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Influence of chickpea genotype and *Bacillus* sp. on protection from Fusarium wilt by seed treatment with nonpathogenic *Fusarium oxysporum*

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Abstract

Seeds of kabuli chickpea cultivars ICCV 4 and PV 61 were treated with conidia of nonpathogenic *Fusarium oxysporum* isolate Fo 90105 suspended in methylcellulose (3×10^6 conidia.seed^{-1}), or with methylcellulose alone, and sown in soil artificially infested with 500 or 1,000 chlamydospores.g^{-1} of *F. oxysporum* f. sp. *ciceris* race 5. At an inoculum concentration of 500 chlamydospores.g^{-1}, seed treatment with Fo 90105 significantly increased the incubation period of the disease by 11 (ICCV 4) or 25 (PV 61) days, and reduced the final disease incidence, disease intensity and the standardized area under the curve of disease intensity over time. This protection from disease was higher and more consistent in PV 61 than in ICCV 4. However, it was annulled with an inoculum concentration of 1,000 chlamydospores.g^{-1}, except for the incubation period in PV 61 which was increased by 10 days. When ICCV 4 seeds were treated with Fo 90105 (3×10^6 conidia.seed^{-1}) and/or *Bacillus* sp. isolate RGAF 51 (1×10^7 cfu.seed^{-1}), then sown in infested soil, there was no influence by the *Bacillus* isolate on protection conferred by Fo 90105. However, the degree of protection by the nonpathogenic *F. oxysporum* was higher and more consistent when plants from treated seeds were grown in sterile sand for 6 days, then transplanted into infested soil.

Introduction

Chickpea (*Cicer arietinum* L.) is the world's third most important legume crop after beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) (Saxena, 1990). Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) Matuo and K. Sato, is a major constraint to production wherever chickpeas are grown, particularly in the Indian Subcontinent and the Mediterranean Basin (Haware, 1990; Nene and Reddy, 1987). Yield losses due to the disease have been estimated to amount 10% in India (Singh and Dahiya, 1973) and Spain (Trapero-Casas and Jiménez-Díaz, 1985) and 40% in Tunisia (Bouslama, 1980).

F. o. ciceris can survive in soil several years by means of chlamydospores (Haware et al., 1996), which markedly reduces the potential of crop rotation as a disease management strategy. The most effective and practical method for control of the disease worldwide is the use of resistant cultivars (Haware, 1990; Jiménez-Díaz et al., 1991; Kraft et al., 1994; Nene and Reddy, 1987), the effectiveness of which is threatened by the occurrence of pathogenic races in *F. o. ciceris* (Haware and Nene, 1982; Jiménez-Díaz et al., 1989; 1993). Because 'kabuli' chickpeas (large, ramhead-shaped, beige seeds) grown in the Mediterranean Basin are susceptible to most *F. o. ciceris* races (Jiménez-Díaz and Trapero-Casas, 1990), efforts must be addressed toward developing new alternatives for more effective disease management.

Nonpathogenic isolates of *F. oxysporum* are among the most promising biocontrol agents against Fusarium wilt diseases caused by formae speciales of *F. oxysporum* (Alabouvette et al., 1987; 1993; Mandeel and Baker, 1991; Ogawa and Komada, 1985; Paulitz et al., 1987). Studies on mechanisms of Fusarium wilt suppressiveness occurring in some natural soils showed the involvement of nonpathogenic *F. oxysporum*, so that the introduction of strains of this fungus into conducive soils made them suppressive to the dis-

ease (Alabouvette, 1986; Paulitz et al., 1987; Schneider, 1984). Recently, Larkin et al. (1996) have concluded that nonpathogenic *F. oxysporum* was the primary antagonist responsible for the suppressiveness to Fusarium wilt of watermelon, which had been induced in soil by successive cultivation (monoculture) of a specific watermelon cultivar. Mechanisms responsible for disease suppression by nonpathogenic *F. oxysporum* include saprophytic competition for nutrients at or near the root surface (Alabouvette, 1986; Alabouvette and Couteaudier, 1992; Lemanceau, 1989), parasitic competition for infection sites on the root (Schneider, 1984), and induced systemic resistance in the host (Matta, 1989; Ogawa and Komada, 1985). Mandeel and Baker (1991) demonstrated that these mechanisms are not neccessarily exclusive of one another. Indeed, they observed that all three mechanisms were active in a single isolate of *F. oxysporum.*

Several studies have shown that prior root inoculation with nonpathogenic strains of *F. oxysporum* or races of *F. oxysporum* f. spp. can protect various hosts from Fusarium wilt diseases after challenge inoculation with the pathogen (Biles and Martyn, 1989; Hervás et al., 1995; Matta, 1989; Ogawa and Komada, 1985). Our previous work showed that treatment of germinated seeds with a conidial suspension of either nonpathogenic races of *F. o. ciceris* or isolates of *F. oxysporum* protected chickpea cultivars from disease caused by the highly virulent *F. o. ciceris* race 5 (Hervás et al., 1995). In that study, a single challenge inoculation was carried out by dipping roots of the inoculated chickpea seedlings in a conidial suspension of the pathogen, then transplanting them into sterilized soil. Single infections occurring in that process do not accurately represent the plant-pathogen interactions taking place during normal growing conditions, thus questioning the extent of disease protection against infections under natural conditions. Similarly, the extent of disease protection can be influenced both by the nature of the biocontrol agent (Biles and Martyn, 1989; Hervás et al., 1995) and the genotype of the host (Hervás et al., 1995), as well as by the inoculum concentration of the pathogen (Hervás et al., 1995; Martyn et al., 1991). Thus, *F. oxysporum* isolate Fo 9009 significantly reduced the amount of disease in chickpea cvs. ICCV 4 and JG 62 after inoculation with *F. o. ciceris* race 5, whereas Fo 90105 protected only 'ICCV 4' against the same pathogen race (Hervás et al., 1995).

In some cases, the association of nonpathogenic *F. oxysporum* and rhizosphere bacteria (fluorescent *Pseudomonas* spp.) suppresses Fusarium wilt diseases more effectively and consistently than each antagonistic microorganism separately (Lemanceau and Alabouvette, 1991; Park et al., 1988). Thus, development of compatible combinations of biocontrol agents is a promising research direction. However, there has been relatively little investigation of the extent to which biocontrol bacteria that inhibit plant pathogens may also inhibit biocontrol fungi (Bin et al., 1991; Dandurand and Knudsen, 1993; Hubbar et al., 1983). Recently, we have isolated *Bacillus* spp. from the chickpea rhizosphere of which culture filtrates inhibit conidial germination and hyphal growth of both *F. o. ciceris* and nonpathogenic *F. oxysporum* (B. Landa, A. Hervás and R.M. Jiménez-Díaz, unpublished). Fravel (1988) discussed the possibility of deleterious effects of antibiotic and antibiotic-like compounds, produced by biocontrol agents, on beneficial microorganisms. Hubbard et al. (1983) stated that seed-colonizing pseudomonads were largely responsible for the failure of *Trichoderma hamatum* (Bonard.) Bainier as a seed protectant in New York soils.

The objectives of this research were: (1) To determine whether or not seed treatment with nonpathogenic *F. oxysporum* isolate Fo 90105 can protect different chickpea cultivars from disease in soil infested with *F. o. ciceris* race 5, and (2) to determine the effect of *Bacillus* sp. isolate RGAF 51 on the biocontrol activity of nonpathogenic *F. oxysporum* when both antagonists are applied jointly as seed treatment.

Materials and methods

Production of bacterial and fungal inoculum

Bacillus sp. isolate RGAF 51 was originally isolated from the rhizosphere of healthy chickpeas grown in *F. o. ciceris*-infested soil from a field plot located at Santaella, Córdoba, southern Spain. This plot has been used repeatedly for Fusarium wilt resistance screening of diverse chickpea germplasm during the last 15 years (Jiménez-Díaz et al., 1991). This bacterial isolate was shown to inhibit *in vitro* growth of *F. o. ciceris, F. o.* f. sp. *melonis* W. C. Snyder and H. C. Hans, *F. o.* f. sp. *phaseoli* J. B. Kendrick and W. C. Snyder, and nonpathogenic *F. oxysporum* (B. Landa, A. Hervás and R. M. Jiménez-Díaz, unpublished). Bacterial cells from cultures in nutrient broth yeast extract medium (NBY) were stored in 25% glycerol at –2 °C. Bacterial inoculum was produced in flasks with nutrient broth

(NB) on a rotatory shaker at 120 rpm and 25 °C for 72 h. Cultures were centrifuged at 10,000 rpm for 20 min and cells were washed three times with sterile distilled water to remove traces of nutrients.

F. o. ciceris (*Foc*) isolate Foc 8012, representative of race 5 of the pathogen (*Foc*-5), was obtained from infected chickpeas in southern Spain and has been used in previous studies (Hervás et al., 1995; Jiménez-Díaz et al., 1991; Jiménez-Díaz et al., 1989; Trapero-Casas and Jiménez-Díaz, 1985). Nonpathogenic *F. oxysporum* isolate Fo 90105 was originally isolated from roots of healthy chickpeas grown in the same infested field plot mentioned above, at Santaella. Isolate Fo 90105 was shown effective in the protection of chickpeas from disease caused by *Foc*-5 (Hervás et al., 1995). Monoconidial fungal isolates were stored and cultured as indicated in previous studies (Hervás et al., 1995).

Inoculum of Fo 90105 consisted of conidia produced in potato-dextrose broth (PDB) incubated on a rotatory shaker at 120 rpm, 25 °C, and a 12-h photoperiod of fluorescent and near-UV light at 36 μE.m^{-2}.s^{-1}. Then, microconidia in the liquid cultures were filtered through eight layers of sterile cheesechoth, pelleted by centrifugation (10,000 rpm, 10 min) and washed three times with sterile distilled water to remove traces of nutrients. Inoculum concentration was adjusted as needed using a haemocytometer. Inoculum of *Foc*-5 consisted of chlamydospores obtained as described by Alexander et al. (1966). A soil extract (1 kg autoclaved sandy loam soil : 1 l sterile distilled water) was filtered, then sterilized through a 0.22-μm Millipore filter and 100 ml of the sterile soil extract in sterile 250-ml Erlenmeyer flasks were infested with 1 g of *Foc*-5 mycelial mass. Mycelial mass was obtained from *Foc*-5 cultures grown in Czapeck medium incubated at 120 rpm under the same conditions as PDB cultures for 7 days. Cultures were filtered through sterile filter paper and washed with sterile distilled water to remove conidia as well as traces of nutrients.The infested soil extract was incubated on a rotatory shaker as before. After 2 months of incubation, examination of the cultures by light microscopy showed that they consisted mostly of chlamydospores. Inoculum concentration was adjusted as needed using a haemocytometer.

Seed treatments

Chickpea cultivars ICCV 4 and PV 61 ('kabuli' type) are, respectively, resistant to races 0 and 1, and moderately resistant to race 0, of *F. o. ciceris* (Jiménez-Díaz et al., 1989; 1993; and unpublished). Seeds of cultivar ICCV 4 were kindly provided by H. A. van Rheenen, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. Seeds were surface-disinfested in 2% NaOCl for 3 min, then washed three times in sterile distilled water, and dried for 6 h under a stream of filtered air. Conidia of *F. oxysporum* isolate Fo 90105 produced in PDB were suspended in 0.5% methylcellulose (Sigma) and their concentration determined with a haemocytometer. The suspension was diluted to 1×10^8 conidia.ml^{-1} and mixed with chickpea seeds (3 ml of the suspension per 100 seeds; 3×10^6 conidia.seed^{-1}) in a 500-ml Erlenmeyer flask. The mixture was rotatory shaken by hand for several minutes until the suspension was totally absorbed by the seeds, then air-dried for 3 h under a stream of filtered air.

Cells of *Bacillus* sp. isolate RGAF 51 produced in NB were suspended in 0.5% methylcellulose and mixed with chickpea seeds (3 ml of the suspension per 100 seeds) and the mixture was treated as for conidial inoculum. For the joint seed treatment with both nonpathogenic *F. oxysporum* and *Bacillus* sp., 1.5 ml of conidial suspension (2×10^8 conidia.ml^{-1}) and 1.5 ml of bacterial suspension were mixed with 100 seeds as described above. Control treatments consisted of nontreated seeds as well as seeds coated with 0.5% methylcellulose.

To assess the amount of fungal and bacterial inoculum on the treated, dried seeds, five seeds were each placed in a separate tube with 10 ml of sterile distilled water and sonicated for 10 min. Serial dilutions of the suspensions were plated on V-8 juice-oxgall-PCNB agar (VOPA), a Fusarium-selective medium (Bouhot and Rouxel, 1971), and PDA. Results indicated that treated seeds contained aproximately 1–3 $\times$ 10^6 cfu.seed^{-1} of *F. oxysporum* isolate Fo 90105 and 1×10^7 cfu.seed^{-1} of *Bacillus* sp. isolate RGAF 51.

Effect of nonpathogenic Fusarium oxysporum *isolate Fo 90105 on Fusarium wilt in different chickpea cultivars*

An experiment was carried out to determine the efficacy of seed treatment with nonpathogenic *F. oxysporum* isolate Fo 90105 to protect 'ICCV 4' and 'PV 61' against disease caused by *Foc*-5. Dry *F. oxysporum*-coated seeds and controls were sown in 15-cm-diameter clay pots (four seeds per pot) filled with an autoclaved soil mixture (clay loam/peat, 2 : 1, vol/vol) artificially infested with 0, 500 or 1,000

chlamydospores.g^{-1}of *Foc*-5. Chlamydospore suspensions obtained as above were mixed with sterile talcum before incorporating them into soil at the appropriate proportion. The inoculum concentration of *Foc*-5 in the infested soil was determined before use by plating on VOPA medium. There were four replicated pots for each cultivar-treatment-inoculum concentration of *Foc*-5 combination. 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 μE.m^{-2}.s^{-1} for 60 days.

Effect of nonpathogenic Fusarium oxysporum isolate *Fo 90105 and* Bacillus *sp. isolate RGAF 51on Fusarium wilt in cultivar ICCV 4*

Two experiments were conducted to determine the efficacy of joint seed treatment with nonpathogenic *F. oxysporum* isolate Fo 90105 and *Bacillus* sp. isolate RGAF 51 to protect against disease caused by *Foc*-5 in susceptible 'ICCV 4' chickpeas. In one experiment, seeds coated with the two microorganisms and control seeds were sown in sterile sand in trays (60 × 40 × 10 cm; two trays per treatment), and seedlings were grown in a growth chamber adjusted as above for 6 days. Then, seedlings were removed from the trays, selected for uniformity and transplanted into 15-cm-diameter clay pots (four plants per pot) filled with the autoclaved soil mixture artificially infested with 0, 1,000 or 2,000 chlamydospores.g^{-1} of *Foc*-5. In another experiment, treated and control seeds were sown ungerminated in clay pots (five seeds per pot) filled with the autoclaved soil mixture infested with 0, 1,000 or 2,000 chlamydospores.g^{-1}of *Foc*-5. After 4–5 days of growth, seedlings were thinned to ensure four plants per pot. In both experiments, the following treatments were included: (i) RGAF 51; (ii) Fo 90105; (iii) RGAF 51 + Fo 90105; (iv) control-methylcellulose; and (v) non-treated control. There were five replicated pots for each treatment-inoculum concentration of *Foc*-5 combination. Plants were grown in a growth chamber adjusted as above for 42 days.

In all experiments, plants were watered as needed and fertilized weekly with 100 ml of Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Root and rhizosphere colonization assay

Seeds of chickpea cvs. ICCV 4 and PV 61 coated with conidia of nonpathogenic *F. oxysporum* isolate Fo 90105 and control were used. Conical plastic tubes (175 by 56 mm (top diameter); Super-Leach Conetainer, Bardi) were filled with about 250 g of an autoclaved soil mixture. In each tube, one seed was sown and covered with 1 cm-thick layer of sterile soil. Tubes in a small frame (35 tubes per frame) were placed in a tray, with their open end in contact with a film of water at the bottom of the tray to maintain the soil moisture, and incubated in a growth chamber at 25 °C and a 14-h photoperiod of fluorescent light at 360 μE.m^{-2}.s^{-1} for 7 days. Then, seedlings were uprooted from the tubes and shaken gently to remove all but the most tightly adhering rhizosphere soil. The principal root of each of six seedlings for each cultivar-treatment combination was sectioned into 5-cm-long pieces, and the segments from 0- to 5-cm root length proximal to the seed were sonicated for 10 min in 10 ml of sterile distilled water to remove the fungus from the root. Serial dilutions of the washings were plated on VOPA and incubated at 25 °C with a 12-h photoperiod of fluorescent and near-UV light at 36 μE.m^{-2}.s^{-1} for 5 days. In addition, isolations were made from five plants per cultivar-treatment combination to determine the ability of the fungus to infect the chickpea roots. Hypocotyl and root tissues were cut into 5-mm-long pieces and, together with cotyledons, surface-disinfested in 0.4% NaOCl for 1 min, plated on VOPA and incubated as above for 3 to 5 days.

Spermosphere colonization assay

Seeds of chickpea cvs. ICCV 4 and PV 61 coated with conidia of nonpathogenic *F. oxysporum* isolate Fo 90105 and control were used. Glass, 9-cm-diameter petri dishes were filled approximately half full with sterile soil and a single treated or control seed was placed on the soil surface in the center of a dish. The seed and the soil surface were overlaid with two layers of sterile filter paper, and additional sterile soil was added onto the filter paper to fill the dishes. Soil was gently compressed and moistened with 5 ml of sterile distilled water. Dishes were placed in plastic bags with moist paper towels and incubated at 25 °C in the dark for 5 days. Then, the upper layer of paper (with the soil above it) was removed from sampled petri dishes. To determine the radial growth of *F. oxysporum* from seed on the filter paper, the lower layer of paper was placed into a sterile petri dish, incubated at 25 °C under fluorescent and near-UV light at 36 μE.m^{-2}.s^{-1} for 24 h, and the diameter of the fungal colony on the

Table 1. Effect of seed treatment with nonpathogenic *Fusarium oxysporum* isolate Fo 90105 on Fusarium wilt developed in chickpea cultivars sown in soil infested with *F. oxysporum* f. sp. *ciceris* race 5

Cultivar	Race 5 (chl/g soil)[w]	Seed treatment[x]	Disease assessment			
			IP (days)[y]	Final DII[y]	SAUDPC[y]	Final DI (%)[y]
ICCV 4	500	Fo 90105	46.8 a	0.11[z] b*	0.07 b*	20.5 b*
		Control-MC	35.4 b	0.49 a*	0.32 a	73.7 a*
	1000	Fo 90105	29.7*	0.95 a	0.61 a	100
		Control-MC	28.3*	0.74 b	0.39 b	97.9
PV 61	500	Fo 90105	46.5 a	0.02 b*	0.01 b*	4.3 b*
		Control-MC	21.7 b	0.50 a	0.36 a	75.2 a
	1000	Fo 90105	30.3 a	0.65	0.45	90.8
		Control-MC	20.0 b	0.70	0.50	89.6

[w] Treated seeds were sown in soil artificially infested with chlamydospores of *F. o. ciceris* race 5 at given inoculum concentrations, and plants were grown at 25 °C and 360 μE.m^{-2}.s^{-1} for 60 days.
[x] Seeds were treated with 0.5% methylcellulose (Control-MC) or with a suspension of 1×10^8 conidia.ml^{-1} of Fo 90105 in 0.5% methylcellulose to give a dose of 1–3 $\times$ 10^6 cfu.seed^{-1} of *F. oxysporum.*
[y] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2-day-intervals. DII values were plotted over time to develop curves of disease increase. IP = Incubation period (number of days until DI $>$ 0). SAUDPC = Standardized area under the curves of DII increase over time; DI = Disease incidence.
[z] Data are the average of three experiments with four replicated pots, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. There were significant cultivar $*$ treatment and inoculum concentration $*$ treatment interactions. Means in a column followed by a different letter are significantly different according to Fisher's protected LSD (P = 0.05). Means in a column followed by an asterisk are significantly lower than the mean for the corresponding treatment at the other inoculum concentration for each cultivar.

paper was measured. There were five replicated dishes per cultivar-treatment combination.

Disease assesment and data analyses

Disease reactions were assessed by the incidence (percentage) and severity of symptoms. Each plant was assessed for symptoms severity at 2 day-intervals using a 0 to 4 rating scale according to 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). Upon termination of experiments, isolations on VOPA were made from stem segments of symptomless plants to determine the occurrence of vascular infections. Stems were cut into 5-mm-long pieces, surface-disinfested in 1% NaOCl for 90 sec, plated on VOPA and incubated at 25 °C with a 12-h photoperiod of fluorescent and near-UV light at 36 μE.$^{-2}$.s^{-1} for 3 to 5 days.

All experiments were conducted following a factorial treatment design, replicated as randomized complete blocks, and they were repeated twice. Similarity among experiments was tested by preliminary analyses of variance using experimental runs as a factor, so that experiment $*$ treatment interaction could be determined. Such an interaction was not significant and allowed combining data for analyses of variance. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analysis of variance. A disease intensity index (*DII*) was determined from data on incidence and severity of symptoms as follows: $DII = (\Sigma\ S_i \times N_i).\ 4^{-1} \times N_t$, where S_i = symptoms severity; N_i = number of plants with $S_i$symptom severity; and N_t = total number of plants. Thus, *DII* expressed the mean value of disease intensity at any given moment as a proportion of the maximum possible disease. *DII* values at each assessment date were plotted over time (days) and the standardized area under the curves of *DII* increase over time (SAUDPC) was calculated (Campbell and Madden, 1990). An incubation period for disease development was established as the number of days taken for $DII > 0$. Data were analyzed using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference test (LSD) at P = 0.05.

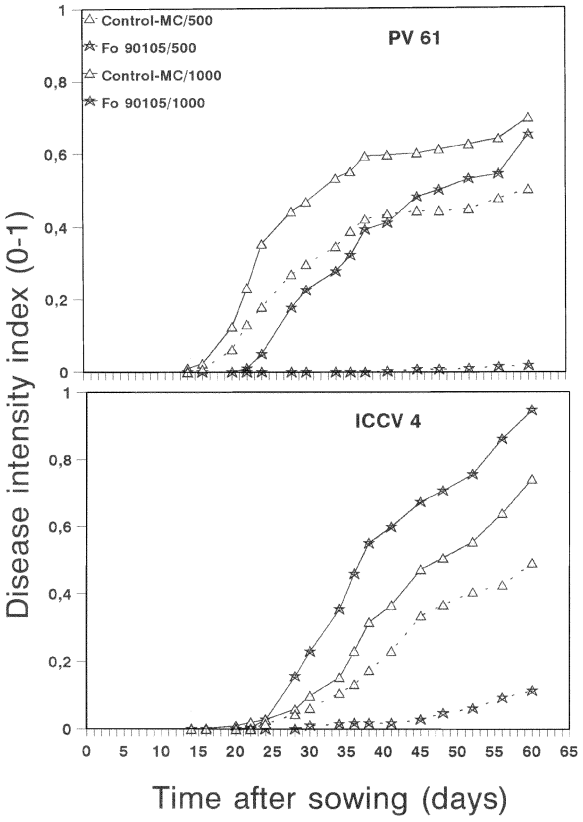

Figure 1. Effect of seed treatment with nonpathogenic *Fusarium oxysporum* isolate Fo 90105 on development of Fusarium wilt in chickpea cvs. ICCV 4 and PV 61 sown in soil artificially infested with 500 or 1,000 chlamydospores.g^{-1} of *F. oxysporum.* f. sp. *ciceris* race 5. Disease was assessed by means of a disease intensity index calculated from data on incidence and severity of symptoms (see Materials and methods). Data are the average of three experiments with four replicated pots, each with four plants.

Results

Effect of nonpathogenic Fusarium oxysporum *isolate Fo 90105 on Fusarium wilt in different chickpea cultivars*

Both seed treatment with nonpathogenic *F. oxysporum* isolate Fo 90105 and inoculum concentration of *Foc*-5 in soil influenced the development of disease in cvs. ICCV 4 and PV 61 (Figure 1, Table 1). There were no symptoms observed in control plants nor in plants from treated seeds grown in noninfested soil (data not shown), and *F. oxysporum* was not isolated from vascular chickpea tissues from these plants. Methylcellulose control plants grown in infested soil showed wilt symptoms characteristic of those caused by *Foc*-5 in susceptible cultivars. In these plants, disease reactions caused by different concentrations of *Foc*-5 varied with the chickpea cultivar. Disease was not significantly ($P = 0.05$) influenced by the inoculum concentration of *Foc*-5 in cultivar PV 61 (Table 1). However, in 'ICCV 4' the final values for *DII* and disease incidence (*DI*) with an inoculum concentration of 1,000 chlamydospores.g^{-1} of *Foc*-5 were significantly ($P < 0.05$) higher than those with 500 chlamydospores.g^{-1} of *Foc*-5 (Table 1). Also, when the inoculum concentration of *Foc*-5 was increased from 500 to 1,000 chlamydospores.g^{-1} the incubation period in methylcelluse controls of 'ICCV 4' significantly ($P < 0.05$) decreased by 7 days.

Disease development in 'PV 61' plants from seed treated with nonpathogenic Fo 90105 varied significantly ($P < 0.05$) with the inoculum concentration of *Foc*-5. Final *DII*, final *DI*, and SAUDPC were significantly higher ($P < 0.05$) with 1,000 than with 500 chlamydospores of *Foc*-5 per g of soil (Table 1). Seed treatment of 'PV 61' with isolate Fo 90105 significantly ($P< 0.05$) reduced the final *DII*, the final *DI*, and SAUDPC, compared with the methylcellulose control at the 500 chlamydospores.g^{-1} concentration of *Foc*-5, but not when the inoculum concentration of *Foc*-5 in soil was 1,000 chlamydospores.g^{-1} of *Foc*-5. However, the incubation period for disease development in soil infested with 1,000 or 500 chlamydospores.g-1 of *Foc*-5 significantly ($P < 0.05$) increased by 10 or 25 days, respectively, as compared with that in the methylcellulose control at the same inoculum concentrations (Table 1).

Treatment of 'ICCV 4' seeds with nonpathogenic isolate Fo 90105 reduced disease development when seeds were sown in soil infested with 500 chlamydospores.g^{-1} of *Foc*-5, with final *DII*, final *DI*, and SAUDPC values significantly ($P < 0.05$) lower, and the incubation period significantly higher, than in plants from methylcellulose control (Figure 1, Table 1). However, when plants were grown in soil infested with 1,000 chlamydospores.g^{-1} of *Foc*-5 there was no suppression of disease in 'ICCV 4' by seed treatment with Fo 90105. Rather, it appeared that seed treatment may have enhanced development of disease at the higher inoculum concentration (Figure 1, Table 1).

Table 2. Effect of seed treatment with *Bacillus* sp. isolate RGAF 51 and nonpathogenic *Fusarium oxysporum* isolate Fo 90105 alone and in combination, on Fusarium wilt developed in chickpea cultivar ICCV 4 sown in soil infested with *F. oxysporum* f. sp. *ciceris* race 5

Race 5 (chl/g soil)[w]	Seed treatment[x]	Disease assessment			
		IP (days)[y]	Final DII[y]	SAUDPC[y]	Final DI (%)[y]
1000	Control-MC	17.6	0.73[z] a	0.53 ab	97.9
	RGAF 51	18.5*	0.61 b	0.46 b	81.7
	Fo 90105	18.4	0.75 a	0.52 ab	93.3
	RGAF 51+ Fo 90105	17.4	0.85 a*	0.62 a	96.7
2000	Control-MC	15.8 b	0.91 a*	0.70 a*	100
	RGAF 51	15.9 b	0.83 a*	0.60 ab*	98.3
	Fo 90105	18.1 a	0.66 b	0.51 b	81.7
	RGAF 51+ Fo 90105	18.1 a	0.68 b	0.52 b	91.6

[w] Treated seeds were sown in soil artificially infested with chlamydospores of *F. o. ciceris* race 5 at given inoculum concentrations, and plants were grown at 25 °C and 360 μE.m^{-2}.s^{-1} for 42 days.
[x] Seeds were treated either with 0.5% methylcellulose (Control-MC), with a suspension of the bacterial isolate RAGF 51 in 0.5% of methylcellulose, with a suspension of 1×10^8 conidia.ml^{-1} of Fo 90105 in 0.5% methylcellulose, or with the two microorganisms to give rise a dose of 1–3 $\times$ 10^6 cfu.seed^{-1} of *F. oxysporum* and 1×10^7 cfu.seed^{-1} of *Bacillus* sp.
[y] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2-day-intervals. DII values were plotted over time to develop curves of disease increase. IP = Incubation period (number of days until DI $>$ 0). SAUDPC = Standardized area under the curves of DII increase over time. DI = Disease incidence.
[z] Data are the average of three experiments with five replicated pots, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. 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 larger than the mean for the corresponding treatment at the other inoculum concentration.

Effect of nonpathogenic Fusarium oxysporum *isolate Fo 90105 and* Bacillus *sp. isolate RGAF 51 on Fusarium wilt in cultivar ICCV 4*

Treatment of 'ICCV 4' seeds with *Bacillus* sp. isolate RGAF 51 and nonpathogenic *F. oxysporum* isolate Fo 90105, alone and in combination, influenced the development of disease caused by *Foc*-5 when ungerminated, treated seeds were sown in infested soil (Figure 2A). There were no symptoms in plants from treated and nontreated seeds sown in noninfested soil (data not shown). Disease development in control plants grown from seeds treated with methylcellulose, as well as in plants from RGAF 51-treated seeds, was significantly ($P < 0.05$) influenced by the inoculum concentration of *Foc*-5. Both the final *DII* and SAUDPC (but not the final *DI*) in soil infested with 2,000 chlamydospores.g^{-1} of *Foc*-5 were significantly higher ($P < 0.05$) than those developed with 1,000 chlamydospores.g^{-1} of *Foc*-5 (Table 2). Seed treatment with the microbial agents influenced disease development compared to that in methylcellulose controls in some cases. When *F. oxysporum*-treated seeds, both alone and in combination with RGAF 51, were sown in soil infested with 2,000 chlamydospores.g^{-1} of *Foc*-5, the incubation period for disease development was significantly higher ($P < 0.05$), and both the final *DII* and SAUDPC were significantly lower ($P < 0.05$), than those developed in methylcellulose control (Table 2). However, there was no significant (P = 0.05) difference in the final *DI* between seed treatment and control. Likewise, there was no significant effect of *F. oxysporum* seed treatment on disease development at 1,000 chlamydospores.g^{-1} of *Foc*-5. Seed treatment with *Bacillus* sp. isolate RGAF 51 reduced disease development at an inoculum concentration of 1,000 chlamydospores.g^{-1}, but not at 2,000 chlamydospores.g^{-1}. Plants from RGAF 51-treated

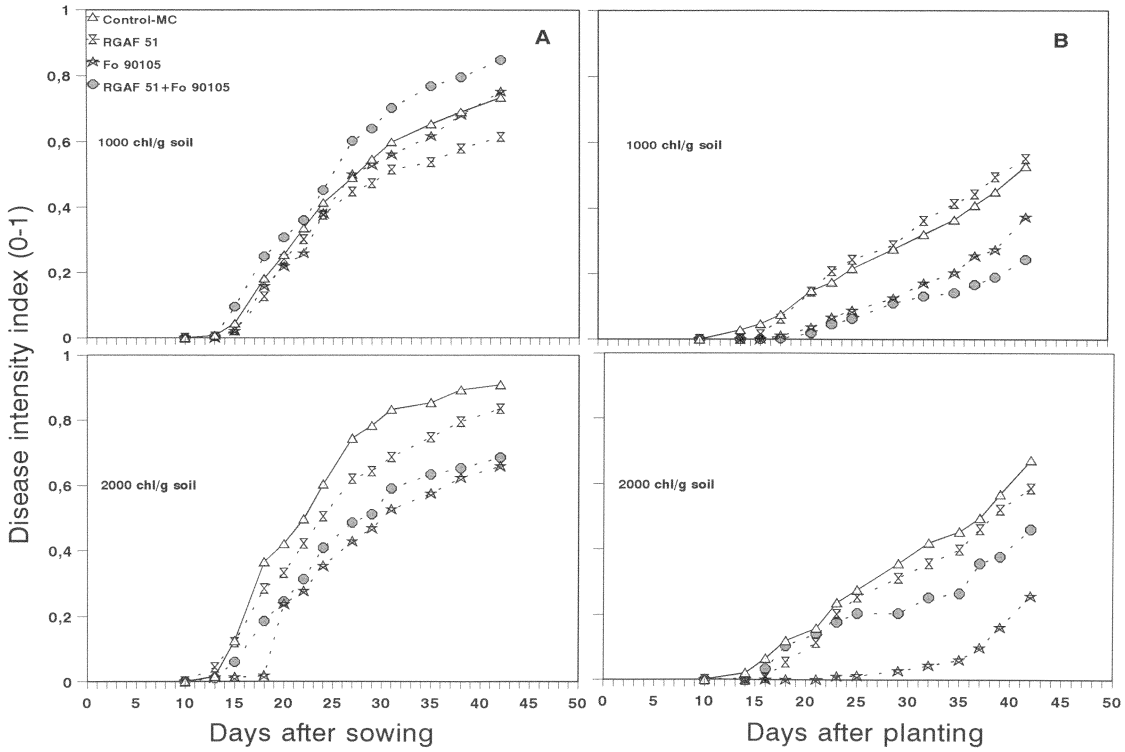

Figure 2. Effect of seed treatment with *Bacillus* sp. isolate RGAF 51 and *Fusarium oxysporum* isolate Fo 90105 alone and in combination, on development of Fusarium wilt in chickpea cv. ICCV 4. (**A**), Treated seeds were sown in soil artificially infested with 1,000 or 2,000 chlamydospores.g^{-1} of *F. oxysporum* f. sp. *ciceris* race 5. (**B**) Treated seeds were sown in sterile sand and after 6 days of growth seedlings were transplanted into soil artificially infested with 1,000 or 2,000 chlamydospores.g^{-1} of *F. o. ciceris* race 5. Disease was assessed by means of a disease intensity index calculated from data on incidence and severity of symptoms (see Materials and methods). Data are the average of three experiments with five replicated pots, each with four plants

seeds at 1,000 chlamydospores.g^{-1}had significantly lower final *DII* but similar SAUDPC and incubation period than those in the methylcellulose controls.

In another experiment, treated and control seeds were not sown in infested soil directly, but rather they were sown in sterile sand and after 6 days of growth seedlings were transplanted into soil infested with *Foc*-5 in pots. With this experimental system, disease developed to a lesser extent than that with direct sowing of treated seeds in infested soil (Figure 2). Also, in this case disease development in plants from seeds coated with isolate RGAF 51 alone was very similar to that in methylcellulose controls (Figure 2B). However, seed treatment with nonpathogenic *F. oxysporum* Fo 90105 alone and in combination with *Bacillus* sp. isolate RGAF 51 markedly influenced the development of disease caused by *Foc*-5 in 'ICCV 4' (Figure 2B). Seed treatment with nonpathogenic Fo 90105 alone and in combination with *Bacillus* sp. isolate RGAF 51 significantly ($P < 0.05$) increased by 6–12 days the incubation period for disease development, and reduced the final *DII* and SAUDPC, as compared to those in the methylcellulose control (Table 3). This protection from disease was found with both (1,000 and 2,000 chlamydospores.g^{-1}) inoculum concentrations of *Foc*-5 assayed. Futhermore, at the end of the experiment a high proportion of plants (28–60%) were symptomless and free from systemic infection by *Foc*-5 (Table 3). Under these experimental conditions, the net reduction in disease incidence by seed treatment with Fo 90105 ranged approximately from 13 to 51%, and surprisingly, it was maximum at the highest inoculum concentration of *Foc*-5 (2,000 chlamydospores.g^{-1}).

Spermosphere and root colonization assays

No fungal growth was observed in the spermosphere of nontreated, control seeds. After 5 days of incubation at 25 °C, seeds of 'ICCV 4' and 'PV 61' chickpeas treated with conidia of nonpathogenic Fo 90105 were

Table 3. Effect of seed treatment with *Bacillus* sp. isolate RGAF 51 and nonpathogenic *Fusarium oxysporum* isolate Fo 90105 alone and in combination, on Fusarium wilt developed in chickpea cultivar ICCV 4 transplanted into soil infested with *F. oxysporum* f. sp. *ciceris* race 5

Race 5 (chl/g soil)[w]	Seed treatment[x]	Disease assessment				Symptomless plants (%)[y]	
		IP (days)[y]	Final DII[y]	SAUDPC[y]	Final DI (%)[y]	Infected	Non-infected
1000	Control-MC	21.2 b	0.53[z] a	0.34 a	72.9 a	12.5	14.6
	RGAF 51	20.4 b	0.55 a	0.35 a	81.7 a	—	18.3
	Fo 90105	26.9 a	0.37 b	0.23 b	60.0 ab	11.7	28.3
	RGAF 51+ Fo 90105	27.5 a	0.24 b	0.21 b	35.0 b	5.0	60.0
2000	Control-MC	21.6 b	0.67 a	0.42 a	97.9 a	—	2.1
	RGAF 51	24.1 b	0.58 a b	0.40 ab	78.3 b	6.7	15.0
	Fo 90105	35.1 a*	0.26 c	0.20 c	46.7 c	—	53.3
	RGAF 51+	22.4 b	0.46 b*	0.30 bc*	65.0 bc	6.7	28.3

[w] Treated seeds were sown in sterile sand and after 6 days of growth in a growth chamber seedlings were transplanted into soil artificially infested with chlamydospores of *F. o. ciceris* race 5 at given inoculum concentrations. Plants were grown at 25 °C and 360 μE.m^{-2}.s^{-1} for 42 days.

[x] Seeds were treated either with 0.5% methylcellulose (Control-MC), with a suspension of the bacterial isolate RAGF 51 in 0.5% of methylcellulose, with a suspension of 1×10^8 conidia.ml^{-1} of Fo 90105 in 0.5% methylcellulose, or with the two microorganisms to give rise a dose of 1–3 $\times$ 10^6 cfu.seed^{-1} of *F. oxysporum* and 1×10^7 cfu.seed^{-1} of *Bacillus* sp.

[y] A disease intensity index (DII) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 2-day-intervals. DII values were plotted over time to develop curves of disease increase. IP = Incubation period (number of days until DI $>$ 0). SAUDPC = Standardized area under the curves of DII increase over time. DI = Disease incidence. The occurrence of vascular infection in symptomless plants was determined by means of isolations from stem segments upon termination of experiments.

[z] Data are the average of three experiments with five replicated pots, each with four plants. Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. There was a significant inoculum concentration $*$ treatment interaction. Means in a column followed by the same 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 larger than the mean for the corresponding treatment at the lower inoculum concentration.

completely coated by dense, white mycelial growth. Radial fungal growth on the filter paper in contact with a treated seed was 16.2 and 14.2 mm for 'ICCV 4' and 'PV 61', respectively.

Results from root colonization assays showed that the mean population size of nonpathogenic isolate Fo 90105 in the chickpea rhizosphere was 11.1×10^2 and 8.7×10^2 cfu/cm of root for 'ICCV 4' and 'PV 61', respectively. Futhermore, isolations from plants of the two cultivars recovered the fungus from cotyledons and the cortical tissue of older portions of the root (aproximately 1–2 cm from cotyledons), but not from the root apex (data not shown).

Discussion

In previous studies (Hervás et al., 1995), prior inoculation of germinated seeds with conidial suspensions of either nonpathogenic races of *F. o. ciceris* or nonpathogenic isolates of *F. oxysporum* protected chickpeas against disease caused by a single, challenge inoculation with *F. o. ciceris* race 5. However, the degree of disease protection was influenced by both the inducing and challenge inoculum concentrations, and it varied with the nature of the inducing agent and genotype of the host. Results from those studies suggested that in 'ICCV 4', protection against *Foc*-5 was conferred best by the nonpathogenic *F. oxysporum* isolate Fo 90105. The present work focused mainly on the ability of isolate Fo 90105 applied to ungerminated seeds to protect chickpeas against disease under conditions highly conducive for Fusarium wilt in soil artificially infested with the highly virulent *Foc*-5. Results support the potential of the nonpathogenic *F. oxysporum* isolate Fo 90105 as biological control agent against Fusarium wilt of chickpea. However, the degree of protection varied with both the chickpea cultivar and the inoculum concentration of *Foc*-5. When treated seeds were sown directly in soil infested with chlamydospores of *Foc*-5, the extent of protection was higher and more consistent in 'PV 61' than in 'ICCV 4' chickpeas.

The net reduction in disease incidence was highest and about 70% in 'PV 61' chickpeas grown in soil infested with the lowest inoculum concentration of *Foc*-5 used in this study. The influence of 'ICCV 4' and 'PV 61' chickpeas on disease protection conferred by nonpathogenic Fo 90105 can not be attributed to differences in the ability of this isolate to colonize the rhizosphere and spermosphere of the two cultivars. Liu et al. (1995) showed that disease suppression mediated by systemic resistance induced in cucumber by seed treatment with some PGPR strains was cultivar specific, although the root colonization capacity of these PGPR strains showed no cultivar specificity. Van Peer et al. (1991) stressed that *Pseudomonas* sp. strain WCS417 suppressed Fusarium wilt in the moderately resistant carnation cv. Pallas more efficiently than in the susceptible cv. Lena. Also, *P. fluorescens* strain WCS374 induced systemic resistance in six radish cultivars differing in susceptibility to *F. o. raphani*, whereas *P. putida* (Trevisan) Migula WCS358 did not (Leeman et al., 1995). The authors suggest that the induction of systemic resistance by *Pseudomonas* spp. is dependent on strain-specific traits. Nonpathogenic *F. oxysporum* isolate Fo 90105 can infect and colonize cotyledonary and cortical root tissues of both 'PV 61' and 'ICCV 4' chickpeas, but it was never isolated from the vascular tissue of infected plants.

The need to separate by a few days the first inoculation with the nonpathogen and the second challenge inoculation with the pathogen has been repeatedly indicated for different Fusarium wilt diseases (Matta, 1989). Our results from experiments with 'ICCV 4' show that the degree of protection conferred by nonpathogenic isolate Fo 90105 against disease caused by *Foc*-5 was higher and more consistent when Fo 90105-treated seeds were sown in sterile sand and the 6-day-old seedlings developed were then transplanted into *Foc*-5 infested soil, than when treated seeds were sown directly in the infested soil. Different mechanisms have been described for biocontrol of Fusarium wilt diseases by nonpathogenic *F. oxysporum* isolates; these include saprophytic competition for nutrients, parasitic competition for infection sites, and induced or enhanced resistance within the host (Alabouvette and Couteaudier, 1992; Mandeel and Baker, 1991; Schneider, 1984). Most of the evidence for enhanced resistance induced by nonpathogenic *Fusarium* spp. was obtained by separation of a biocontrol agent from an infection court and a subsequent decrease of disease following the challenge inoculation with the pathogen (Biles and Martyn, 1989; Ogawa and Komada, 1985). Our results do not necessarily suggest induction of resistance. Rather, the time period required between the antagonist and challenge inoculations may be necessary for the antagonist to more fully colonize the emerging plant root before being attacked, and it would be consistent with competition as a mechanism.

Our results indicate that seed treatment with *Bacillus* sp. isolate RGAF 51 did not significantly influence either the development of disease caused by *Foc*-5 in 'ICCV 4' or the degree of protection achieved by the nonpathogenic isolate Fo 90105 in the same cultivar. Although this bacterial isolate can inhibit *in vitro* growth of both *Foc*-5 and nonpathogenic *F. oxysporum* (B. Landa, A. Hervás and R. M. Jiménez-Díaz, unpublished), the joint application of nonpathogenic Fo 90105 and *Bacillus* sp. RGAF 51 did not modify the biocontrol activity of the former isolate. These results are similar to those of Bin et al. (1991) who observed that *P. fluorescens* suppressed radial growth and hyphal density of *T. harzianum* Rifai on agar and in sterile bulk soil. However, in nonsterile soil, the biocontrol efficacy of *T. harzianum* was not significantly affected by treatment with the bacteria. The biocontrol mechanism of *P. fluorescens* neither inhibited nor enhanced the biocontrol activity of *T. harzianum* (Dandurand and Knudsen, 1993). On the contrary, the association of nonpathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. suppressesed Fusarium wilt diseases more efficiently and consistently than each antagonistic microorganim separately (Lemanceau and Alabouvette, 1991; Park et al., 1988).

Despite the fact that the specific mechanism or mechanisms operating in our system have not been determined yet, we believe that nonpathogenic *F. oxysporum* isolate Fo 90105 shows potential as a biocontrol agent for Fusarium wilt of chickpea. However, such a potential may be undermined by high inoculum concentration of the pathogen or high disease pressure. Further studies, including a wider range of inoculum concentration of races of the pathogen and field trials in *F. oxysporum* f. sp. *ciceris*-infested soil, are underway to confirm this potential.

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