Antagonistic Activity of Bacteria from the Chickpea Rhizosphere Against *Fusarium oxysporum* f.sp. *ciceris*

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The antagonistic activity against *in vitro* growth of *Fusarium oxysporum* f.sp. *ciceris* was determined for 74 bacterial isolates obtained from the rhizosphere of chickpeas grown in two field soils with different histories of Fusarium wilt, and for seven isolates of *Pseudomonas* spp. from culture collections. Twenty-four isolates of *Bacillus* spp. and *Pseudomonas chlororaphis* 30-84 showed a strong antagonism against three races (0, 1 and 5) of *F.o. ciceris* tested. Three selected *Bacillus* isolates and *P. chlororaphis* 30-84 were further tested against 30 isolates of races 0, 1 and 5 of *F.o. ciceris*, races 0, 1 and 2 of *F.o. melonis, F.o. phaseoli* and nonpathogenic *F. oxysporum. Bacillus* isolates differed in their antagonistic activity and were less inhibitory to mycelial growth of *F.o. ciceris* than to that of other fungal isolates. Furthermore, the extent of growth inhibition of *F.o. ciceris* was influenced both by bacterial isolates and by race of the pathogen. Cell-free culture filtrates of four *Bacillus* isolates inhibited conidial germination and hyphal growth of *F.o. ciceris* and nonpathogenic *F. oxysporum.* Joint seed+soil treatment with some selected antagonistic *Bacillus* spp. isolates suppressed disease caused by the highly virulent *F.o. ciceris* race-5 in cv. ICCV 4 and cv. PV 61 chickpeas. However, the degree of protection was influenced by the host genotype and the inoculum concentration of the pathogen.

KEY WORDS: Antagonism; *Bacillus* spp.; biological control; chickpea; *Cicer arietinum*; Fusarium wilt; fluorescent pseudomonads.

INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend:: Fr. f.sp. *ciceris* (Padwick) Matuo & K. Sato (*F.o. ciceris*), is a major constraint to chickpea (*Cicer arietinum* L.) production throughout the world and particularly in the Indian subcontinent and the Mediterranean basin (8,15). Yield losses caused by the disease amounted to 10% in India (17) and Spain (19) and up to 40% in Tunisia (3). Fusarium wilt is best controlled by the use of race-specific resistant cultivars, the efficacy of which against virulent races of pathogen might be enhanced by the use of antagonistic microorganisms. Rhizosphere bacteria have proved to be effective biocontrol agents against root diseases of many crop plants (21,22), their antibiotic production now recognized as an important factor in disease suppression (6,23). So far, antibiotic production has not been shown to be involved in the suppression of Fusarium wilt diseases. Also, in most cases the antagonistic ability of the putative biocontrol rhizosphere bacteria is determined against just one or a few isolates of

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the target pathogen, and neither the variability in the pathogen population nor the beneficial rhizosphere bacteria are usually considered in terms of antagonistic activity.

The objectives of this research were to (i) select bacteria from the chickpea rhizosphere with antagonistic activity against *F.o. ciceris*; (ii) assess the degree of specificity of antagonism against a number of isolates representative of the known races of *F.o. ciceris* as well as of nonpathogenic *F. oxysporum* from chickpea roots; and (iii) determine the ability of selected bacteria to suppress Fusarium wilt caused by a highly virulent race of *F.o. ciceris*. An additional objective was to determine the antagonistic activity of a number of strains of fluorescent pseudomonads from culture collections against the above fungi.

MATERIALS AND METHODS

Bacterial and fungal isolates

Seventy-four bacterial isolates were obtained from the rhizosphere of chickpeas grown in soil from two field plots (A and B) located at Santaella, Córdoba, southern Spain. Plot A has been used repeatedly for Fusarium wilt resistance screening of diverse chickpea germplasm during the last 15 years (12), while plot B has been under barley monoculture for the same period. In addition, the following strains of fluorescent pseudomonads were used in this study: *Pseudomonas chlororaphis* (former *P. aureofaciens* Kluyver) strains 30-84, Q2-87, Q65c-80, and Q69c-80; *P. fluorescens* strains 2-79 and Q29z-80; and *P. putida* (Trevisan) Migula N1R. Strains of *P. chlororaphis* and *P. fluorescens* were kindly provided by Dr. D.M. Weller (USDA-ARS, Pullman, WA, USA); these strains had been isolated originally from wheat rhizosphere. *P. putida* strain N1R, originally isolated from soil of Salinas Valley, CA, USA, was kindly provided by the late Dr. R. Baker (University of Colorado, Fort Collins, CO, USA). Bacteria were stored in nutrient broth yeast medium (NBY) with 25% glycerol at -20°C.

The isolates of *F.o. ciceris, F.o. melonis* W.C. Snyder & H.C. Hans, *F.o. phaseoli* J.B. Kendrick & W.C. Snyder and of nonpathogenic *F. oxysporum* used in this study are summarized in Table 1. Monoconidial cultures of these isolates were stored in sterile soil tubes at 4°C. Active cultures were obtained from small aliquots of a soil culture plated on potato-dextrose agar (PDA). Fungal cultures were incubated at 25°C a 12-h photoperiod of fluorescent and near-UV light at 36 μ E.m $^{-2}$.s^{-1}

Selection of bacteria for ability to inhibit in vitro *growth of* Fusarium oxysporum *f.sp.* ciceris

A total of 81 bacterial isolates, including 43 isolates from field A and 31 isolates from field B and the seven fluorescent pseudomonads, were assayed in dual cultures on PDA for their ability to inhibit *in vitro* hyphal growth of isolates of *F.o. ciceris* races 0 (Foc 7802), 1 (Foc 7989) and 5 (Foc 8012). Four bacterial isolates, spotted at equidistant points along the perimeter of the plate, were tested per plate; they were first incubated in the dark at 28°C. After 48 h a 6-mm plug from the leading edge of a 7-day-old culture of *F.o. ciceris* on PDA was placed in the center of the plate. Plates without bacteria were used as control. Plates were incubated at 28°C for 5 days, after which the length of hyphal growth toward the bacteria (Ri) and that on a control plate (Rc) were measured. Inhibition of fungal growth was recorded as the relative growth ratio R = Ri/Rc (7). There were five replicated plates in a completely randomized design for each bacterium–fungal isolate combination.

TABLE 1. Isolates of *Fusarium oxysporum* f.sp. *ciceris*, *F.o. melonis*, *F.o. phaseoli* and nonpathogenic *F. oxysporum* used in this study

Isolates of *F. oxysporum*	Number of isolates	Origin	Source*
F.o. ciceris			
Race 0	1	California, USA	I.W. Buddenhagen, UCD
	4	Spain	This laboratory
	1	Tunisia	H.M. Halila, INRAT
Race 1	1	India	M.P. Haware, ICRISAT
	1	California, USA	This laboratory
	1	California, USA	I.W. Buddenhagen, UCD
	2	Morocco	This laboratory
	2	Spain	This laboratory
Race 2	1	India	M.P. Haware, ICRISAT
Race 3	1	India	M.P. Haware, ICRISAT
Race 4	1	India	M.P. Haware, ICRISAT
Race 5	2	California, USA	This laboratory
	2	Spain	This laboratory
F.o. phaseoli	1	–	ATCC
	1	Colombia	H.F. Schwartz, UC
	1	California, USA	M.J. Silbernagel, USDA
F.o. melonis			
Race 0	1	Italy	Petoseed
Race 1	1	California, USA	Petoseed
Race 2	1	California, USA	Petoseed
Nonpathogenic *F. oxysporum*	1	France	C. Alabouvette, INRA
	3	Spain	This laboratory

*ATCC, American Type Culture Collection, Rockville, MD, USA.
ICRISAT, International Crops Research Institute for the Semiarid Tropics, Patancheru, A.P., India.
INRA, Institut National de la Recherche Agronomique, Dijon, France.
INRAT, Institut National de la Recherche Agronomique de Tunisia, Ariana, Tunisia.
Petoseed, Petoseed Ibérica S.A., Almería, Spain.
UC, University of Colorado, Fort Collins, CO, USA.
UCD, University of California, Davis, CA, USA.
USDA, USDA-ARS, Prosser, WA, USA.

Twenty-four of the most promising bacterial isolates were characterized to species by the conventional API 50 CH-Bacillus, API 20E and API 20NE identification systems.

Specificity of bacterial antagonistic activity against isolates of Fusarium oxysporum

Four bacterial isolates, namely, *P. chlororaphis* 30-84, RGAF 6a, RGAF 12 and RGAF 51, were selected to determine the degree of their antagonistic specificity against a collection of 30 isolates of *F. oxysporum* of diverse pathogenicity and geographical origin (Table 1). Fungal growth inhibition was assayed in a dual culture experiment on PDA as described previously.

Effect of bacterial culture filtrates on microconidia germination and hyphal growth of Fusarium oxysporum

Four isolates of *Bacillus* spp., namely, RGAF 6a, RGAF 6b, RGAF 12 and RGAF 51, were selected because of their ability to inhibit the hyphal growth of *F.o. ciceris* in dual cultures. Isolate RG 56 of *Stenotrophomonas maltophilia* [synonym *Xanthomonas maltophilia* (Hugh) Swings *et al.*] (16) was also selected as a representative of bacterial isolates non-inhibitory to *F.o. ciceris*. Bacterial isolates were grown in potato–dextrose broth (PDB) at 25°C on a rotatory shaker at 120 rpm for 72 h. PDB without bacteria was used as control. Cultures were centrifuged at 10,000 rpm for 20 min and the supernatants were filtered through sterile 0.45- and 0.30-μm-pore-size Millipore filters.

Conidia of Foc 8012 and of nonpathogenic *F. oxysporum* (Fo 90105) were obtained as described in previous work (9), and their concentration was adjusted to 10^5 conidia per ml using a hemocytometer. A 0.5-ml aliquot of the microconidia suspension was mixed with 0.5 ml of a cell-free bacterial culture filtrate. For the control treatment, 0.5 ml of PDB was added instead of the cell-free bacterial culture filtrate. A 40-μl drop of the conidia-bacterial culture filtrate mixture was placed on a glass ring slide and incubated at 25°C in a moist chamber for 12 h. Then, a drop of acid fuchsine in lactophenol was added to kill and stain conidia, and the mixture was examined with a light microscope to determine conidia germination in five randomly chosen microscope fields (out of 100 conidia) per ring slide preparation. Four 40-μl drops were assayed for each bacterium–fungal isolate combination.

The ability of cell-free culture filtrates to inhibit hyphal growth of *F.o. ciceris* race 0 (Foc 7802), race 5 (Foc 8012) and nonpathogenic *F. oxysporum* (Fo 90105) was assessed using PDA cultures. A 6-mm plug of the medium was removed from the center of a plate and replaced with a similar plug from the leading edge of a 7-day-old fungal colony on PDA. Six equidistant 4-mm-diam wells were made 2.5 cm from the center of the plate and numbered from 1 to 6. A 40-μl drop of the cell-free culture filtrate (crude filtrate) was placed in well-1 and similar drops of 1/2, 1/4, 1/8 and 1/16 dilutions (crude filtrate/sterile PDB) were placed in well-2, -3, -4 and -5, respectively. A 40-μl drop of PDB placed in well-6 served as control. There were four replicated plates in a completely randomized design for each bacterium–fungal isolate combination. Plates were incubated at 25°C for 5 days. Hyphal growth inhibition was determined by the R values 5 days after fungal inoculation.

In planta *assay for suppression of Fusarium wilt of chickpea*

Chickpea cultivars ICCV 4 and PV 61 ('kabuli' type, ramhead-shaped, beige seeds) are, respectively, resistant to races 0 and 1 and moderately resistant to race 0 of *F.o. ciceris* (11,13, and unpublished). Cultivar ICCV 4 was kindly provided by H.A. van Rheenen, International Crops Research Institute for the Semiarid Tropics (ICRISAT), Hyderabad, India. Seeds were surface disinfested in 2% NaOCl for 3 min, washed three times in sterile distilled water, and air dried under sterile conditions. Five bacterial isolates, namely, RGAF 6a, RGAF 6b, RGAF 7, RGAF 12 and RGAF 51 were tested for suppression of disease caused by *F.o. ciceris* race 5 (Foc 8012) by means of soil and/or seed treatment. Bacteria were cultured for 3 days in PDB, as described previously. Bacterial cells were harvested by centrifugation (10,000 rpm for 20 min), and resuspended twice in a sterile

aqueous solution of 0.1 M $MgSO_4$.

For seed treatment, 1.5 ml of a bacterial cell suspension was mixed with 10 g of chickpea seeds agitating thoroughly until the suspension was absorbed by the seeds completely. The control treatment consisted of seeds treated with 0.1 M $MgSO_4$ only. Bacteria were enumerated on the treated seeds by dilution plating onto BV8A, a *Bacillus*-semiselective medium (20). Treated seeds yielded approximately 10^6–10^7 cfu per seed. For soil treatment, 100 g of heat-sterilized silica sand were infested with 10 ml of a bacterial suspension, and the mixture was dried up at 30° for 4–5 days. Bacteria on the infested silica sand were enumerated by dilution plating onto BV8A yielding approximately 10^7-5.10^8 cfu per g of silica sand.

Two experiments (I, II) were conducted using chlamydospores of Foc 8012 as inoculum, obtained as described by Alexander *et al.* (1). In exp. I, cv. ICCV 4 and bacterial isolates RGAF 6a, RGAF 6b, RGAF 7, RGAF 12 and RGAF 51 were used. Conical plastic tubes (175 mm long, 56 mm top diameter; Super-Leach Container, Bardi, Peralta (Navarra), Spain) were filled with an autoclaved soil mixture (clay loam and peat, 2:1, v/v) infested with 0, 500, 1,000 or 2,000 chlamydospores of Foc 8012 per g of soil. Seeds were treated as above and one treated or nontreated seed was sown per tube.

In exp. II, bacterial isolates RGAF 6a, RGAF 7 and RGAF 51 and cvs. ICCV 4 and PV 61 were used. Bacterial isolates were applied as seed (SE), soil (SO) and SE+SO treatments. For SO and SE+SO treatments, bacteria were delivered into soil at a rate of 3–5 g of treated silica sand per kg of autoclaved soil mixture. The final bacterial concentration in soil determined as above was 3.10^5–5.10^6 cfu per g of soil. Conical tubes were filled with the soil mixture non-infested or infested with bacteria alone (controls), or infested with the pathogen or bacteria+pathogen at an inoculum concentration of 500 and 2,000 chlamydospores of Foc 8012 per g of soil. Seed treatment was performed as above and one treated or nontreated seed was sown per tube. There were five tubes for each combination of experimental factors. Plants were grown in a growth chamber adjusted to 25°C, 90% r.h., and a 14-h photoperiod of fluorescent light at 360 $\mu E.m^{-2}.s^{-1}$ for 40 (exp. I) or 30 (exp. II) days. Plants were watered as needed and fertilized weekly with 25 ml of Hoagland's nutrient solution (10).

Disease reactions were assessed by the severity of symptoms at 2–3-day intervals using a 0 to 4 rating scale according to 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). Data on disease severity were used to calculate the following: (i) Incubation Period determined as the time from sowing to appearance of first symptoms; (ii) Final Disease Severity; and (iii) Standardized Area Under the Disease Progress Curve (SAUDPC) (4).

Seeds of cvs. ICCV 4 and PV 61 treated with bacterial isolates RGAF 6a, RGAF 7 and RGAF 51 were used to determine the extent of root colonization. One treated (SE and SE+SO treatments) or nontreated (SO treatment) seed was sown per conic plastic tube and plants were grown for 10 days in a growth chamber adjusted as above. Plants were removed from the tubes 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 segments in 10 ml of sterile 0.1 M $MgSO_4$ was sonicated for 15 min to release the rhizosphere bacteria from roots. Appropriate dilutions of the suspension were plated onto BV8A and incubated at 30°C for 48 h to determine the number of cfu per g of root. There were three replications in a completely randomized design, with two seedlings for each cultivar–bacterial isolate–

bacteria application treatment combination.

Statistical analysis

All *in vitro* experiments were designed as factorial treatments. Data were analyzed by analysis of variance using Statistix (NH Analytical Software, Roseville, MN, USA). Growth ratios were compared using Fisher's protected least significant difference test (LSD) at P=0.05. When the bacteria–fungal isolates interaction was significant, an orthogonal-single-degree of freedom contrast for *F.o. ciceris* isolates was performed at P=0.05. All *in planta* experiments were conducted as a completely randomized design repeated once. Similarity among experiments tested by preliminary analysis of variance using experimental runs as blocks and Barlett's test of equal variances (18), allowed combining data for analyses of variance. The means for the incubation period, the final disease severity, and SAUDPC were compared using Fisher's protected LSD test at P=0.05.

RESULTS

Selection of bacteria for ability to inhibit in vitro *growth of* Fusarium oxysporum *f.sp.* ciceris

Twenty-four of the 81 bacterial isolates from the chickpea rhizosphere and *P. chlororaphis* 30-84 inhibited the *in vitro* hyphal growth of *F.o. ciceris* isolates Foc 7802, 7989 and 8012 with R values lower than 0.7 (data not shown). These bacterial isolates were selected as antagonists for subsequent assays. Some isolates of *Bacillus* spp., *P. fluorescens* and *S. maltophilia* from the chickpea rhizosphere showed a lesser ability to inhibit *F.o. ciceris* and were not selected for further assays (data not shown). The 24 antagonistic bacterial isolates of the above were identified as *Bacillus subtilis* (Ehrenberg) Cohn (isolates RGAF 1, 3, 9, 99 and RG 32), *B. amyloliquefaciens* Fukumoto (isolate RGAF 4), *B. licheniformis* (Weigmann) Chester (isolates RGAF 45 and 47), *B. polymyxa* (Prazmowski) Macé (isolate RGAF 84) and *Bacillus* sp. (isolates RGAF 6a, 6b, 7, 11, 12, 14, 46, 48, 49, 50, 51, 82, 101, 102 and 103). Of those 24 antagonistic *Bacillus* spp. isolates, 18 (75%) originated from chickpeas grown in soil from the Fusarium wilt-sick plot used for resistance screening during the last 15 years, as compared with only six bacterial antagonists (25%) that originated from chickpeas grown in soil from a plot under barley monoculture.

Specificity of bacterial antagonistic activity against isolates of Fusarium oxysporum

Pseudomonas chlororaphis 30-84 and *Bacillus* spp. isolates RGAF 6a, 12 and 51, which inhibited *in vitro* growth of *F.o. ciceris*, also inhibited it with isolates of *F.o. phaseoli, F.o. melonis* and nonpathogenic *F. oxysporum* (Fig. 1). However, the extent of growth inhibition of *F.o. ciceris* was significantly lower (P<0.05) than that of other *formae speciales* and of nonpathogenic *F. oxysporum*. Bacterial isolates differed significantly (P<0.05) in the extent of growth inhibition of the fungal isolates, with *Bacillus* sp. RGAF 51 showing the strongest activity (Fig. 1).

When the antagonistic activity of the four bacterial isolates was tested against different *F.o. ciceris* races, the extent of hyphal growth inhibition was influenced both by bacterial isolates and by races of the fungus. Bacterial isolates differed significantly (P<0.05) in their ability to inhibit *F.o. ciceris* races 0, 1, 2, 3 and 5 (Fig. 2). Isolate *Bacillus* sp. RGAF 51 showed the strongest inhibition to all races except for race 4; and races 1, 2 and 3 were

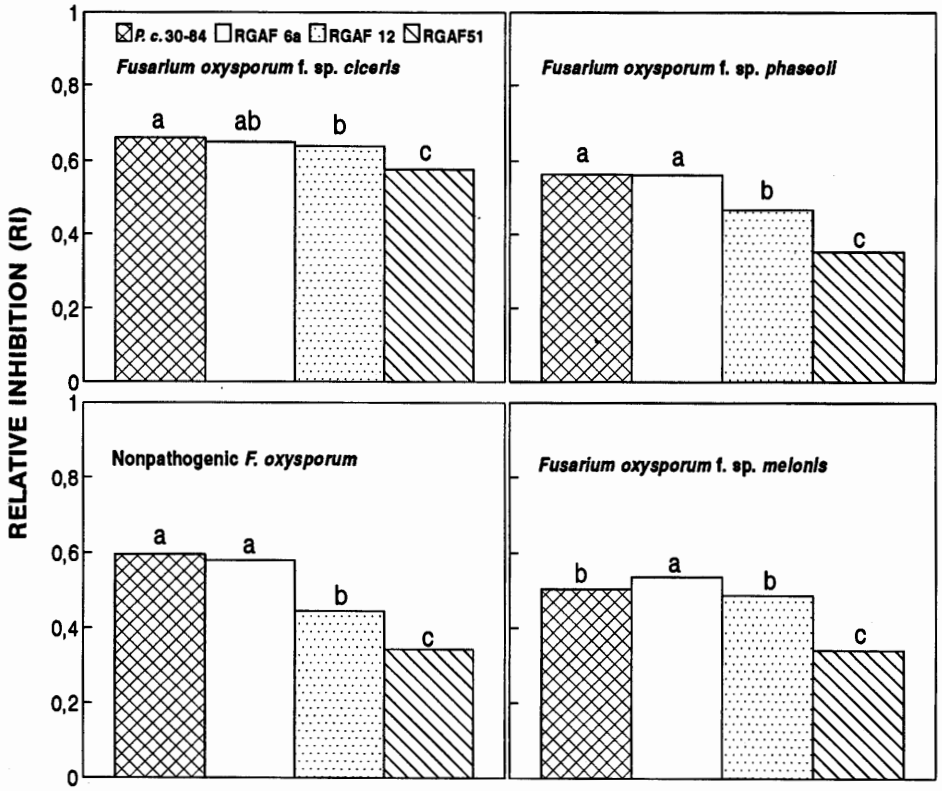

Fig. 1. Effect of *Pseudomonas chlororaphis* strain 30-84 (*P.c.* 30-84) and *Bacillus* sp. isolates RGAF 6a, RGAF 12 and RGAF 51 on inhibition of hyphal growth of 20 isolates of *Fusarium oxysporum* f.sp. *ciceris*, three isolates each of *F.o. melonis* and *F.o. phaseoli*, and four isolates of nonpathogenic *F. oxysporum* on PDA. Inhibition of growth is expressed as the ratio of the radius of hyphal growth in the direction of the bacterium relative to the radius of growth on a control plate without bacteria (R ratio). Each R value is the mean of five replications for each bacterial isolate, as described in Materials and Methods. Means for a group of *F. oxysporum* isolates with a common letter do not differ significantly (P=0.05) according to Fisher's protected LSD test. LSD values for *F.o. ciceris*, *F.o. melonis*, *F.o. phaseoli* and nonpathogenic *F. oxysporum* are 0.012, 0.027, 0.023 and 0.027, respectively.

inhibited to the same extent by *P. chlororaphis* 30-84 and *Bacillus* spp. isolates RGAF 6a and 12. Results from orthogonal-single-degree of freedom contrasts (not shown) showed that R values did not vary when *F.o. ciceris* isolates were grouped according to the disease syndrome induced (yellowing syndrome: race 0 *vs* wilt syndrome: races 1–6). On the contrary, the geographical origin of *F.o. ciceris* isolates influenced the growth inhibition by the four bacterial isolates, the isolates from Spain differing significantly ($P<0.05$) from those from California and India, which also differed between them.

Effect of bacterial culture filtrates on microconidia germination and hyphal growth of Fusarium oxysporum

Cell-free filtrates of bacterial cultures significantly inhibited conidial germination and hyphal growth of *F.o. ciceris* and of nonpathogenic *F. oxysporum* (Table 2). Cell-free cul-

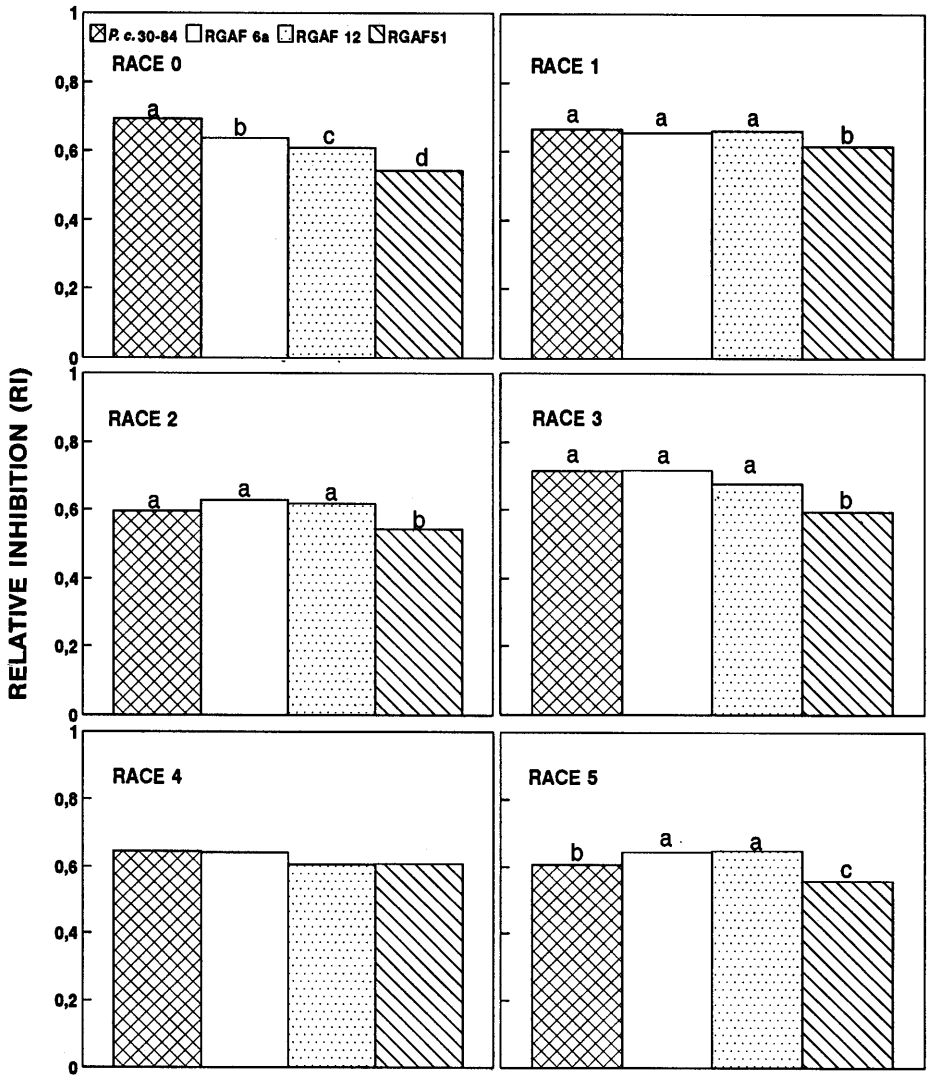

Fig. 2. Effect of *Pseudomonas chlororaphis* strain 30-84 (*P.c.* 30-84) and *Bacillus* sp. isolates RGAF 6a, RGAF 12 and RGAF 51 on inhibition of hyphal growth of races 0, 1, 2, 3, 4 and 5 of *Fusarium oxysporum* f.sp. *ciceris* on PDA. Inhibition of growth is expressed as the ratio of the radius of hyphal growth in the direction of the bacterium relative to the radius of growth on a control plate without bacteria (R ratio). Each R value is the mean of five replications for each bacterial isolate, as described in Materials and Methods. Within a race, means with the same (or no) letter do not differ significantly (P=0.05) according to Fisher's protected LSD test. LSD values for races 0, 1, 2, 3, 4 and 5 were 0.023, 0.020, 0.040, 0.068, 0.055 and 0.026, respectively.

ture filtrates from the selected *Bacillus* spp. isolates RGAF 6a, RGAF 6b, RGAF 12 and RGAF 51 significantly (P<0.05) inhibited conidia germination of both *F.o. ciceris* race

TABLE 2. Effect of cell-free culture filtrates of *Bacillus* isolates RGAF 6a, RGAF 6b, RGAF 12 and RGAF 51 and *Stenotrophomonas maltophilia* RG 56 on microconidia germination and hyphal growth of isolates of *Fusarium oxysporum* f.sp. *ciceris* (Foc 7802 and Foc 8012) and nonpathogenic *F. oxysporum* (Fo 90105)

Bacterial isolates	Germination (%)[z]		Relative inhibition of hyphal growth (%)[y]		
	Fungal isolate		Fungal isolate		
	Foc 8012	Fo 90105	Foc 7802	Foc 8012	Fo 90105
RGAF 6a	26.50 b B	55.25 c A	0.87 bc A	0.79 c B	0.86 bc A
RGAF 6b	25.20 b B	40.17 d A	0.88 b	0.84 b	0.89 b
RGAF 12	9.60 c	11.22 e	0.81 d	0.78 c	–[x]
RGAF 51	9.97 c B	19.10 f A	0.84 cd	0.79 c	0.84 c
RG 56	88.22 a A	78.30 b B	0.90 b	0.88 b	0.85 c
Control	87.00 a	89.60 a	1 a	1 a	1 a

Within columns, means with a common lower-case letter do not differ significantly (P=0.05) according to Fisher's protected LSD test. Comparisons between fungal isolates within a bacterial isolate are indicated by capital letters. Only significant differences are indicated.

[z]Data on microconidia germination are means of four replications. Percentages of conidia germination were transformed into arcsin $(Y/100)^{1/2}$ for analyses of variance.

[y]Growth inhibition is expressed as the ratio of the radius of hyphal growth in the direction of the well with crude filtrate relative to the radius of hyphal growth in the direction of the control well (R ratio). Each R value is the mean of four replications for each bacterial isolate, as described in Materials and Methods.

[x]Treatment not performed.

5 (Foc 8012) and nonpathogenic *F. oxysporum* (Fo 90105) isolates. Inhibition of conidia germination by isolates RGAF 6a, RGAF 6b and RGAF 51 was significantly (P<0.05) stronger for *F.o. ciceris* race 5 than for nonpathogenic *F. oxysporum* (Table 2). *S. maltophilia* isolate RG 56 inhibited only conidia germination of the nonpathogenic isolate. Crude cell-free culture filtrates from the four *Bacillus* spp. isolates and their 1/2–1/16 dilutions significantly (P<0.05) inhibited hyphal growth of *F.o. ciceris* isolates Foc 8012 and Foc 7802 (race 0) and of nonpathogenic Fo 90105 on PDA (Table 2). However, for *S. maltophilia* RG 56, only crude filtrate inhibited hyphal growth of *F.o. ciceris* isolates, while all dilutions (1/2 to 1/16) of the same crude filtrate inhibited hyphal growth of the nonpathogenic *F. oxysporum* isolate. In all cases, inhibition diminished significantly with a linear trend (P=0.05) as dilution of the cell-free culture filtrates increased (from 1/1 to 1/16) (data not shown).

In planta *assay for disease suppression*

In exp. I, seed treatment with *Bacillus* spp. isolates RGAF 6a, RGAF 6b, RGAF 7, RGAF 12 and RGAF 51 did not significantly (P=0.05) modify the development of disease caused by *F.o. ciceris* race 5 in cv. ICCV 4 (data not shown). In exp. II, there was variance heterogeneity between the two chickpea cultivars (ICCV 4 or PV 61) according to Barlett's test of equal variances (18). Therefore, separate analyses were performed for each cultivar. When isolates RGAF 6a, RGAF 7 and RGAF 51 were compared using SE, SO, or SE+SO treatments, both the bacterial isolate and the SE+SO treatment influenced development of disease caused by *F.o. ciceris* race 5 in cvs. ICCV 4 and PV 61 (Table 3). The degree of disease suppression by bacterial isolates was influenced by the inoculum

TABLE 3. Development of Fusarium wilt in susceptible chickpea cvs. ICCV 4 and PV 61 grown in soil infested with different inoculum densities of *Fusarium oxysporum* f.sp. *ciceris* race 5 after seed, soil, and seed+soil treatment with *Bacillus* spp. isolates RGAF 6a, RGAF 7 and RGAF 51

Bacterial treatment	Bacterial isolate	Inoculum density of *F.o.* f.sp. *ciceris*					
		500 cfu/g soil			2,000 cfu/g soil		
		IP[z] (days)	FDS[y] (0-4)	SAUDPC[x]	IP (days)	FDS (0-4)	SAUDPC
				ICCV 4			
Seed	RGAF 6a	21.9[w]	2.9	2.06	18.3	3.7	2.86
	RGAF 7	21.0	3.2	1.96	18.4	3.4	2.52
	RGAF 51	22.2	2.1	1.31	20.8	3.2	2.11
	Control	19.4	3.1	2.14	17.8	3.4	2.42
Soil	RGAF 6a	18.9	2.5	1.72	18.7	3.0	2.27
	RGAF 7	19.7	2.9	1.91	20.3	3.1	2.32
	RGAF 51	20.6	2.7	1.66	19.2	3.0	2.38
	Control	19.4	3.1	2.14	17.8	3.4	2.42
Seed + soil	RGAF 6a	20.1	3.0 bc	1.90 ab	21.0	2.4 b	1.77
	RGAF 7	19.3	4.0 a	2.75 a	17.6	3.8 a	2.63
	RGAF 51	23.3	2.3 c	1.37 b	20.0	3.3 ab	2.30
	Control	19.4	3.1 b	2.14 ab	17.8	3.4 a	2.42
				PV 61			
Seed	RGAF 6a	17.0	3.8	2.91	16.9	4.00	3.08
	RGAF 7	17.2	3.9	3.23	17.1	4.00	3.41
	RGAF 51	16.4	3.9	3.20	16.2	4.00	3.37
	Control	17.5	3.9	3.27	17.2	3.72	2.99
Soil	RGAF 6a	17.2	3.6	2.77	17.2	4.00	3.28
	RGAF 7	17.5	3.9	3.15	18.7	4.00	3.33
	RGAF 51	17.3	3.7	3.11	16.2	4.00	3.28
	Control	17.5	3.9	3.27	18.2	3.72	2.99
Seed + soil	RGAF 6a	17.2	3.4 b	2.32 c	18.2	4.00	3.32
	RGAF 7	16.8	3.5 b	2.72 bc	18.4	4.00	3.17
	RGAF 51	16.4	3.9 a	3.16 ab	17.0	3.95	3.19
	Control	17.5	3.9 a	3.27 a	16.2	3.72	2.99

[z] Incubation period determined as the time from sowing to appearance of first symptoms.

[y] Final disease severity assessed on a rating scale of 0–4 according to 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) 30 days after sowing.

[x] Standarized area under disease progress curve (SAUDPC) determined by the trapezoidal integration method (6).

[w] Data are means of ten replicated plants. Within columns and bacterial treatment, means with a common letter do not differ significantly (P=0.05) according to Fisher's protected LSD test.

concentration of the pathogen. SE+SO treatment with isolates RGAF 51 and RGAF 6a significantly (P<0.05) reduced the final disease severity in cv. ICCV 4 with inoculum concentrations of 500 and 2,000 chlamydospores per g of soil, respectively. On the contrary, in cv. PV 61 little disease suppression, although statistically significant (P<0.05), was achieved by the same treatments with isolates RGAF 6a and RGAF 7, and only when chickpeas were grown in soil infested with the lowest inoculum concentration of the pathogen (500 chlamydospores per g of soil).

TABLE 4. Populations of *Bacillus* spp. isolates RGAF 6a, RGAF 7 and RGAF 51 recovered from 10-day-old cv. PV 61 and cv. ICCV 4 chickpeas after seed, soil, and seed+soil treatments with the bacteria

Bacterial isolate	Bacterial treatment	Means[z] (log cfu/g fresh root tissue)	
		cv. ICCV 4	cv. PV 61
RGAF 6a	Seed	4.38 b	5.37 c
	Soil	4.90 a	5.66 b
	Seed + soil	4.91 a	5.90 a
RGAF 7	Seed	4.56 b	5.29 c
	Soil	4.68 b	5.87 b
	Seed + soil	5.28 a	6.28 a
RGAF 51	Seed	4.92 b	5.81 c
	Soil	5.09 a	6.09 b
	Seed + soil	5.11 a	6.27 a

[z]Populations of *Bacillus* spp. were determined as described in Materials and Methods. Data are means of three root samples (four petri dishes per replication).

[y]Within columns, means with the same letter do not differ significantly (P=0.05) according to Fisher's protected LSD test. Only comparisons among bacterial treatments within a bacterial isolate are indicated.

The three bacterial isolates tested were able to colonize the chickpea rhizosphere of cvs. ICCV 4 and PV 61 (Table 4); however, the rhizosphere population density was higher in PV 61 than in ICCV 4. Bacterial treatment significantly influenced (P<0.05) the rhizosphere colonization by *Bacillus* isolates RGAF 6a, RGAF 7 and RGAF 51, the highest levels of rhizosphere colonization being achieved when bacteria were applied as SE+SO treatment to cv. PV 61 or as SO and SE+SO treatments to cv. ICCV 4.

DISCUSSION

The goal of this work was to select bacteria from the chickpea rhizosphere antagonistic against *F.o. ciceris* and able to suppress Fusarium wilt in chickpeas. Our reasoning is that the rhizosphere environment may provide an opportunity to select strains effective as biocontrol agents in the same environment as where they will be used eventually (5,21). Approximately 32% of 74 bacterial isolates from the chickpea rhizosphere inhibited *in vitro* growth of *F.o. ciceris* in dual cultures, which appears to be a higher proportion than that inhibitory of *P. megasperma* Dreschsler f.sp. *medicaginis* obtained by Myatt *et al.* (14) from a similar environment. In our work, the greatest proportion (75%) of antagonistic bacteria were isolated from a Fusarium wilt-sick plot used repeatedly for resistance screening of chickpea germplasm and where a diversity of *F.o. ciceris* races occurs (11). Weller *et al.* (24) demonstrated that wheat roots grown in a take-all-suppressive soil yielded higher numbers of take-all-suppressive bacterial strains than those grown in take-all-conducive soils.

In most cases, bacteria effective as biocontrol agents of fungal plant diseases belong to the genera *Bacillus*, *Pseudomonas* and *Streptomyces* (5). In our study, the 24 bacterial isolates from the chickpea rhizosphere that we selected as antagonists of *F.o. ciceris* are *Bacillus* spp., while isolates of *S. maltophilia* and *P. fluorescens* showed a lesser inhibitory capacity against *F.o. ciceris* in dual cultures. However, *S. maltophilia* was a better biocontrol agent against Verticillium wilt of oilseed rape (*Verticillium dahliae* Kleb.) than was

B. subtilis or *P. fluorescens* (2). Results from bioassays in dual cultures suggest that production of antibiotics and/or other antifungal substances by these bacteria may be involved in the inhibition of hyphal growth of fungal isolates. This is supported by the following: (*a*) for all bacterial isolates selected initially, there was no direct contact between fungal mycelium and bacterial colonies, so that the inhibition of fungal growth was due to substances that diffused into the agar medium; (*b*) the PDA medium used for dual cultures is rich in nutrients and thus competition for them might be excluded; and (*c*) antibiosis is the general mode of antagonism observed for *Bacillus* spp. Most *Bacillus* spp. produce antibiotics, many of which have antifungal activity (5). The involvement of antifungal compounds produced by bacteria in the inhibition of fungal growth was confirmed by the ability of cell-free culture filtrates of bacteria to inhibit *in vitro* conidia germination and hyphal growth of *F.o. ciceris* and nonpathogenic *F. oxysporum*.

It is important to note that the antagonistic activity of isolates of *Bacillus* spp. from the chickpea rhizosphere was effective against a diversity of pathogenic *F. oxysporum* isolates that include different races of *F.o. ciceris, F.o. phaseoli* and *F.o. melonis*. Also, selected isolates of *Bacillus* spp. and *S. maltophilia* isolate RG 56 showed antagonistic activity against a beneficial rhizosphere fungi. Thus, cell-free culture filtrates inhibited conidial germination and hyphal growth of nonpathogenic *F. oxysporum* isolate Fo 90105, which was shown effective in the protection of cv. ICCV 4 chickpeas from disease caused by *F.o. ciceris* race 5 (9). Fravel (6) discussed the possibility of deleterious effects of antibiotic and antibiotic-like compounds, produced by biocontrol agents, on beneficial microorganisms. The effect of bacteria that inhibit *F.o. ciceris* on suppression of Fusarium wilt by nonpathogenic *F. oxysporum* is currently being investigated.

Bacillus spp. form spores that are resistant to unfavorable environmental conditions, and thus can be adapted to formulation and application in the field. Therefore, the production of antibiotics and of spores by *Bacillus* spp. suggests that these species may be attractive biological control agents of plant diseases caused by phytopathogenic fungi. Our results indicate that joint seed and soil treatments with *Bacillus* spp. isolates RGAF 6a and RGAF 51 can protect cv. ICCV 4 chickpeas from disease caused by a high inoculum concentration of the highly virulent race 5 of *F.o. ciceris*, whereas seed or soil treatments alone with the same bacterial isolates did not modify the development of Fusarium wilt. These bacterial isolates significantly inhibited growth of *F.o. ciceris* race 5 both directly and by means of cell-free culture filtrates, which suggests a correlation between antagonistic activity and disease suppression. Although the degree of disease suppression was low, it was significant and consistent. Such a low degree of suppression may result from the high disease potential imposed on the experimental system, *i.e.*, a large amount of highly virulent inoculum and the optimum environment for disease development. Also, results may be influenced by the final amount of antagonistic bacteria established in the chickpea rhizosphere, as both seed and soil treatments were required for a significant effect on disease. Hervás *et al.* (9) found that chickpea cultivar and inoculum concentration of *F.o. ciceris* race 5 can influence the degree of protection from the disease provided by nonpathogenic *F. oxysporum*. Results from the present study also showed that the degree of disease suppression varied with both the antagonistic bacterial isolate and the chickpea cultivar, as well as with the inoculum concentration of the pathogen. Futher studies, including a large number of bacterial isolates, host genotypes and inoculum concentration of the pathogen are under way to confirm this effect.

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