bs	23.4 a	0.73	0.42	95.3
		Th	20.6 ab	0.65 *	0.38 *	89.1
		Bs + Th	23.4 a	0.68 *	0.36 *	87.5
	6700	Control	18.1 b	0.90 ab	0.50 a	98.4
		Bs	22.5 a	0.66 c	0.37 b	92.2
		Th	19.8 b	0.83 b	0.53 a	96.9
		Bs + Th	18.5 b *	0.96 a	0.58 a	100
PV 61	1300	Control	20.0 b	0.83 a *	0.49 a	95.3 ab
		Bs	24.7 a	0.56 b *	0.29 b *	73.4 c
		Th	18.6 b	0.92 a *	0.57 a	100 a
		Bs + Th	23.7 a	0.62 b *	0.33 b *	76.6 bc
	6700	Control	18.8	0.94	0.58	100
		Bs	19.8*	0.88	0.53	95.3
		Th	17.8	0.99	0.65	100
		Bs + Th	20.6*	0.95	0.83	98.4

[a] Seven-day-old seedlings were transplanted into soil artificially infested with chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5 at the given inoculum concentrations. Plants were grown in a growth chamber adjusted to 25°C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 35 days.

[b] Seeds were sown in sterile soil amended with a commercial formulation (Kodiak, HB) of *Bacillus subtilis* isolate GB03 (1×10^6 cfu/g), with a commercial formulation (T-22G) of *Trichoderma harzianum* strain KRL-AG2 (1×10^5 cfu/g), or with the two microorganisms. For control treatment, seeds were sown in sterile soil.

[c] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2- or 3-day intervals. DII were plotted over time to develop curves of disease increase. IP, Incubation period (number of days until DII > 0). SAUDPC, Standardized area under the disease progress curves of DII increase over time. DI, Disease incidence (%). Data are the average of four blocks with four replicated pots/block, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were arcsine transformed $(Y/100)^{1/2}$ for analyses of variance. There was variance heterogeneity between the two chickpea cultivars ('ICCV 4' or 'PV 61') according to the Bartlett's test of equal variances. There was a significant inoculum concentration × treatment interaction. Means in a column followed by the same letter or no letter for each inoculum concentration are not significantly different according to Fisher's protected LSD ($P = 0.05$). Means in a column followed by an asterisk are significantly smaller than the mean for the corresponding treatment at the other inoculum concentration.

porum f. sp. *ciceris* race 5. At this inoculum concentration, the IP was significantly lower ($P < 0.05$), and the final DII and SAUDPC were significantly higher ($P < 0.05$) than those at the lowest inoculum concentration of the pathogen.

Treatment of 'ICCV 4' seedlings with *B. subtilis* alone or in combination with *T. harzianum* significantly delayed ($P < 0.05$) the IP by 3 days compared with the control at the lowest inoculum concentration of the pathogen (Table 2), but had no effect on the final DII, SAUDPC, and final DI. On the other hand, at the highest inoculum concentration of *F. oxysporum* f. sp. *ciceris* race 5 only *B. subtilis* alone was able to significantly ($P < 0.05$) delay the IP by 4 days and reduce both the final DII and SAUDPC by 26% compared with the control. There was no effect on the final DI. Disease development in plants treated with *B. subtilis* alone was not significantly influenced by the pathogen's inoculum concentration. However, in plants treated with *T. harzianum* alone or in combination with *B. subtilis,* both the final DII and SAUDPC were significantly higher ($P < 0.05$) with the pathogen at 6.7×10^3 cfu/g soil than at 1.3×10^3 cfu/g soil.

Experiment II: Effect of B. subtilis and nonpathogenic F. oxysporum alone and in combination on Fusarium wilt development in chickpea cvs. 'ICCV 4' and 'PV 61'. Colonization of chickpea seedling roots by either *B. subtilis* alone or the nonpathogenic *F. oxysporum* Fo 90105 alone, prior to transplanting them into soil infested with *F. oxysporum* f. sp. *ciceris* race 5, reduced to some extent SAUDPC and the final DI and DII in 'ICCV 4' and 'PV 61.' However, the net disease suppression by the biocontrol treatments varied with the cultivars (Fig. 2, Table 3). Symptoms did not develop in controls, nor in plants treated with the biocontrol agents grown in noninfested soil (data not shown). In control plants grown in soil infested with the pathogen, disease developed to reach near the maximum level (Fig. 2, Table 3). Also, as observed in the previous experiment, treatment of 'PV 61' plants with *B. subtilis* alone as well as the nonpathogenic *F. oxysporum* Fo

90105 alone delayed the IP by 3 days (significant at $P < 0.05$) and affected the final DI, DII, and SAUDPC (Table 3). For *B. subtilis*, the final DII was reduced by 31.5%, SAUDPC by 42.1%, and the final DI by 25%, over the control. For the nonpathogenic *F. oxysporum* alone, the final DII was reduced by 23.9%, SAUDPC by 26.3%, and the final DI by 18.8%, over the control. However, disease after the joint treatment with the two biocontrol agents was not significantly different ($P = 0.05$) from that in the control.

For 'ICCV 4,' both of these biocontrol treatments alone were able to affect SAUDPC (Table 3). *Bacillus subtilis* alone reduced the SAUDPC by 12.9% compared to the control, while the nonpathogenic *F. oxysporum* Fo 90105 did by 20.4%. Although there were no significant differences between these two biocontrol treatments in their reduction of SAUDPC, only that achieved by the nonpathogenic *F. oxysporum* was significantly ($P < 0.05$) lower than the control.

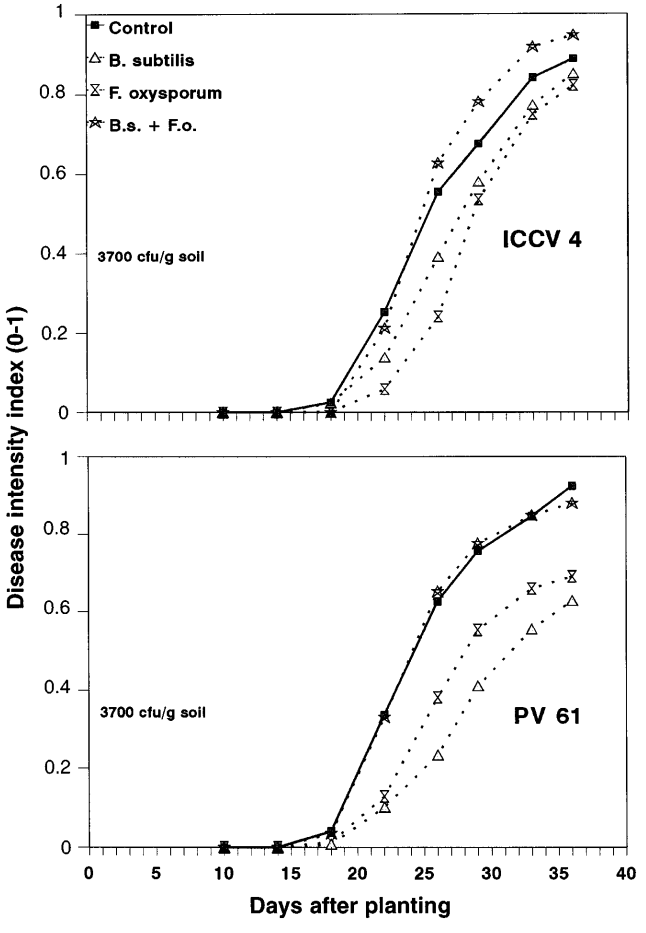

FIG. 2. Effect of *Bacillus subtilis* isolate GB03 and nonpathogenic *Fusarium oxysporum* isolate Fo 90105, alone and in combination, on the development of Fusarium wilt in susceptible chickpea cvs. 'ICCV 4' and 'PV 61.' Seeds were sown in sterile soil amended with a commercial formulation (Kodiak, HB) of *B. subtilis*, with a nonpathogenic *F. oxysporum*, or with the two microorganisms. Seven-day-old seedlings were transplanted into soil artificially infested with 3.5×10^3 cfu/g of *Fusarium oxysporum* f. sp. *ciceris* race 5. Disease was assessed by means of a disease intensity index calculated from data on incidence and severity of symptoms. Data are the average of four replications with four pots per replication and four plants per pot.

TABLE 3

Effect of *Bacillus subtilis* (Bs) Isolate GB03 and Nonpathogenic *Fusarium oxysporum* (Fo) Isolate Fo 90105, Alone and in Combination, on Fusarium Wilt Development in Susceptible Chickpeas 'ICCV 4' and 'PV 61' Transplanted into Soil Infested with *Fusarium oxysporum* f. sp. *ciceris* Race 5

		Disease assessment[b] (36 days)			
Cultivar	Treatment[a]	IP (days)	Final DII	SAUDPC	Final DI (%)
ICCV 4	Control	22.2	0.91	0.54 ab	98.4
	Bs	23.4	0.85	0.47 bc	95.3
	Fo	24.3	0.83	0.43 c	100
	Bs + Fo	23.2	0.95	0.62 a	100
PV 61	Control	20.7 c	0.92 a	0.57 a	100 a
	Bs	24.1 a	0.63 b	0.33 b	75.0 b
	Fo	23.7 ab	0.70 b	0.42 b	81.2 b
	Bs + Fo	21.7 bc	0.88 a	0.59 a	93.7 ab

[a] Seeds were sown in sterile soil amended with either a commercial formulation (Kodiak, HB) of *Bacillus subtilis* isolate GB03 (1×10^6 cfu/g), with nonpathogenic *Fusarium oxysporum* isolate Fo 90105 (1×10^5 cfu/g) or with both microorganisms. For control treatment, seeds were sown in sterile soil. Seven days after seeding in a growth chamber, seedlings were transplanted into soil artificially infested with chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5 at 3.7×10^3 cfu/g soil. Plants were grown in a growth chamber adjusted to 25°C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 36 days.

[b] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2- or 3-day intervals. DII were plotted over time to develop curves of disease increase. IP, Incubation period (number of days until DII > 0). SAUDPC, Standardized area under the disease progress curves of DII increase over time. DI, Disease incidence (%). Data are the average of four blocks with four replicated pots/block, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were arcsine transformed $(Y/100)^{1/2}$ for analyses of variance. There was variance heterogeneity between the two chickpea cultivars ('ICCV 4' or 'PV 61') according to the Bartlett's test of equal variances. Means in a column followed by the same letter or no letter are not significantly different according to Fisher's protected LSD ($P = 0.05$).

Experiment III: Effect of B. subtilis, nonpathogenic F. oxysporum, and T. harzianum alone and in combination on Fusarium wilt development in chickpea cvs. 'ICCV 4' and 'PV 61'. As for the previous experiment, disease in control plants grown in pathogen-infested soil developed to its maximum level. Root colonization by *B. subtilis*, nonpathogenic *F. oxysporum* Fo 90105, or *T. harzianum* alone, or in combinations, prior to transplanting seedlings to soil infested with *F. oxysporum* f. sp. *ciceris* race 5 had variable effects on the development of Fusarium wilt in 'ICCV 4' and 'PV 61' (Fig. 3,

Table 4). For 'PV 61' only the *B. subtilis* + *T. harzianum* treatment significantly delayed ($P < 0.05$) the IP by 3 days compared with the control (Table 4). The final DII also was significantly ($P < 0.05$) reduced but only by treatments *T. harzianum* (14.1%) and *B. subtilis* + *T. harzianum* (16.2%). In addition, all the biocontrol treatments were able to reduce the SAUDPC by 8.6 to 28.4%, but only the nonpathogenic *F. oxysporum* Fo 90105 alone, *T. harzianum* alone, *B. subtilis* + nonpathogenic *F. oxysporum*, and *B. subtilis* + *T. harzianum* were significantly ($P < 0.05$) different from the control. None of these treatments had any affect on the final DI.

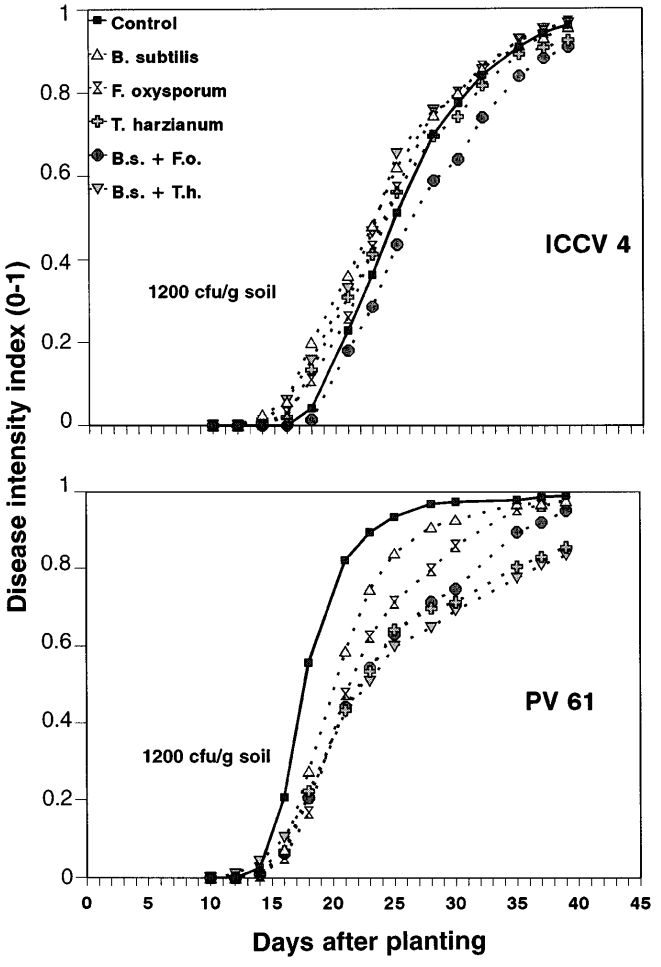

FIG. 3. Effect of *Bacillus subtilis* isolate GB03, nonpathogenic *Fusarium oxysporum* isolate Fo 90105, *Trichoderma harzianum* strain KRL-AG2, Bs + Fo, or Bs + Th on the development of Fusarium wilt in susceptible chickpea cvs. 'ICCV 4' and 'PV 61.' Seeds were sown in sterile soil amended with a commercial formulation (Kodiak, HB) of *B. subtilis*, with a nonpathogenic *F. oxysporum*, or with a commercial formulation (T-22G) of *T. harzianum*, either alone or in combination. Seven-day-old seedlings were transplanted into soil artificially infested with 1.2×10^3 cfu/g of *Fusarium oxysporum* f. sp. *ciceris* race 5. Disease was assessed by means of a disease intensity index calculated from data on incidence and severity of symptoms. Data are the average of four replications with four pots per replication and four plants per pot.

TABLE 4

Effect of *Bacillus subtilis* (Bs), Nonpathogenic *Fusarium oxysporum* (Fo) Isolate Fo 90105, *Trichoderma harzianum* (Th), Bs + Fo, or Bs + Th on Fusarium Wilt Development in Susceptible Chickpeas 'ICCV 4' and 'PV 61' Transplanted into Soil Infested with *Fusarium oxysporum* f. sp. *ciceris* Race 5

		Disease assessment[b] (39 days)			
Cultivar	Treatment[a]	IP (days)	Final DII	SAUDPC	Final DI (%)
ICCV 4	Control	20.2 ab	0.96	0.61	100 a
	Bs	17.7 c	0.95	0.63	100 a
	Fo	18.8 bc	0.97	0.62	100 a
	Th	19.8 abc	0.92	0.61	100 a
	Bs + Fo	21.9 a	0.91	0.58	96.9 b
	Bs + Th	19.1 bc	0.96	0.65	100 a
PV 61	Control	16.2 b	0.99 a	0.81 a	100
	Bs	17.1 b	0.98 a	0.74 ab	100
	Fo	17.7 ab	0.97 a	0.68 bc	100
	Th	18.0 ab	0.85 b	0.60 c	93.7
	Bs + Fo	17.0 b	0.95 a	0.61 c	100
	Bs + Th	19.3 a	0.83 b	0.58 c	93.7

[a] Seeds were sown in sterile soil amended with a commercial formulation (Kodiak, HB) of *Bacillus subtilis* isolate GB03 (1×10^6 cfu/g), with nonpathogenic *Fusarium oxysporum* isolate Fo 90105 (1×10^5 cfu/g), or with a commercial formulation (T-22G) of *Trichoderma harzianum* strain KRL-AG2 (1×10^5 cfu/g), either alone or in combination. For the control treatment, seeds were sown in sterile soil. Seven days after seeding in a growth chamber, seedlings were transplanted into soil artificially infested with chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5 at 1.2×10^3 cfu/g soil. Plants were grown in a growth chamber adjusted to 25°C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 39 days.

[b] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2- or 3-day intervals. DII were plotted over time to develop curves of disease increase. IP, Incubation period (number of days until DII > 0). SAUDPC, Standardized area under the disease progress curves of DII increase over time. DI, Disease incidence (%). Data are the average of four blocks with four replicated pots/block, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were arcsine transformed $(Y/100)^{1/2}$ for analyses of variance. There was variance heterogeneity between the two chickpea cultivars ('ICCV 4' or 'PV 61') according to the Bartlett's test of equal variances. Means in a column followed by the same letter or no letter are not significantly different according to Fisher's protected LSD ($P = 0.05$).

For 'ICCV 4,' the biocontrol treatments generally had no effect on the IP, the final DII, SAUDPC, or the final DI, except that the *B. subtilis* treatment significantly decreased the IP over the control, whereas *B. subtilis* + nonpathogenic *F. oxysporum* significantly decreased the final DI (6.2%) (Table 4).

DISCUSSION

Bacillus subtilis, nonpathogenic *F. oxysporum*, and *T. harzianum* each were effective in suppressing Fu-

sarium wilt in chickpea to a degree. This is in agreement with previous studies where these species individually or in combination with other antagonists have been used to suppress this and other diseases caused by *Fusarium* spp. (Datnoff *et al.*, 1995; Hervás *et al.*, 1995, 1997; Kaur and Mukhopadhyay, 1992; Landa *et al.*, 1997a,b; Lemanceau and Alabouvette, 1993; Lemanceau *et al.*, 1993; Park *et al.*, 1988). All three microorganisms were able to significantly delay the onset of disease (the time to first observable visible symptoms of Fusarium wilt) by about 3 days. In one experiment, *B. subtilis* was able to delay the onset of disease by 4 to 5 days. If the incubation period can be delayed by at least 3 days, the final disease severity index (DII), SAUPDC, and more importantly the final disease incidence (DI) can be reduced. In this study, the final disease incidence was reduced between 18 and 25%. This would be an important disease parameter to manage when trying to improve chickpea yields under field conditions.

The two chickpea cultivars used varied in the level of wilt suppression achieved when their roots were colonized by the different antagonists. The extent of disease protection was always higher and more consistent in 'PV 61' than in 'ICCV 4.' Hervás *et al.* (1997) made the same observation in a related study when seeds of these two cultivars were treated with conidia of the nonpathogenic *F. oxysporum* isolate Fo 90105. Apparently, this cultivar reaction is not unique to only the nonpathogenic *F. oxysporum* since the same phenomenon occurred when roots of 'PV 61' seedlings were colonized by either *B. subtilis* or *T. harzianum*. Although both 'ICCV 4' and 'PV 61' are susceptible to the highly virulent race 5 of *F. oxysporum* f. sp. *ciceris*, they differ genetically. 'ICCV 4' was bred for resistance to race 1 of the pathogen (Kumar *et al.*, 1985) and must carry two genes in the recessive form conferring resistance against races 0 and 1 (Jiménez-Díaz *et al.*, 1993; Upadhyaya *et al.*, 1983a,b). In contrast, 'PV 61' has not been genetically manipulated and carries no major gene for resistance to races 1 or 5 of *F. oxysporum* f. sp. *ciceris* (R. M. Jiménez-Díaz, unpublished). Thus, it is likely that the two cultivars differ in residual, incomplete resistance, as well as in specific resistance. Several authors have observed that the efficiency in disease suppression can vary among cultivars differing in resistance to the pathogen. Van Peer *et al.* (1991) showed that *Pseudomonas* sp. strain WCS417 suppressed Fusarium wilt development in the moderately resistant carnation cv. 'Pallas' more efficiently than in the susceptible cv. 'Lena,' but Leeman *et al.* (1995) showed that *P. fluorescens* WCS374 protected each of six radish cultivars differing in susceptibility to *F. oxysporum* Schlechtend.: Fr. f. sp. *raphani* Kendrick & Snyder. More recently, Smith *et al.* (1997) demonstrated that tomato lines differing in resistance to *Pythium torulosum* Coker & F. Patterson also differ in support of biocontrol by *Bacillus cereus* UW85. However, lines representing the lowest and highest levels of resistance to the pathogen are equally nonsupportive of biocontrol, suggesting that the two traits are independent. The disease suppression conferred to 'PV 61' by *B. subtilis*, nonpathogenic *F. oxysporum*, and *T. harzianum* but not to 'ICCV 4' cannot be attributed solely to the differences in the ability of these microorganisms to colonize the rhizosphere of the two cultivars, especially since root colonization by these microorganisms showed no cultivar specificity. Possibly, induction of host resistance by these microorganisms before the parasitic phase of *F. oxysporum* f. sp. *ciceris* is partly responsible for the reduction in overall wilt development in 'PV 61.' Liu *et al.* (1995) showed that disease suppression mediated by systemic resistance induced in cucumber by PGPR strains was cultivar specific, although root colonization by these strains was not. However, modification by the plant of factors such as antibiotics that contribute to efficacy of biocontrol cannot be ruled out (Smith *et al.*, 1997).

In our experiments, the nonpathogenic *F. oxysporum*, *B. subtilis*, or *T. harzianum*, individually, was able to suppress wilt development to the same general degree even though the particular mode of action probably differs among all three microorganisms. For example, one reported mode of action for *B. subtilis* is antibiosis, while for *T. harzianum* competition for infection sites, mycoparasitism, and/or antibiosis have been implicated. The mode of action for the nonpathogenic *F. oxysporum* probably is competition for infection sites, nutrients, and/or induced resistance. Although our expectations that the combination of these antagonistic microorganisms would be more effective than each alone was not supported in these studies, the combination of *B. subtilis* + *T. harzianum* was able to effectively reduce the disease assessments in these experiments. However, in each of the experiments this combination treatment was not better than *T. harzianum* alone or *B. subtilis* alone. These results are somewhat similar to those of Datnoff *et al.* (1995), where Fusarium crown and root rot of tomato were reduced in commercial field situations with a combination of species of *Trichoderma* and *Glomus*, and the effect of this combination was not better than that of either species alone. In another study, the association of nonpathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. suppressed Fusarium wilt more efficiently and consistently than each antagonistic microorganism separately (Lemanceau and Alabouvette, 1993; Park *et al.*, 1988). In contrast, *P. fluorescens* neither inhibited nor enhanced the biocontrol activity of *T. harzianum* (Dandurand and Knudsen, 1993).

Success of establishment of microorganisms in the rhizosphere might be dependent on the delivery system. In our studies, chickpea seeds were sown into soil

amended with the antagonistic microorganisms, and seedlings were grown for at least 7 days to allow good root colonization before being transplanted and challenged by the pathogen. This transplant system is not relevant to commercial chickpea cropping practices; however, it was selected to utilize the full potential of these antagonistic microorganisms to protect the plant from *F. oxysporum* f. sp. *ciceris*. Our reasoning was that seed treatment with the antagonists might not be efficient enough to protect the root infection courts. Loper *et al.* (1984) demonstrated that colonization of roots by selected microbes placed on seeds can occur in a log-normal distribution; consequently, microbial populations decrease on the root as the root moves away from the court of microbial activity. This relates to results by Hervás *et al.* (1997), who recently demonstrated that colonization of chickpea roots and protection against Fusarium wilt were more efficient in a transplant system as opposed to a seed treatment. Similarly, Landa *et al.* (1997a) demonstrated that seed + soil treatment was more efficient for establishing species of *Bacillus* for biological control than either seed or soil treatment alone.

Mixtures of compatible biocontrol agents are an ecologically sound approach to biocontrol of soilborne diseases, especially when used in combination with limited or partial resistance. Mixtures of different species of microorganisms may result in better plant colonization, may be better adapted to environmental changes, may present a large number of pathogen suppressive mechanisms, and/or may protect against a broad range of pathogens (Duffy and Weller, 1995). However, several areas need improvement if the maximum potential of mixtures of microorganisms is to be attained. These will include improved formulations, more efficacious strains, and appropriate adjuvants for biocontrol agents. Better delivery systems also are needed as well as more information about specific cultivar responses to the presence of beneficial microorganisms.

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