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Integrated Management of Fusarium Wilt of Chickpea with Sowing Date, Host Resistance, and Biological Control

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ABSTRACT

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A 3-year experiment was conducted in field microplots infested with *Fusarium oxysporum* f. sp. *ciceris* race 5 at Córdoba, Spain, in order to assess efficacy of an integrated management strategy for Fusarium wilt of chickpea that combined the choice of sowing date, use of partially resistant chickpea genotypes, and seed and soil treatments with biocontrol agents *Bacillus megaterium* RGAF 51, *B. subtilis* GB03, nonpathogenic *F. oxysporum* Fo 90105, and *Pseudomonas fluorescens* RG 26. Advancing the sowing date from early spring to winter significantly delayed disease onset, reduced the final disease intensity (amount of disease in a microplot that combines disease incidence and severity, expressed as a percentage of the maximum possible amount of disease in that microplot), and increased chickpea seed yield. A significant linear relationship was found between disease development over time and weather variables at the experimental site, with epidemics developing earlier and faster as mean temperature increased and accumulated rainfall decreased. Under conditions highly conducive for Fusarium wilt development, the degree of disease control depended primarily on choice of sowing date, and to a lesser extent on level of resistance of chickpea genotypes to *F. oxysporum* f. sp. *ciceris* race 5, and the biocontrol treatments. The main effects of sowing date, partially resistant genotypes, and biocontrol agents were a reduction in the rate of epidemic development over time, a reduction of disease intensity, and an increase in chickpea seedling emergence, respectively. Chickpea seed yield was influenced by all three factors in the study. The increase in chickpea seed yield was the most consistent effect of the biocontrol agents. However, that effect was primarily influenced by sowing date, which also determined disease development. Effectiveness of biocontrol treatments in disease management was lowest in January sowings, which were least favorable for Fusarium wilt. Sowing in February, which was moderately favorable for wilt development, resulted in the greatest increase in seed yield by the biocontrol agents. In March sowings, which were most conducive for the disease, the biocontrol agents delayed disease onset and increased seedling emergence. *B. subtilis* GB03 and *P. fluorescens* RG 26, applied either alone or each in combination with nonpathogenic *F. oxysporum* Fo 90105, were the most effective treatments at suppressing Fusarium wilt, or delaying disease onset and increasing seed yield, respectively. The importance of integrating existing control practices, partially effective by themselves, with other control measures to achieve appropriate management of Fusarium wilt and increase of seed yield in chickpea in Mediterranean-type environments is demonstrated by the results of this study.

Additional keywords: *Cicer arietinum*, metalaxyl, principal components analysis, quantitative epidemiology, seed treatments, weather conditions.

In sustainable agriculture, diseases of grain legumes should be managed by integrated disease management (IDM) strategies that involve the use of additive or synergistic combinations of biotic, cultural, and chemical control measures (7,21).

Chickpea (*Cicer arietinum* L.) is an important source of human food and animal feed that also helps in the management of soil fertility, particularly in dry lands (43). In the European Union, chickpea production is concentrated in the Mediterranean Basin, with Spain being the principal producer. Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the most important limiting factors of chickpea production in the Mediterranean Basin and the Indian Subcontinent (19). Fusarium wilt epidemics cause significant annual losses of chickpea yields (12,19) that may reach 100% under conditions favorable for disease (2,12,37). In Spain, annual yield losses of 12 to 15% are common (45).

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Management of Fusarium wilt of chickpea is difficult to achieve and no single control measure is fully effective. Currently, the use of resistant cultivars appears to be the most practical and economically efficient control measure for management of Fusarium wilt of chickpea and is also a key component in IDM programs (19,21,22). Good progress has been made in the development of high-yielding, well-adapted 'kabuli' (large, ramhead-shaped, beige seeds) chickpea lines, with combined complete or partial resistance to both Fusarium wilt and Ascochyta blight (*Didymella rabiei* [anamorph *Ascochyta rabiei*]) diseases (38). However, effectiveness of Fusarium wilt resistance can be curtailed by the occurrence of pathogenic races in *F. oxysporum* f. sp. *ciceris* (15,20). Eight races (designated 0, 1A, 1B/C, 2, 3, 4, 5, and 6) of *F. oxysporum* f. sp. *ciceris* have been identified (15,20,25). Race 0, the least virulent of the eight races, and race 1B/C induce progressive foliar yellowing compared with the severe wilting induced by races 1A to 6 (20).

Crop rotation, soil solarization, pathogen-free seed, removal of infested plant debris, and fungicide seed treatment are among the disease control measures that have also been employed to control Fusarium wilt, but have met with only limited success (13,16,19,23). Where land is not limiting, avoiding heavily infested fields can greatly reduce the impact of the disease (13).

Choice of proper sowing time can be useful for the management of Fusarium wilt of chickpea. For chickpeas in southern Spain, advancing the sowing date from early spring to early winter significantly delays epidemic onset, slows epidemic development, and reduces the final disease incidence and severity, and yield loss (36,37). In addition, chickpea winter sowing enables the reproductive phase of the crop to match with more favorable thermal and moisture supply regimes resulting in an increase in yield (43). However, the benefits of early sowing can be annulled if the cultivar sown is highly susceptible to wilt or a highly virulent race of the pathogen is dominant in the soil (36,37).

Biological management of Fusarium wilt of chickpea also has been addressed using bacterial and fungal antagonists in recent years (2,17,18,31,32,47). Isolates of *Pseudomonas* spp., *Bacillus* spp., *Paenibacillus* spp., and nonpathogenic (NP) isolates of *F. oxysporum* isolated from the chickpea rhizosphere were effective in suppressing Fusarium wilt of chickpea under controlled conditions (17,18,32). Therefore, biological control offers potential for suppression of Fusarium wilt under field conditions, particularly when used in combination with cultivars with partial resistance to the disease and choice of sowing date.

The objective of this research was to identify the benefits of integrating several control measures including choice of sowing time, partially resistant cultivars, and biological control, which previously were shown to be useful in the management of Fusarium wilt of chickpeas when used individually. Studies were conducted during three consecutive years in artificially infested field microplots in southern Spain that have been used in previous work (36,37).

MATERIALS AND METHODS

Microorganisms and production of inocula. The NP isolate *F. oxysporum* Fo 90105 was used in this study. The NP *F. oxysporum* Fo 90105 was isolated from roots of healthy chickpeas grown in a field naturally infested with *F. oxysporum* f. sp. *ciceris* at Santaella, Córdoba, in southern Spain. Isolate Fo 90105 was effective against Fusarium wilt of chickpea under controlled conditions (17,18). The isolate was stored in sterile soil in glass tubes at 4°C. Active cultures were obtained from small aliquots of soil plated on fresh potato dextrose agar (PDA) (250 g of unpeeled potatoes, 20 g of agar, and 20 g of glucose per liter of distilled water) and incubated at 25°C with a 12-h photoperiod of fluorescent and near-UV light at 36 µE m^{-2} s^{-1}.

Three bacterial isolates, *Pseudomonas fluorescens* RG 26, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03, were evaluated. *P. fluorescens* RG 26 and *B. megaterium* RGAF 51 were isolated from the chickpea rhizosphere and investigated for their ability to suppress Fusarium wilt of chickpea under controlled conditions in previous studies (18,31,32). Bacterial cells from cultures in Luria-Bertani broth medium (Difco Laboratories, Detroit) were stored in 25% glycerol at –80°C. Active cultures were obtained by streaking bacteria from stock cultures onto King's B agar (KBA) (27) (*P. fluorescens* RG 26) or PDA (Difco Laboratories; pH 7) (*B. megaterium* RGAF 51) plates and incubating at 28°C for 2 days. Inoculum of *B. subtilis* GB03 was obtained from the commercial formulation Kodiak HB provided by Gustafson, Inc., Dallas, TX, which contained 6 × 10^9 endospores per g of the formulation.

Chickpea genotypes. Four kabuli chickpea lines (CA-252.10.1.1M [CA-252] and CA-255.2.5.0M [CA-255]) and cultivars ('ICCV-4' and 'PV-61') were selected for the study based on their known reaction to races of *F. oxysporum* f. sp. *ciceris*. Seeds of cvs. ICCV-4 and PV-61 are small (<30 g per 100 seeds) and lines CA-252 and CA-255 are large (>44 g per 100 seeds) seeded chickpeas. Cultivar ICCV-4 is resistant to race 0 and susceptible to race 5 of *F. oxysporum* f. sp. *ciceris* (20,29) and was provided by H. A. van Rheenen, International Crops Research Institute for the Semiarid Tropics (ICRISAT), Hyderabad, India. Cultivar PV-61 is a commercial Spanish landrace moderately resistant to race 0 and susceptible to race 5 of the pathogen (20). Lines CA-252 and CA-255 are resistant to race 0 and partially resistant to race 5 of *F. oxysporum* f. sp. *ciceris* (38; R. M. Jiménez-Díaz, *unpublished data*). In addition, cv. JG-62 ('desi' type, small, angular, colored seeds) was used as a susceptible check; 'JG-62' is resistant to race 0 but highly susceptible to race 5 of *F. oxysporum* f. sp. *ciceris* (24).

Seed and soil treatment with biocontrol agents. Inoculum of NP *F. oxysporum* Fo 90105 for seed treatment was produced as described previously (18). Briefly, microconidia were produced in 100 ml of fresh potato dextrose broth (PDB) (250 g of unpeeled potatoes and 20 g of glucose per liter of distilled water) in 250-ml Erlenmeyer flasks incubated on an orbital shaker for 7 days at the same conditions described previously. Conidia in the liquid cultures were filtered through eight layers of sterile cheesecloth, harvested by centrifugation (10,000 × *g* for 10 min), and washed twice with sterile distilled water to remove traces of nutrients. Inoculum concentration was adjusted as needed with a hemacytometer. Conidial suspensions were stored at 4°C until use (less than 3 h). For soil treatment, inoculum of NP *F. oxysporum* Fo 90105 was increased in a cornmeal-sand (CMS) mixture (17,45) incubated at 25°C with a 12-h photoperiod of fluorescent and near-UV light at 36 µE m^{-2} s^{-1} for 2 weeks. Inoculum concentration of NP *F. oxysporum* Fo 90105 in the CMS mixture was estimated before soil treatment.

Bacterial inocula used to treat seed and soil was produced as described by Landa et al. (32). Briefly, inoculum of *B. megaterium* RGAF 51 was produced in 100 ml of PDB, pH 7, in 250-ml Erlenmeyer flasks, on an orbital shaker at 125 rpm and 28°C for 3 days. Inoculum of *P. fluorescens* RG 26 was produced on KBA plates incubated at 28°C for 2 days. Bacterial cells were harvested by centrifugation (10,000 × *g* for 20 min) and washed twice with sterile 0.1 M $MgSO_4$. Bacterial concentration in the suspension was adjusted to approximately 5 × 10^8 cells per ml by measuring absorbance at 600 nm (A_{600}) in a spectrophotometer and using standard curves for each bacterial isolate. *B. subtilis* GB03 spores were suspended in sterile 0.1 M $MgSO_4$ and applied to the seeds at a rate of 4 mg of Kodiak formulation per gram of seed, according to supplier's recommendations. For bacterial soil treatment, 25 g of heat-sterilized (110°C, 3 h) talcum powder was infested with 10 ml of a bacterial suspension and the mixture was dried at 30°C in an incubator for 5 to 7 days. Bacterial inoculum density in the infested talcum powder was determined before soil treatment.

Before treatment with biocontrol agents, seeds were surface disinfested in 2% NaOCl for 3 min, washed three times in sterile distilled water, and dried under a stream of filtered air for 3 h. Seeds were treated at the time with a biocontrol agent (2.0 ml of biocontrol agent suspension in 0.1 M $MgSO_4$ per 100 g of chickpea seeds) and metalaxyl (Apron 20, Syngenta Agro, Madrid, Spain; 300 mg a.i. per kg of seed) fungicide for control of Pythium seed rot and pre-emergence damping-off (26). Treatments were applied in 500-ml Erlenmeyer flasks to obtain an inoculum density of approximately 1 to 5 × 10^7 CFU/seed for bacterial isolates and 1 to 5 × 10^5 CFU/seed for NP *F. oxysporum* Fo 90105. Seeds of lines CA-252 and CA-255 (large seeded) received an additional 1 ml of 0.1 M $MgSO_4$ to facilitate homogeneous distribution of microbial inoculum. Seeds were rotatory-shaken by hand until the inoculum suspension was totally absorbed and then dried under a stream of filtered air for 3 h. The control treatment consisted of seeds treated with 0.1 M $MgSO_4$ and metalaxyl only. Inoculum density of biocontrol agents on the treated chickpea seeds was determined before sowing.

For soil treatment, appropriate amounts of talcum powder infested with the bacteria, NP *F. oxysporum* Fo 90105 inoculum in CMS, or 1.5 g of Kodiak were mixed with 250 g of autoclaved

(121°C, 1 h, twice, on two consecutive days) sand in a plastic bag to reach an inoculum density of approximately 1 to 5 × 10^7 CFU per g of sand for bacterial isolates, and 1 to 5 × 10^6 CFU per g of sand for NP *F. oxysporum* Fo 90105. In addition, when treatments with single biocontrol agents were compared with treatments combining two of them, bags with a bacterium or NP *F. oxysporum* Fo 90105 treatment received the appropriate amount of noninfested CMS or noninfested talcum powder, respectively. Control and Kodiak treatments received similar amounts of both noninfested CMS and talcum powder.

Assessment of inoculum density of biocontrol agents on treated seeds, talcum powder, and CMS substrates. Immediately following seed treatment, three sets of five treated, dried chickpea seeds were placed in tubes with 10 ml of 0.1 M $MgSO_4$, sonicated for 10 min, and vortexed for 1 min. Serial dilutions of the suspensions were plated onto each of three replicate plates of (i) V8 juice-oxgall-PCNB agar, a Fusarium-semiselective medium (5) for assessing inoculum density of the NP *F. oxysporum* Fo 90105; (ii) salt V8 agar *Bacillus*-semiselective medium (BSV8A) (46) for *B. megaterium* RGAF 51 and *B. subtilis* GB03; and (iii) modified KBA (9) for assessing *P. fluorescens* RG 26. For treatments combining two biocontrol agents (i.e., NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26), serial dilutions were plated onto the corresponding media at the same time. Cultures were incubated under the same conditions described previously, which are optimal for each microorganism. The inoculum assessment was designed as a 7 × 3 factorial treatment (7 biocontrol agent treatments × 3 culture media) with three replicates in a completely randomized design. Seeds treated with metalaxyl and biocontrol agents yielded 1.2 to 9.5 × 10^7 CFU of *B. megaterium* RGAF 51 per seed, 0.2 to 7.0 × 10^7 CFU of *B. subtilis* GB03 per seed, 1.4 to 8.3 × 10^4 CFU of *P. fluorescens* RG 26 per seed, and 1.5 to 9.8 × 10^5 CFU of NP *F. oxysporum* Fo 90105 per seed, depending upon chickpea genotypes, sowing dates, and year of experiments. There were no bacterial or fungal colonies based on dilution plating of the control treatment. An unexpected low inoculum density occurred in the treatment with *P. fluorescens* RG 26, which prompted us to study the effect of metalaxyl on viability of biocontrol agents.

To assess the amount of fungal and bacterial inocula on the infested talcum powder and CMS, four 1-g samples of each substrate were placed into a 250-ml Erlenmeyer flask containing 100 ml of sterile 0.1% water agar and the mixture was stirred in a blender for 1 min. Appropriate dilutions of the suspensions were plated onto each of four plates of the respective semiselective media and incubated at optimum conditions for each microorganism, as described previously. The inoculum assessment was designed as a 7 × 3 factorial treatment (7 biocontrol agents × 3 culture media) with four replicates in a completely randomized design. The infested talcum powder yielded 1.2 to 7.3 × 10^9 CFU/g of *B. megaterium* RGAF 51 and 0.2 to 2.3 × 10^9 CFU/g of *P. fluorescens* RG 26. The infested CMS yielded 3.2 to 10.5 × 10^7 CFU/g of NP *F. oxysporum* Fo 90105, depending upon date and year of experiments.

Effect of seed storage and treatment with metalaxyl on survival of biocontrol agents on seeds. Seeds of cv. PV-61 were treated with 0 or 3 g a.i. of metalaxyl per kg of seed and with one of the biocontrol agents or a combination of them: *B. megaterium* RGAF 51, *B. subtilis* GB03, NP *F. oxysporum* Fo 90105, *P. fluorescens* RG 26, NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26. The treated seeds were stored in sterile paper bags at 4°C for 200 days. Survival of biocontrol agents on the treated seeds was assessed at 15- to 30-day intervals by transferring four seeds to 10 ml of 0.1 M $MgSO_4$ in test tubes and processing the samples as described previously. The experiment had a 7 × 2 factorial design (7 biocontrol agent treatments × 2 levels of fungicide treatment). Each treatment was replicated three times (three independent seed samples) with four replicate plates per sample in a completely randomized design within each of nine sampling dates.

Design of field experiment. An experiment was conducted in microplots established on 30 October 1986 (36,37) in a field with sandy loam soil (pH 8.5, 1.4% organic matter) at the Alameda del Obispo Research Station near Córdoba during three consecutive seasons (harvest years 1997, 1998, and 1999). The microplots (1.25 × 1.25 m, 50 cm deep) were established in a field plot fumigated with methyl bromide plus chloropicrin (80 g/m^2) and were artificially infested with *F. oxysporum* f. sp. *ciceris* race 5 (isolate Foc 8012). Race 5 is the most virulent of the pathogen races present in Spain, for which no commercial kabuli cultivar with complete resistance is available (22; R. M. Jiménez-Díaz *unpublished data*). Inoculum of the pathogen was originally increased in the CMS mixture incubated as described earlier for NP *F. oxysporum* Fo 90105. The upper 15-cm layer in each microplot was mixed thoroughly with the infested CMS on 14 December 1986. This field has been used repeatedly for studies on the epidemiology of Fusarium wilt of chickpea (36,37) and screening of diverse chickpea germ plasm during the last 10 years (R. M. Jiménez-Díaz, *unpublished data*), which favored a high and homogenously distributed population of the pathogen in soil.

The experiment consisted of a completely randomized design, arranged in four replicate blocks, and was conducted in three consecutive harvest seasons. In each season, the treatments consisted of three dates of sowing, four chickpea genotypes, and four biocontrol agents (192 microplots in total). For each sowing date, genotype × biocontrol agent treatment combinations were randomly allocated to a set of microplots. Microplots were sown on 7 January, 6 February, and 18 March 1997 (year 1); 9 January, 11 February, and 19 March 1998 (year 2); and 8 January, 10 February, and 18 March 1999 (year 3). Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were included in years 1 and 2. Line CA-255 was not included in year 3. The following biocontrol treatments were tested in the year indicated: (i) *B. megaterium* RGAF 51, NP *F. oxysporum* Fo 90105, and *B. subtilis* GB03 (year 1); (ii) NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 (year 2); and (iii) NP *F. oxysporum* Fo 90105, *P. fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 (year 3). In each microplot, three rows manually opened 0.4-m apart and 0.2 m from the closest microplot edge barrier were sown to a chickpea genotype. In addition, four seeds of 'JG-62' were sown at each of four spots between the rows. The cv. JG-62 is highly susceptible to *F. oxysporum* f. sp. *ciceris* race 5 and was used as a susceptible internal control in the microplots. Each biocontrol microorganism was jointly applied to the seed and furrow at sowing by carefully spreading 250 g of the infested sand along the length of the sowing furrow and then placing 15 seeds of each genotype equidistant over the sand.

Before each sowing date, soil in a microplot was fertilized with 35 g of an 8-15-15 fertilizer basal dressing (Fertiberia, Madrid, Spain) and thoroughly mixed with the soil. Weeds in the microplots were removed by hand, and dimethoate 40% wt/vol (Rometan, ARAGRO, Madrid, Spain) and clorpirifos 48% wt/vol (Dursban 48, Syngenta Agro, Madrid, Spain) insecticides were applied for control of leaf miner (*Hylemiya* sp.) and cotton worm (*Spodoptera littoralis*) as needed, according to farmers' practices (10). Plants in the microplots were treated periodically with clortalonil 50% (Bravo 50, Dow Agrosciences, Madrid, Spain) to prevent infections by *D. rabiei*. Daily mean temperature and rainfall data were recorded by a weather station at the experimental site.

Disease assessment and chickpea seed yield. Disease reactions were assessed by the incidence and severity of symptoms at 7- to 10-day intervals. Severity of symptoms in individual plants of a microplot was assessed on a 0-to-4 rating scale based on the percentage of foliage with yellowing or necrosis in acropetal pro-

gression (0 = 0%, 1 = 1 to 33%, 2 = 34 to 66%, 3 = 67 to 100%, and 4 = dead plant). Incidence of foliar symptoms, I (0-to-1 scale), and severity data, S (0-to-4 scale), were used to calculate a disease intensity index (DII) (32,36) by the equation DII = $(I \times S)/4$. Thus, DII expresses the mean value of disease intensity at any given moment as a proportion of the maximum possible amount of disease. Disease progress curves were obtained from the accumulated DII over time in days from the date of sowing. Disease progress curves, 192 in years 1 and 2, and 144 in year 3, were characterized by means of four associated variables: (i) final disease intensity (DII_{final}) = DII observed at the final date of disease assessment; (ii) standardized area under disease progress curve (SAUDPC) calculated by the trapezoidal integration method standardized by duration time in days; (iii) $t_{0.05}$ = time in days to initial symptoms (disease onset), estimated as the number of days from sowing to reach a DII level of 0.05; and (iv) $t_{0.4}$ = number of days to reach a DII level of 0.4. Additionally, the percentage of total seedling emergence (SLE) was calculated. Chickpea yield in microplots was determined by mid-July every crop season. Seed yield (SY) (total seed weight per row) and 100-seed weight per row were determined for each microplot. Also, to quantify the effect of biocontrol agents, the four variables associated with disease progress curves, seed yield, and 100-seed weight in a microplot were expressed in relative units by dividing each variable value by the average variable value in the corresponding biocontrol nontreated (control) microplot.

Data analyses. All analyses were conducted using the Statistical Analysis Software System (version 8.0; SAS Institute, Cary, NC). Data of inoculum density on chickpea seeds were analyzed by standard analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS. Inoculum density data were log-transformed before analysis. Mean comparisons among treatments were performed using Fisher's protected least significant difference (LSD) test at $P = 0.05$. Furthermore, to describe the effect of time of storage on viability of biocontrol microorganisms on chickpea seeds, the negative asymptotic model, $\log(\text{CFU/seed}) = I_i - \{I_f/[1 + \exp(b - r \times t)]\}$, was fitted to inoculum density data by nonlinear regression analyses. In this model, I_i is the initial inoculum density, I_f is the difference between I_i and the final inoculum density on seeds, b is a parameter related to the time when there is a decrease in the inoculum viability (i.e., the smaller the value, the more rapid the loss of viability), and r is the rate of loss of viability. Analyses were performed using the least-squares program for nonlinear models (NLIN) procedure of SAS. The coefficient of determination (R^2), the mean square error, and the asymptotic standard error associated with the estimated parameter were used to assess the appropriateness of a model to describe the data. The standard errors of parameters obtained from regression analysis were used to compare the effect of experimental factors on viability of biocontrol microorganisms on chickpea seeds (6).

The effect of sowing date, host cultivar, and biocontrol agents on chickpea SY, SLE, and disease development was determined by multivariate ANOVA (MANOVA) and univariate ANOVA using the GLM procedure of SAS. The 1997, 1998, and 1999 experiments were analyzed separately. A factorial treatment design was used for analyses of experiments in which sowing date, chickpea cultivar, and biocontrol treatment were factors. In addition, principal component analysis was performed as described by Navas-Cortés et al. (36) with the FACTOR procedure of SAS. Principal component analysis produced a set of variables that were linear combinations of the original variables. After the initial factor extraction, an orthogonal varimax rotation was used to estimate the factor loadings. The first three factors associated with the four curve elements ($t_{0.05}$, $t_{0.4}$, DII_{final}, and SAUDPC), SLE, and SY were used to characterize each epidemic.

Multiple regression models were developed to quantify the effects of environmental variables on the time to disease onset and subsequent disease development. Cumulative rainfall and average daily temperature were selected as predictor variables. Appropriateness of a model to describe the data was evaluated according to criteria indicated previously. Analyses were performed with the least-squares program for linear models procedure of SAS.

RESULTS

The amount of Fusarium wilt and SY varied among the 3 years of the study. Fusarium wilt intensity was lowest in 1997 but increased in 1998 and 1999; accordingly, SY was highest in 1997 and decreased in 1998 and 1999. For each year, there was a trend ($P < 0.05$) for disease intensity to increase, and SY to decrease, as sowing date was delayed from winter (January) to early spring (March) (Table 1). No effect on 100-seed weight could be associated with sowing date, biocontrol agents, or year of experiment (data not shown). Overall, delaying the sowing date from January to February increased SAUDPC by 25% and reduced the time for disease onset ($t_{0.05}$) by 29 days and SY by 46%. Similarly, delaying the sowing date from February to March increased SAUDPC by 15% and reduced the $t_{0.05}$ and SY by 9 days and 78%, respectively (Table 1). Chickpea genotypes and treatment with the biocontrol agents also influenced disease intensity and SY, but to a lesser extent compared with sowing dates. Fusarium wilt developed faster and reached higher intensity in 'ICCV-4' compared with that of partially resistant lines CA-255 and CA-252, with disease development in 'PV-61' being intermediate between 'ICCV-4' and the partially resistant lines. Seed yield loss was highest in 'ICCV-4', and decreased in CA-252, CA-255, and 'PV-61', in that order (Table 1). As predicted, disease reaction in the highly susceptible check 'JG-62' was very severe; all plants died within 44 to 87 days after sowing, before maturity, indicating that inoculum of *F. oxysporum* f. sp. *ciceris* race 5 was uniformly spread throughout the microplot experimental field. In general, treatments with the biocontrol agents reduced disease intensity, delayed disease development, and increased SY to some extent.

For each year of the experiment, changes in Fusarium wilt progression and chickpea SY were related primarily to date of sowing, susceptibility of chickpea genotype, and to a lesser extent, treatment with the biocontrol agents and their interactions (Table 2). Initial MANOVA revealed a significant effect of sowing date (Wilks' $\lambda < 0.0382$, $P < 0.0001$), chickpea genotype (Wilks' $\lambda < 0.5462$, $P < 0.0001$), and the interaction of these two variables, for each year of the experiment (Wilks' $\lambda < 0.4900$, $P < 0.0001$). In addition, there was a significant effect on disease development and SY by treatment with the biocontrol agents (Wilks' $\lambda = 0.8045$, $P = 0.0353$) and their interaction with sowing date (Wilks' $\lambda = 0.6382$, $P = 0.0048$) in 1998 and 1997, respectively. In 1999, effects by all main factors (Wilks' $\lambda < 0.6724$, $P < 0.0024$) and their interactions (Wilks' $\lambda < 0.4570$, $P < 0.0001$) were significant (Table 2). The disease progress curve elements ($t_{0.05}$, $t_{0.4}$, DII_{final}, and SAUDPC) and variables associated with chickpea growth (SY and SLE) used in MANOVA did not contribute equally to variation in effects by main factors and their interactions. Eigenvectors of characteristic roots in MANOVA indicated that DII_{final} and SAUDPC were the dependent variables with the greatest influence. On the other hand, SLE and SY had the lowest weights (data not shown).

Similar to MANOVA, based on univariate ANOVA using the disease progress curve elements, SLE, and SY as dependent variables, with few exceptions, sowing date, chickpea genotype, and their interaction had a significant ($P < 0.05$) effect on disease development and chickpea growth. Treatment with the biocontrol agents also influenced SLE significantly ($P < 0.05$) in all years of the experiment, as well as $t_{0.05}$ in 1999 and SY in 1997 (Table 2). Major differences in the effects of main factors concerned the influence of sowing dates on epidemic development over time ($t_{0.05}$ and $t_{0.4}$), chickpea genotypes on disease intensity (DII_{final} and SAUDPC), and treatment with the biocontrol agents on disease

TABLE 1. Mean, standard error (SE), and range of values of disease progress curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, chickpea seedling emergence (SLE), and seed yield (SY) used to characterize epidemics of Fusarium wilt in four chickpea genotypes treated with biocontrol agents and sown in *Fusarium oxysporum* f. sp. *ciceris* race 5-infested microplots in 1997, 1998, and 1999[a]

			Chickpea genotype								
			ICCV-4		PV-61		CA-252		CA-255		
Year	Sowing date	Variable[b]	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Range[c]
1997	January	DII_{final}	0.54	0.02	0.50	0.01	0.52	0.03	0.55	0.02	0.46–0.57
		SAUDPC	0.15	<0.01	0.17	<0.01	0.20	0.02	0.20	0.01	0.13–0.22
		$t_{0.05}$	86.75	1.36	96.05	0.54	95.52	0.74	95.25	0.29	80.04–97.25
		$t_{0.4}$	117.04	0.72	117.72	0.39	115.23	1.86	115.51	0.74	114.36–119.54
		SLE	94.54	0.95	96.11	0.99	92.22	0.14	76.07	2.11	70.56–96.67
		SY	262.22	6.81	405.36	11.51	320.86	42.01	270.29	12.57	215.30–443.63
	February	DII_{final}	0.65	0.03	0.55	<0.01	0.57	0.02	0.56	<0.01	0.51–0.71
		SAUDPC	0.27	0.01	0.27	<0.01	0.26	0.01	0.23	<0.01	0.21–0.30
		$t_{0.05}$	54.60	2.15	51.32	0.31	61.62	3.29	63.87	0.94	48.09–66.17
		$t_{0.4}$	88.30	0.98	87.60	0.86	90.81	1.17	92.08	0.86	82.64–98.47
		SLE	58.33	14.96	81.94	4.95	69.91	5.52	65.14	3.03	46.67–87.78
		SY	126.39	25.89	308.71	6.44	147.37	9.27	185.82	28.62	61.88–356.90
	March	DII_{final}	0.90	0.01	0.84	0.03	0.79	0.03	0.77	0.03	0.74–0.95
		SAUDPC	0.46	0.03	0.39	0.02	0.32	0.03	0.37	0.02	0.27–0.55
		$t_{0.05}$	37.24	0.38	34.91	2.34	41.94	1.49	41.34	0.57	33.35–44.64
		$t_{0.4}$	60.51	3.74	66.17	3.89	74.54	3.06	67.45	2.52	55.39–82.76
		SLE	79.44	5.42	88.61	2.00	85.14	3.37	62.50	2.59	58.33–92.78
		SY	29.73	10.64	55.96	12.21	66.59	18.15	50.05	9.32	13.23–93.80
1998	January	DII_{final}	0.74	0.02	0.77	0.03	0.68	0.04	0.82	0.03	0.61–0.90
		SAUDPC	0.02	0.02	0.29	0.02	0.21	0.01	0.29	0.03	0.18–0.38
		$t_{0.05}$	77.06	1.34	82.21	0.90	88.90	1.92	89.63	0.72	67.75–96.21
		$t_{0.4}$	117.75	3.48	119.16	3.27	128.69	2.03	117.87	3.93	106.21–133.32
		SLE	72.50	3.78	82.08	1.94	78.89	3.05	73.61	2.51	55.00–83.33
		SY	136.22	13.69	264.21	16.70	193.44	26.82	107.52	14.70	81.76–331.30
	February	DII_{final}	0.87	0.04	0.87	0.06	0.89	0.09	0.96	0.02	0.78–0.98
		SAUDPC	0.36	0.02	0.35	0.02	0.30	0.03	0.40	0.02	0.22–0.45
		$t_{0.05}$	55.86	0.47	53.40	0.53	58.98	1.75	49.48	1.02	41.78–61.06
		$t_{0.4}$	93.19	4.01	95.10	2.31	100.76	2.61	89.65	2.63	78.58–110.60
		SLE	77.78	3.21	83.89	0.68	75.28	4.17	75.42	4.07	68.89–88.33
		SY	64.62	25.52	104.01	22.16	59.68	22.74	23.93	7.36	4.68–153.50
	March	DII_{final}	0.83	0.05	0.84	0.06	0.81	0.06	0.80	0.08	0.73–0.93
		SAUDPC	0.33	0.02	0.37	0.03	0.35	0.02	0.38	0.04	0.26–0.58
		$t_{0.05}$	54.42	2.82	47.04	2.75	52.34	1.35	55.06	1.45	38.52–57.71
		$t_{0.4}$	76.94	1.28	70.58	2.97	74.01	1.08	75.35	3.54	61.51–83.64
		SLE	49.03	10.03	60.28	1.85	46.81	5.21	44.72	8.31	20.56–81.11
		SY	7.78	3.49	3.56	2.67	4.41	2.21	7.55	3.53	0.00–17.78
1999	January	DII_{final}	0.86	<0.01	0.75	0.03	0.74	0.02	...[d]	...	0.69–0.91
		SAUDPC	0.34	<0.01	0.29	0.01	0.31	<0.01	...	...	0.25–0.37
		$t_{0.05}$	67.49	2.73	68.41	2.18	75.10	2.20	...	...	57.23–79.02
		$t_{0.4}$	97.20	0.80	101.46	1.87	100.96	1.28	...	...	94.17–104.71
		SLE	61.81	4.48	69.86	2.36	65.97	4.57	...	...	47.78–80.00
		SY	16.76	5.03	58.68	12.78	33.38	2.19	...	...	6.48–88.58
	February	DII_{final}	0.86	0.05	0.75	0.07	0.74	0.03	...	...	0.67–0.89
		SAUDPC	0.34	0.02	0.29	0.02	0.31	0.04	...	...	0.25–0.44
		$t_{0.05}$	53.87	2.25	50.11	1.30	53.48	1.51	...	...	37.43–57.48
		$t_{0.4}$	82.45	0.77	85.86	1.62	82.28	1.77	...	...	71.80–91.94
		SLE	75.56	5.05	71.39	3.62	65.28	2.63	...	...	58.89–82.22
		SY	18.04	3.28	48.76	3.81	18.50	3.12	...	...	2.10–82.58
	March	DII_{final}	0.93	0.03	0.59	0.08	0.60	0.07	...	...	0.48–0.96
		SAUDPC	0.42	0.02	0.25	0.06	0.28	0.05	...	...	0.20–0.45
		$t_{0.05}$	47.27	2.95	45.82	2.18	50.29	3.03	...	...	36.43–58.64
		$t_{0.4}$	61.87	1.69	69.91	0.89	68.39	2.29	...	...	56.81–77.49
		SLE	59.86	14.52	62.96	13.90	56.53	16.22	...	...	53.33–67.41
		SY	6.49	4.74	na[e]	na	na	na	...	...	0.10–22.45

[a] Experiments were conducted during three consecutive seasons in microplots established on 30 October 1986 (36,37) in a field plot that was fumigated and artificially infested with *F. oxysporum* f. sp. *ciceris* race 5. Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were sown on 7, 9, and 8 January 1997, 1998, and 1999, respectively; 6, 11, and 10 February 1997, 1998, and 1999, respectively; and 18, 19, and 18 March 1997, 1998, and 1999, respectively. Chickpea seeds and soil in sowing furrows of microplots were treated with nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999.

[b] DII_{final} = disease intensity index determined at the final date of disease assessment (0 to 1); SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method standardized by duration time in days; $t_{0.05}$ = time in days to initial symptoms, estimated as the number of days to reach DII = 0.05; $t_{0.4}$ = time in days to reach DII = 0.4; SLE (%); and SY (grams per microplot).

[c] Based on values obtained from 132 (global), 48 (1997 and 1998), and 36 (1999) disease progress curves.

[d] This chickpea line was not included in year 3 of experiments.

[e] na = data not available due to severe attack by pod borer (*Heliothis* spp.) during pod filling.

intensity and SLE. Seed yield contributed equally to differences among levels of all three main effects (Table 2).

Univariate ANOVAs indicated that, overall, the effect of sowing date accounted for the largest amount of variation (59.1%) followed by chickpea genotype (13.1%). Conversely, treatment with the biocontrol agents (2.4%) and interactions between main factors (4.5 to 9.4%) accounted for a lower amount of variance (Table 2). An exception to that general trend occurred in 1999, for which chickpea genotype and the interaction among the three main factors accounted for the largest amount of variation in disease intensity (DII_{final} and SAUDPC) and SLE, respectively (Table 2). To better estimate differences among effects of main factors and their interactions, additional multivariate analysis of principal components was conducted.

Principal components analysis. Means, standard errors, and range values of the disease progress curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, and chickpea SLE and SY included in the factor analysis are listed in Table 1. Pearson's correlation coefficients among the four curve elements were relatively high and significant (P < 0.001) (Table 3) (DII_{final} versus SAUDPC [r > 0.75] and $t_{0.05}$ versus $t_{0.4}$ [r > 0.86]). Overall, disease intensity variables DII_{final} and SAUDPC were negatively correlated with epidemic positional variables $t_{0.05}$ and $t_{0.4}$ and chickpea growth variables SLE and SY. However, some exceptions were observed when each year of the experiment was analyzed separately. Thus, SLE was not significantly ($P \geq 0.05$) correlated with any of the four curve elements in 1999, but SLE was negatively correlated with SAUDPC (r = –0.38, P = 0.008) in 1998, and positively correlated (P < 0.05) with $t_{0.05}$, $t_{0.4}$, and SY (0.63 > r > 0.28) in 1997 and 1998. Seed yield was negatively correlated with DII_{final} and SAUDPC (–0.86 > r > –0.58) in 1997 and 1998 and positively correlated with $t_{0.05}$ and $t_{0.4}$ (0.86 > r > 0.42) in each of the 3 years of the experiment; i.e., chickpea SY decreased as the onset of Fusarium wilt occurred earlier and epidemics developed more severely (Table 3).

In the principal components analysis, the first three factors accounted for at least 88.9% of the total variance when the 124 epidemics from the 3 years of the experiment were used in a single analysis, or the epidemics of a single season were considered separately (Table 4). Variation attributable to factors 4, 5, and 6 was marginal; therefore, only factors 1, 2, and 3 were extracted from the disease progress data (Table 4). As a result, the dimensionality of the curve elements, SLE, and SY was effectively reduced to three descriptive variables. Factors were a combination of all curve elements, SLE, and SY in the analysis and the corresponding values in the eigenvectors for each variable were used to interpret the epidemiological significance of the factors (Table 4). Factor 1 was dominated by high negative weights (<–0.72) for SAUDPC and DII_{final} in all experimental periods and high positive weights (>0.75) for SY in the global analysis, and in analyses of years 1997 and 1999. Factor 1 represents the amount of disease developed and chickpea SY yield obtained. Factor 2 was dominated by high positive weights (>0.76) for $t_{0.05}$ and $t_{0.4}$ in each of the 3 years of the experiment and also for SY in 1998. This factor can be interpreted as a positional factor for epidemic development over time, and chickpea SY in 1998. Factor 3, which accounted for the lowest percentage of the cumulative explained variance (≤18.2%), identifies the uniqueness of SLE (Table 4). A severe pod borer (*Heliothis* spp.) attack to the crop planted in March 1999 occurred during pod filling, in particular in microplots

TABLE 2. Effects of sowing date, chickpea genotype, and biocontrol agent and their interactions on disease progress curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, chickpea seedling emergence (SLE), and seed yield (SY) in microplot experiments in 1997, 1998, and 1999[a]

	Multivariate ANOVA		Disease progress curve element and associated chickpea growth variable[b]											
			DII_{final}		SAUDPC		$t_{0.05}$		$t_{0.4}$		SLE		SY	
Source	Wilks' λ	P	SS (%)	$P > F$	SS (%)	$P > F$	SS (%)	$P > F$	SS (%)	$P > F$	SS (%)	$P > F$	SS (%)	$P > F$
1997														
Sowing date (Sd)	0.0143	0.0001	85.60	0.0001	79.73	0.0001	81.31	0.0001	95.31	0.0001	30.10	0.0001	73.27	0.0001
Genotype (G)	0.2787	0.0001	4.71	0.0002	2.00	0.0826	17.01	0.0001	0.79	0.0412	23.54	0.0001	12.69	0.0001
Treatment (Ba)	0.8147	0.0642	0.69	0.3895	0.25	0.8374	<0.01	0.9475	0.06	0.8946	6.03	0.0008	1.59	0.0195
Sd × G	0.3168	0.0001	2.71	0.0628	9.25	0.0001	0.94	0.0001	1.54	0.0153	8.93	0.0005	6.27	0.0001
Sd × Ba	0.6382	0.0048	1.01	0.6002	0.18	0.9960	0.21	0.2166	0.08	0.9892	14.39	0.0001	2.15	0.0386
G × Ba	0.6505	0.2753	1.45	0.6809	3.59	0.2102	0.18	0.6301	0.64	0.6509	4.89	0.1248	1.16	0.5933
Sd × G × Ba	0.4909	0.6455	3.86	0.4939	5.01	0.5200	0.34	0.7698	0.58	0.5347	12.13	0.0152	2.86	0.4424
1998														
Sowing date (Sd)	0.0278	0.0001	47.62	0.0001	34.84	0.0001	88.61	0.0001	90.49	0.0001	64.48	0.0001	68.75	0.0001
Genotype (G)	0.5461	0.0001	7.97	0.0325	12.31	0.0315	2.03	0.0029	1.82	0.0292	6.81	0.0010	11.09	0.0001
Treatment (Ba)	0.8045	0.0353	3.41	0.2804	4.60	0.3384	0.07	0.9099	0.26	0.7236	7.92	0.0003	1.47	0.2054
Sd × G	0.4899	0.0001	11.21	0.0554	6.16	0.6036	4.61	0.0001	1.69	0.2032	1.47	0.7162	9.24	0.0002
Sd × Ba	0.7287	0.1573	4.66	0.5121	12.20	0.1818	0.56	0.6689	0.91	0.5888	5.90	0.0266	2.65	0.2197
G × Ba	0.7109	0.6877	10.19	0.2521	7.05	0.8129	1.09	0.5536	1.03	0.8069	6.71	0.0608	3.12	0.3686
Sd × G × Ba	0.5185	0.7867	14.93	0.5340	22.85	0.5350	3.03	0.2566	3.80	0.3798	6.71	0.5368	3.69	0.8563
1999														
Sowing date (Sd)	0.0381	0.0001	6.61	0.0007	1.53	0.4377	79.55	0.0001	93.58	0.0001	6.57	0.0001	36.22	0.0001
Genotype (G)	0.2692	0.0001	54.43	0.0001	40.50	0.0001	2.54	0.0591	2.31	0.0012	1.97	0.0158	30.87	0.0001
Treatment (Ba)	0.6723	0.0023	0.63	0.6887	5.69	0.1111	3.76	0.0401	0.15	0.8192	4.26	0.0006	1.97	0.5346
Sd × G	0.3952	0.0001	23.95	0.0001	19.24	0.0007	1.69	0.4281	0.64	0.4125	2.44	0.0359	10.90	0.0208
Sd × Ba	0.4569	0.0001	1.96	0.5930	23.97	0.0006	3.25	0.2927	1.36	0.2183	3.41	0.0272	6.24	0.3349
G × Ba	0.3566	0.0001	4.95	0.0805	3.92	0.6434	2.08	0.5769	0.65	0.6696	23.51	0.0001	4.02	0.6134
Sd × G × Ba	0.1536	0.0001	7.48	0.1474	5.15	0.9308	7.14	0.1959	1.31	0.7682	57.84	0.0001	9.78	0.5415

[a] Experiments were conducted during three consecutive seasons in microplots established on 30 October 1986 (36,37) in a field plot that was fumigated and artificially infested with *Fusarium oxysporum* f. sp. *ciceris* race 5. Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were sown on 7, 9, and 8 January 1997, 1998, and 1999, respectively; 6, 11, and 10 February 1997, 1998, and 1999, respectively; and 18, 19, and 18 March 1997, 1998, and 1999, respectively. Chickpea seeds and soil in sowing furrows of microplots were treated with nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999.

[b] DII_{final} = disease intensity index determined at the final date of disease assessment (0 to 1); SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method standardized by duration time in days; $t_{0.05}$ = time in days to initial symptoms, estimated as the number of days to reach DII = 0.05; $t_{0.4}$ = time in days to reach DII = 0.4; SLE (%); and SY (grams per microplot). SS (%) = relative percentage of the sum of squares accounting for the main effects and their interactions in analysis of variance (ANOVA). $P > F$ = probability values associated with F tests.

sowed to chickpea cv. PV-61 and line CA-252, which made the corresponding data for SY useless. Those data were not included in the principal component analysis.

A biplot display representing Fusarium wilt epidemics was developed for each of the 3 years of the experiment projected on the plane of factors 1 and 2 (Fig. 1). Factor 1 was negatively and positively correlated with disease intensity (DII_{final} and SAUDPC) and SY, respectively. Factor 2 was positively correlated with time to epidemic development ($t_{0.05}$ and $t_{0.4}$). In 1998, factor 2 also was positively correlated with SY. According to the position of epidemics projected on the X (factor 2)-Y (factor 1) plane, epidemic development was delayed from left to right along the *x* axis and

TABLE 3. Correlation analysis of disease curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, chickpea seedling emergence (SLE), and seed yield (SY) used to characterize epidemics of Fusarium wilt in four chickpea genotypes treated with biocontrol agents and sown in microplots infested with *Fusarium oxysporum* f. sp. *ciceris* race 5 in 1997, 1998, and 1999[a]

Year of experiment	Variable[b]	SAUDPC	$t_{0.05}$	$t_{0.4}$	SLE	SY
Global	DII_{final}	0.826***[c]	–0.498***	–0.398***	–0.214*	–0.701***
	SAUDPC	...	–0.681***	–0.685***	–0.345***	–0.701***
	$t_{0.05}$	...	...	0.906***	0.279**	0.688***
	$t_{0.4}$	...	...	...	0.385***	0.686***
	SLE	...	...	...	...	0.518***
1997	DII_{final}	0.926***	–0.815***	–0.885***	–0.168 NS	–0.859***
	SAUDPC	...	–0.861***	–0.944***	–0.274 NS	–0.816***
	$t_{0.05}$	...	...	0.969***	0.289*	0.817***
	$t_{0.4}$	...	...	...	0.322*	0.857***
	SLE	...	...	...	...	0.450**
1998	DII_{final}	0.746***	–0.599***	–0.497***	0.042 NS	–0.571***
	SAUDPC	...	–0.683***	–0.740***	–0.381**	–0.647***
	$t_{0.05}$	...	...	0.886***	0.370*	0.771***
	$t_{0.4}$	...	...	...	0.630***	0.861***
	SLE	...	...	...	...	0.577***
1999	DII_{final}	0.759***	0.123 NS	0.094 NS	–0.021 NS	–0.044 NS
	SAUDPC	...	–0.043 NS	–0.208 NS	–0.177 NS	–0.273 NS
	$t_{0.05}$	...	...	0.861***	0.077 NS	0.429**
	$t_{0.4}$	...	...	...	0.175 NS	0.657***
	SLE	...	...	...	...	0.228 NS

[a] Experiments were conducted during three consecutive seasons in microplots established on 30 October 1986 (36,37) in a field plot that was fumigated and artificially infested with *F. oxysporum* f. sp. *ciceris* race 5. Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were sown on 7, 9, and 8 January 1997, 1998, and 1999, respectively; 6, 11, and 10 February 1997, 1998, and 1999, respectively; and 18, 19, and 18 March 1997, 1998, and 1999, respectively. Chickpea seeds and soil in sowing furrows of microplots were treated with nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999.

[b] DII_{final} = disease intensity index determined at the final date of disease assessment; SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method standardized by duration time in days; $t_{0.05}$ = time in days to initial symptoms, estimated as the number of days to reach DII = 0.05; $t_{0.4}$ = time in days to reach DII = 0.4; and SY (grams per microplot).

[c] Pearson's correlation coefficients and level of significance: NS = not significant ($P \geq 0.05$); and *, **, and *** indicate significant at $P < 0.05$, 0.01, and 0.001, respectively, based on values obtained from 48 (1997 and 1998) and 36 (1999) disease progress curves.

TABLE 4. Eigenvectors and eigenvalues of principal components derived from disease curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, chickpea seedling emergence (SLE), and seed yield (SY) used to characterize epidemics of Fusarium wilt in four chickpea genotypes treated with biocontrol agents and sown in microplots infested with *Fusarium oxysporum* f. sp. *ciceris* race 5 in 1997, 1998, and 1999[a]

	Experimental period											
	Global			1997			1998			1999		
Variable[b]	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
DII_{final}	–0.95*[c]	–0.24	–0.09	–0.87*	–0.47	–0.01	–0.84*	0.40	0.22	–0.83*	–0.30	–0.26
SAUDPC	–0.74*	–0.56	–0.17	–0.72*	–0.65	–0.08	–0.85*	–0.29	–0.36	–0.78*	–0.34	–0.25
$t_{0.05}$	0.35	0.90*	0.08	0.47	0.86*	0.16	0.32	0.90*	0.15	0.15	0.96*	–0.07
$t_{0.4}$	0.29	0.91*	0.23	0.62	0.76*	0.18	0.37	0.77*	0.48	0.51	0.83*	0.04
SLE	0.17	0.15	0.97*	0.10	0.11	0.99*	0.01	0.29	0.94*	0.18	–0.06	0.97*
SY	0.75*	0.42	0.39	0.78*	0.44	0.35	0.37	0.77*	0.37	0.90*	0.13	0.01
Eigenvalues	4.12	0.83	0.66	4.64	0.22	0.94	4.03	0.43	1.10	3.47	1.25	0.62
Variance	2.23	2.19	1.19	2.49	2.16	1.15	1.80	2.31	1.44	2.41	1.84	1.08
C.E. variance (%)[d]	68.60	82.47	93.39	77.26	80.95	96.68	67.14	74.35	92.61	57.76	78.53	88.85

[a] Experiments were conducted during three consecutive seasons in microplots established on 30 October 1986 (36,37) in a field plot that was fumigated and artificially infested with *F. oxysporum* f. sp. *ciceris* race 5. Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were sown on 7, 9, and 8 January 1997, 1998, and 1999, respectively; 6, 11, and 10 February 1997, 1998, and 1999, respectively; and 18, 19, and 18 March 1997, 1998, and 1999, respectively. Chickpea seeds and soil in sowing furrows of microplots were treated with nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999.

[b] DII_{final} = disease intensity index determined at the final date of disease assessment; SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method standardized by duration time in days; $t_{0.05}$ = time in days to initial symptoms, estimated as the number of days to reach DII = 0.05; $t_{0.4}$ = time in days to reach DII = 0.4; and SY (grams per microplot).

[c] Curve elements are based on values obtained from 48 (1997 and 1998) and 28 (1999) disease progress curves. * Indicates values of curve elements dominating principal components F1, F2, and F3.

[d] Percent cumulative explained variance.

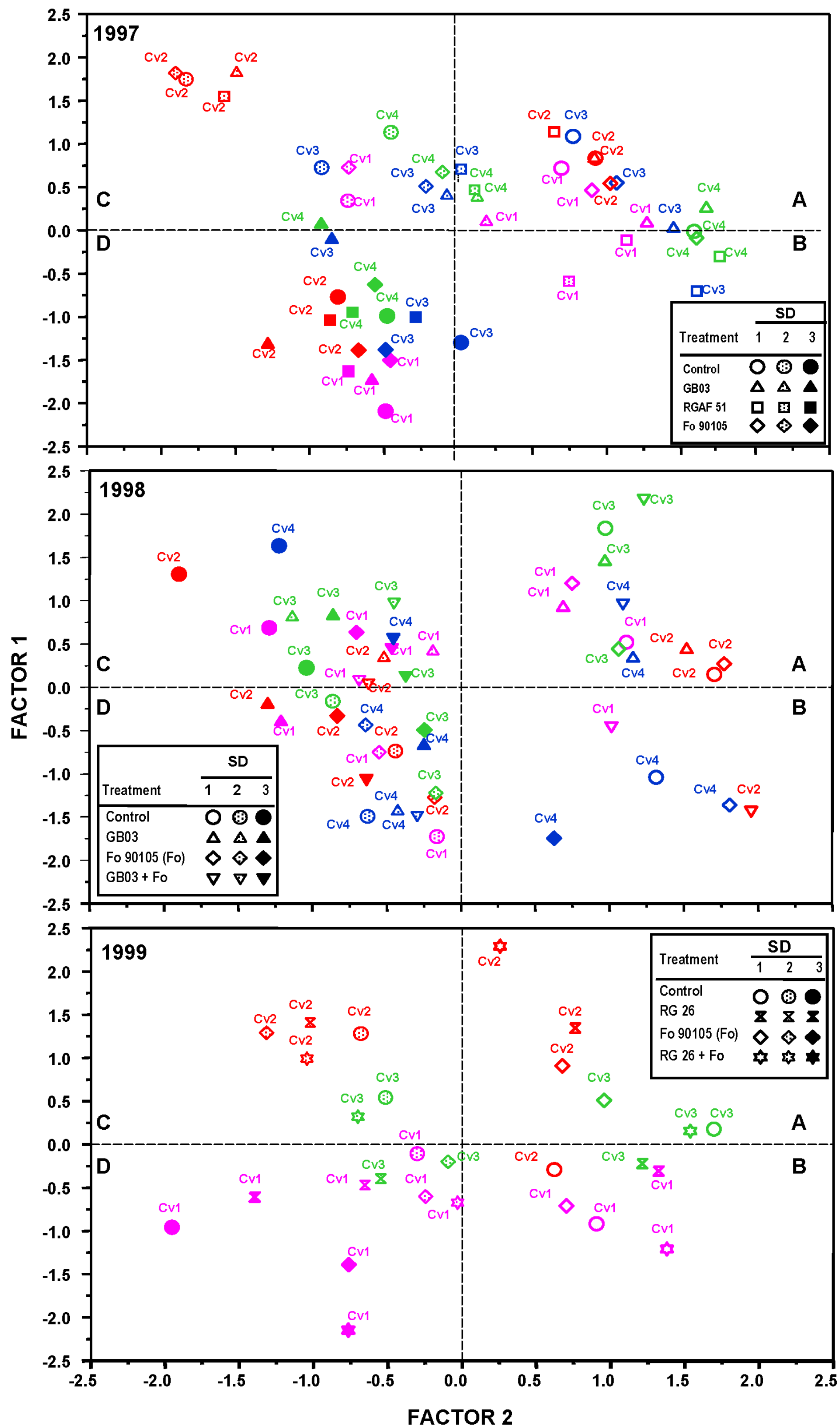

Fig. 1. Projection of factor scores on the plane of factors 1 and 2 from principal component analysis for the years 1997 (upper panel), 1998 (middle panel), and 1999 (lower panel). Chickpea cvs. ICCV-4 (Cv1, pink symbols) and PV-61 (Cv2, red symbols) and lines CA-252 (Cv3, green symbols) and CA-255 (Cv4, blue symbols) were sown in microplots artificially infested with *Fusarium oxysporum* f. sp. *ciceris* race 5 on 7, 9, and 8 January 1997, 1998, and 1999, respectively (1st sowing date; SD 1; open symbols); 6, 11, and 10 February 1997, 1998, and 1999, respectively (2nd sowing date; SD 2; dashed symbols); and 18, 19, and 18 March 1997, 1998, and 1999, respectively (3rd sowing date; SD 3; closed symbols). The biocontrol treatments included nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999. According to the position of projected epidemics along the *x* axis, epidemic development is delayed from left to right. Similarly, position of projected epidemics along the *y* axis indicates that the overall disease intensity increases and total chickpea seed yield decreases from top to bottom along the axis. Disease intensity of an epidemic increases and total chickpea seed yield decreases progressively from **A to D**. **A,** Epidemics with the least disease intensity, the most delayed disease onset, and highest seed yield. **D,** Epidemics with the highest disease intensity, earliest disease onset, and least seed yield.

the overall disease intensity increased, and SY decreased from top to bottom along the *y* axis. Thus, epidemics with the least disease intensity, most-delayed onset, and highest SY were grouped at the top right quadrant (A), while those with the highest disease intensity, earliest disease onset, and lowest SY were located at the bottom left quadrant (D). Therefore, chickpea SY and disease intensity increased progressively from epidemics projected on quadrant A to those projected on quadrants B, C, and D, in this sequence (Fig. 1).

For 3 years of the experiment, Fusarium wilt epidemics were located along the *x* axis according to the time of sowing and, to a lesser extent, to chickpea genotype and treatment with biocontrol agent. Epidemics that developed on sowings in early winter (January) were located to the far right and were characterized by the largest delay in disease onset and slowest disease progression. Conversely, epidemics occurring in microplots established in late winter (February) and early spring (March) were placed to the middle or to the far left of the *x* axis and developed earlier compared with epidemics occurring in microplots sown in January. In general, epidemics affecting crops sown at a given date were located from left to right along the *x* axis according to increasing susceptibility of chickpea genotypes to *F. oxysporum* f. sp. *ciceris* race 5. Thus, epidemics started earlier and progressed faster on 'PV-61', 'ICCV-4', CA-252, and CA-255, in that order (Fig. 1). Location of projected epidemics along the *y* axis was influenced also by combinations of chickpea genotypes and treatment with biocontrol agents, and to a lesser extent by sowing date. In 1997, most of the Fusarium wilt epidemics in microplots sown in January and February were located above $y = 0$ (Fig. 1), indicating less disease compared with that developed on microplots sown in March for which epidemics were mostly located below $y = 0$ (Fig. 1). In 1997 and 1999, epidemics developed in microplots sown at a given date were located from top to bottom along the *y* axis according to chickpea genotype, so that disease intensity was lowest on 'PV-61' and decreased progressively on CA-252, CA-255, and 'ICCV-4' in this sequence. Conversely, SY was highest on 'PV-61' and decreased in the same genotype sequence that disease intensity increased. Location of epidemics along the *y* axis according to chickpea genotypes was particularly clear in 1999, for which most epidemics that developed on 'ICCV-4' were located below $y = 0$, and those that developed on 'PV-61' and CA-252 were located above $y = 0$ (Fig. 1).

Location of projected Fusarium wilt epidemics along factors 1 and 2 associated with biocontrol agents was observed in microplots sown in February, in particular. In 1997, epidemics on the nontreated control sown in February were located in the middle section of quadrant C, except for cv. PV-61, which was located to the far left of quadrant C. In contrast, epidemics that developed on biocontrol-treated microplots sown to 'ICCV-4', CA-252, and CA-255 were located near $x = 0$, either to the right of quadrant C (NP *F. oxysporum* Fo 90105) or the left of quadrant A (*B. subtilis* GB03 and *B. megaterium* RGAF 51), suggesting that treatments with those biocontrol agents were effective in delaying disease onset in the February 1997 sowing. In 1998, epidemics that developed in microplots sown in February and treated with *B. subtilis* GB03 either singly or combined with NP *F. oxysporum* Fo 90105 were located in quadrant C, compared with those in nontreated microplots that were located in quadrant D. An exception to that was line CA-255, for which epidemics were located at quadrant D in the February 1998 sowing. Thus, those latter biocontrol agents were effective in suppressing disease intensity and, to a lesser extent, delayed epidemic onset. In that same year, epidemics developed in microplots sown in March and treated with *B. subtilis* GB03 either singly or combined with NP *F. oxysporum* Fo 90105 were located close to $x = 0$, $y = 0$ in quadrants C and D. This indicated a delay in disease onset and disease progression, as well as an increase in SY by treatments with those biocontrol agents, compared with epidemics in the nontreated control that were located to the far left of axis *x* in quadrant C. No clear pattern associated with biocontrol agents was discernible in 1999. In 1999, the position of projected epidemics along *x* and *y* axes was dominated by chickpea genotypes, irrespective of sowing dates (Fig. 1).

Beneficial effects of treatment with biocontrol agents. The ability of biocontrol agents to reduce DII_{final} and SAUDPC, delay disease onset ($t_{0.05}$), slow the rate of disease progression ($t_{0.4}$), or increase chickpea SLE and SY was expressed as the percentage of microplots treated with a biocontrol agent exhibiting >0%, >15%, and >30% of biocontrol effect. The biocontrol effect reflected either the percentage of reduction in DII_{final} and SAUDPC or the increase in $t_{0.05}$, $t_{0.4}$, chickpea SLE, and SY that occurred in a treated microplot relative to the average value of a variable obtained in the untreated, control microplots. Results are presented for the effects of sowing date, chickpea genotype, or biocontrol agent, each of which were pooled within the two other main factors in the study (Table 5).

Irrespective of the experimental factor combination, chickpea SY and the curve element SAUDPC were the variables most influenced by the biocontrol agents. Overall, the proportion of microplots across years of experiment and main factors ($n = 396$) with a decrease larger than 15% in DII_{final} and SAUDPC was 27.1 and 16.1%, respectively, and the proportion with an increase larger than 15% in $t_{0.05}$, $t_{0.4}$, SLE, and SY was 18.6, 10.0, 17.3, and 34.2%, respectively. Of particular importance was the increase in SY reached in 23.5 and 15.1% of the 396 treated microplots, which translated, respectively, into an increase of >50 and >100% of the SY achieved in the nontreated control microplots. Overall, the proportion of microplots across years and main factors with an increase in SY larger than 15% ranged from 25.4 to 45.8%, and the proportion with an increase larger than 30% ranged from 17.0 to 39.6% (Table 5).

Significant differences in biocontrol efficacy by treatments with biocontrol agents occurred among the 3 years in the study. The highest proportion of 15% reduction in DII_{final} (17.4%) and SAUDPC (32.6%) occurred in 1998; whereas a 15% increase in the delay of disease onset (34.6%), SLE (31.8%), and SY (39.3%) occurred in 1999. In 1997, the effects of the biocontrol agents were less apparent, so that a lesser degree of reduction in disease intensity, disease progression, and increase in SY occurred compared with those in 1998 and 1999 (Table 5).

Sowing date also influenced the proportion of cases with disease suppression larger than 15 or 30%. Sowing in January gave rise to a 15% reduction in DII_{final} in 17.7% of the cases and in SAUDPC in 29.2% of the cases. Interestingly, delaying the sowing date from January to February resulted in the highest proportion of 15 and 30% increase in SY (42.0 and 34.4%, respectively), even though the proportion of 15% reduction in disease intensity was slightly lower compared with the January sowings (13.0% of cases for DII_{final} and 27.5% of cases for SAUDPC). In March sowings, when the environment was the most conducive to disease, biocontrol agents were more effective in delaying disease onset. The proportion of 15% increase was 26.7 and 14.7% of cases for $t_{0.05}$ and $t_{0.4}$, respectively. Also a 15% increase in chickpea SLE and SY occurred in 22.9 and 26.7% of cases, respectively (Table 5).

Chickpea genotypes differed in their ability to support biological control. The highest proportion of 15% decrease in disease intensity occurred in CA-255 (21.1 and 31.0% of cases for DII_{final} and SAUDPC, respectively); however, CA-255 had the lowest proportion of 15% increase in $t_{0.05}$ (14.1% of cases) and the lowest increase in SY (25.4% of cases) and SLE (7.0% of cases). The beneficial effect of biocontrol agents was most consistent with 'ICCV-4', with intermediate disease suppression (13.1 and 29.0% of cases with 15% decrease for DII_{final} and SAUDPC, respectively), but the longest delay in disease progression (24.3 and 15.9% of cases with 15% increase on $t_{0.05}$ and $t_{0.4}$, respectively)

and the highest proportion of 15% increase in SLE (32.7% of cases) and SY (40.2% of cases). Reaction in CA-252 and 'PV-61' was intermediate with a 15% reduction in SAUDPC in 22.4 and 27.1% of cases, respectively, a 15% increase in $t_{0.05}$ in 16.8 and 17.8% of cases, respectively, and a 15% increase in SY in 30.8 and 37.4%, respectively (Table 5).

Overall, in the 3 years of the experiment, *B. subtilis* GB03 either alone or in combination with NP *F. oxysporum* Fo 90105, was the most effective biocontrol agent in suppressing disease. Thus, treatment with *B. subtilis* GB03 resulted in a 15% reduction of DII_{final} and SAUDPC in 15.8 and 31.6% of cases, respectively; *B. subtilis* GB03 plus NP *F. oxysporum* Fo 90105 led to a 15% reduction in DII_{final} and SAUDPC in 20.8 and 33.3% of cases, respectively. Treatment with *P. fluorescens* RG 26, either alone or in combination with NP *F. oxysporum* 90105, delayed disease onset and increased SLE the most. Thus, treatment with *P. fluorescens* RG 26 increased $t_{0.05}$ and SLE by 15% in 37.1 and 40.0% of cases, and by 30% in 8.6 and 28.6% of cases, respectively. Conversely, treatment with *P. fluorescens* RG 26 plus NP *F. oxysporum* Fo 90105 increased $t_{0.05}$ and SLE by 15% in 38.9 and 36.1% of cases and by 30% in 19.4 and 25.0% of cases, respectively. In parallel to those effects, treatment with these biocontrol agents resulted in the highest proportion of microplots with 15 and 30% increase in SY, which ranged from 31.6 to 45.8% and from 24.2 to 39.6%, respectively (Table 5). On the other hand, treatment with *B. megaterium* RGAF 51 provided the lowest proportion of cases with 15% disease suppression and SY increase, and treatment with NP *F. oxysporum* Fo 90105 resulted in intermediate values in the proportion of cases with 15% disease suppression and SY increase (Table 5).

Relationships between Fusarium wilt intensity and weather variables. Weather conditions differed among the years of the experiment but the greatest differences occurred among sowing dates within each harvest year (Fig. 2). Differences in temperature occurred mainly during the first month after sowing in each experimental period. Mean temperature in 1997, 1998, and 1999 averaged 10.8, 10.4, and 9.4°C in January; 13.4, 13.3, and 9.9°C in February; 16.7, 16.0, and 14.5°C in March; and ranged from 18.9 to 23.8°C, 15.3 to 26.9°C, and 17.7 to 29.2°C from April to mid-July, at harvest, respectively. Also, important differences in monthly rainfall occurred among years of experiment and among sowing dates within a year. Cumulative monthly rainfall in January 1997, 1998, and 1999 was 231, 146, and 30 mm, respectively. In February, cumulative rainfall was 0, 85, and 15 mm; in March, total rainfall was 0, 26, and 0 mm; and from mid-April to mid-July, cumulative rainfall was 166, 161, and 66 mm (Fig. 2).

Over the 3 years of the experiment, the average mean daily temperature (AMT) at the time of disease onset ($t_{0.05}$) and the time of DII = 0.4 ($t_{0.4}$) was 16.7 ± 1.5°C and 18.9 ± 1.9°C in January sowings, 17.0 ± 2.1°C and 19.8 ± 1.8°C in February sowings, and 19.3 ± 0.5°C and 21.3 ± 1.4°C in March sowings, respectively. Average accumulated rainfall (AR) from sowing to $t_{0.05}$ and from $t_{0.05}$ to $t_{0.4}$ was 151 ± 105 and 32 ± 25 mm for sowings in January, 15 ± 15 and 49 ± 31 mm for sowings in February, and 50 ± 32 and 48 ± 46 mm for sowings in March, respectively.

In the 3 years of the experiment, a highly significant multiple linear relationship ($P < 0.002$) was found between AMT and AR as independent variables and both time to disease onset ($t_{0.05}$) and time elapsed from onset to DII = 0.4 ($t_{0.05-0.4}$); i.e., disease started earlier and developed faster as AMT increased and AR decreased (data not shown). The multiple linear regression model accounted for 93, 91, and 75% of the variation on $t_{0.05}$ in 1997, 1998, and 1999, respectively; and for 24, 81, and 50% of the variation on $t_{0.05-0.4}$ in 1997, 1998, and 1999, respectively. In the 3 years of the study, the average mean temperature accounted for the highest amount of variance in the multiple regression model, which ranged from 52 to 99%, except for $t_{0.05-0.4}$ in 1997 for which AMT and AR accounted for 11 and 89% of variance, respectively. The

TABLE 5. Effects of year of experiment, sowing date, chickpea genotype, and biocontrol agent treatment on the percentage of number of cases in the study for which the disease curve elements DII_{final}, SAUDPC, $t_{0.05}$, and $t_{0.4}$, chickpea seedling emergence (SLE), and seed yield (SY) exhibited a percentage higher than 0, 15, or 30% of reduction (DII_{final}, SAUDPC) or increase ($t_{0.05}$, $t_{0.4}$, SLE, and SY) compared with the control that was not treated with biocontrol agents[a]

		Disease progress curve element and associated variables[b]																	
	No. of	DII_{final}			SAUDPC			$t_{0.05}$			$t_{0.4}$			SLE			SY		
Source	cases	>0	>15	>30	>0	>15	>30	>0	>15	>30	>0	>15	>30	>0	>15	>30	>0	>15	>30
Year of experiment																			
1997	144	47.5	16.3	0.0	49.6	23.4	4.3	42.6	11.3	1.4	50.4	7.8	2.1	45.4	9.2	5.0	39.7	29.1	21.3
1998	144	50.7	17.4	2.1	52.1	32.6	16.7	49.3	13.9	2.8	54.9	13.2	1.4	32.6	14.6	4.9	38.9	35.4	29.9
1999	108	39.3	14.0	2.8	47.7	24.3	9.3	65.4	34.6	11.2	57.1	8.6	0.0	55.1	31.8	23.4	41.1	39.3	34.6
Sowing date																			
January	132	46.2	17.7	0.8	52.3	29.2	13.8	44.6	13.8	3.1	54.6	4.6	0.0	54.6	18.5	6.9	44.6	33.8	26.9
February	132	45.0	13.0	1.5	51.9	27.5	9.9	56.5	15.3	2.3	51.9	10.7	1.5	35.1	10.7	4.6	45.0	42.0	34.4
March	132	48.1	17.6	2.3	45.8	24.4	6.9	52.7	26.7	8.4	55.0	14.7	2.3	40.5	22.9	18.3	29.8	26.7	22.9
Chickpea genotype																			
ICCV-4	108	45.8	13.1	0.9	55.1	29.0	10.3	61.7	24.3	7.5	58.9	15.9	4.7	61.7	32.7	18.7	45.8	40.2	35.5
PV-61	108	46.7	16.8	0.9	43.9	27.1	8.4	36.4	17.8	5.6	57.5	7.5	0.0	43.9	9.3	4.7	47.7	37.4	30.8
CA-252	108	41.1	15.0	3.7	46.7	22.4	7.5	51.4	16.8	1.9	42.5	5.7	0.0	40.2	16.8	12.1	30.8	30.8	20.6
CA-255	72	54.9	21.1	0.0	56.3	31.0	16.9	57.7	14.1	2.8	57.7	11.3	0.0	19.7	7.0	1.4	32.4	25.4	23.9
Biocontrol agent																			
GB03	96	54.7	15.8	0.0	56.8	31.6	12.6	50.5	10.5	2.1	53.7	12.6	3.2	43.2	13.7	7.4	41.1	31.6	24.2
RGAF 51	48	40.4	14.9	0.0	42.6	14.9	0.0	38.3	10.6	2.1	46.8	8.5	0.0	53.2	8.5	2.1	40.4	25.5	17.0
RG 26	36	42.9	11.4	0.0	57.1	31.4	8.6	68.6	37.1	8.6	60.0	11.4	0.0	51.4	40.0	28.6	45.7	42.9	37.1
Fo 90105	132	45.8	17.6	1.5	48.1	26.0	10.7	49.6	17.6	3.1	50.8	6.9	1.5	40.5	15.3	8.4	33.6	30.5	26.0
GB03 + Fo 90105	48	52.1	20.8	6.3	52.1	33.3	18.8	45.8	16.7	2.1	60.4	14.6	0.0	27.1	8.3	2.1	47.9	45.8	39.6
RG 26 + Fo 90105	36	30.6	11.1	2.8	38.9	22.2	5.6	66.7	38.9	19.4	60.0	8.6	0.0	55.6	36.1	25.0	41.7	41.7	36.1

[a] Experiments were conducted during three consecutive seasons in microplots established on 30 October 1986 (36,37) in a field plot that was fumigated and artificially infested with *Fusarium oxysporum* f. sp. *ciceris* race 5. Chickpea cvs. ICCV-4 and PV-61 and lines CA-252 and CA-255 were sown on 7, 9, and 8 January 1997, 1998, and 1999, respectively; 6, 11, and 10 February 1997, 1998, and 1999, respectively; and 18, 19, and 18 March 1997, 1998, and 1999, respectively. Chickpea seeds and soil in sowing furrows of microplots were treated with nonpathogenic (NP) *F. oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, and *B. subtilis* GB03 in 1997; NP *F. oxysporum* Fo 90105, *B. subtilis* GB03, and NP *F. oxysporum* Fo 90105 plus *B. subtilis* GB03 in 1998; and NP *F. oxysporum* Fo 90105, *Pseudomonas fluorescens* RG 26, and NP *F. oxysporum* Fo 90105 plus *P. fluorescens* RG 26 in 1999.

[b] DII_{final} = disease intensity index determined at the final date of disease assessment (0 to 1); SAUDPC = area under disease intensity progress curve estimated by the trapezoidal integration method standardized by duration time in days; $t_{0.05}$ = time in days to initial symptoms, estimated as the number of days to reach DII = 0.05; $t_{0.4}$ = time in days to reach DII = 0.4; SLE (%); and SY (grams per microplot).

rate of decrease of $t_{0.05}$ over AMT was low in 1999, and increased significantly ($P < 0.05$) in 1998 and 1997. In contrast, the rate of increase of $t_{0.05}$ over AR was the highest in 1999, and decreased significantly ($P < 0.05$) in 1997 and 1998. On the other hand, for $t_{0.05–0.4}$, both the rate of decrease over ATM and the rate of increase over AR were the highest in 1998, and decreased (not significantly) in 1997, but significantly ($P < 0.05$) in 1999 (data not shown).

Viability of biocontrol agents on chickpea seeds during storage. Mean population density of *B. megaterium* RGAF 51 on chickpea seeds was log 7.7 CFU/seed, while that of *B. subtilis* GB03 was log 6.4 CFU/seed (Fig. 3). The population of those bacteria on chickpea seeds remained stable over 200 days of storage (Fig. 3) as indicated by lack of significant differences among sampling dates. That occurred regardless of treatment with metalaxyl fungicide (300 mg a.i. per kg of seed). Co-inoculation of seeds with *B. subtilis* GB03 and NP *F. oxysporum* Fo 90105 did not influence survival of *B. subtilis* GB03, because population densities of the bacterium on singly- and double-inoculated seeds did not differ significantly. In contrast, the population size of NP *F. oxysporum* Fo 90105 and *P. fluorescens* RG 26 on chickpea seeds declined over time during storage. The net reduction in viability of biocontrol agents varied with agent, treatment with metalaxyl, and whether the agents were applied singly or in combination (Fig. 3). The reduction in viability over time was adequately described by a negative asymptotic model (Fig. 3; Table 6), i.e., viability of the microorganisms remained stable for a certain period immediately after storage, decreased to some extent, and thereafter stabilized to a final population density.

The viability of NP *F. oxysporum* Fo 90105 inoculum on chickpea seeds decreased slightly (about log 0.5 CFU/seed) after 200 days of storage. Decline in viability started after 75 to 80 days of storage, and it was not affected by co-inoculation with *B. subtilis* GB03 (Fig. 3; Table 6). In contrast, mixing NP *F. oxysporum* Fo 90105 with *P. fluorescens* RG 26 enhanced the decline in viability of the fungus over time of storage, with an earlier (immediately after seed treatment) and higher loss of viability (about log 3.3 CFU/seed in 200 days). That effect resulted in estimates of I_f and b parameters significantly ($P < 0.05$) higher and smaller than those of the biocontrol agents mentioned previously, respectively (Table 6). Treating seeds with metalaxyl significantly reduced survival of NP *F. oxysporum* Fo 90105 on seeds compared with seeds not treated with the fungicide. Thus, the population density of NP *F. oxysporum* Fo 90105 decreased significantly ($P < 0.05$) immediately after treatment with metalaxyl (Fig. 3; Table 6), and the decrease resulted in estimates of the I_i parameter being significantly ($P < 0.05$) smaller for the metalaxyl-treated seeds than for nontreated ones, irrespective of whether seeds were co-inoculated or not with another biocontrol agent (Table 6).

Similarly, treatment of seeds with NP *F. oxysporum* Fo 90105 inoculated singly or jointly with *B. subtilis* GB03, and treated with metalaxyl, resulted in an earlier and higher decline in viability compared with that of fungal inoculum on seeds without metalaxyl. This resulted in estimates of I_f and b parameters of metalaxyl-treated seeds that were significantly ($P < 0.05$) higher and smaller than those of the corresponding treatments without metalaxyl, respectively.

The viability of *P. fluorescens* RG 26 inoculum on seeds declined after 30 to 40 days of storage and diminished to about log 3.0 CFU/seed in 200 days, regardless of whether seeds were inoculated with the bacterium singly or jointly with NP *F. oxysporum* Fo 90105 (Fig. 3; Table 6). Treatment with metalaxyl dramatically affected the survival of *P. fluorescens* RG 26 on seeds (Fig. 3; Table 6). Viable inoculum of this bacterium after fungicide treatment was about log 3.0 CFU/seed lower than on untreated seeds (i.e., I_i estimates for seeds not treated with metalaxyl were significantly higher [$P < 0.05$] than those treated with metalaxyl). However, the variation of *P. fluorescens* RG 26 inoculum density on seeds over time of storage was not influenced by joint application of bacterial inoculum with NP *F. oxysporum* Fo 90105 and/or treatment with metalaxyl (Fig. 3; Table 6).

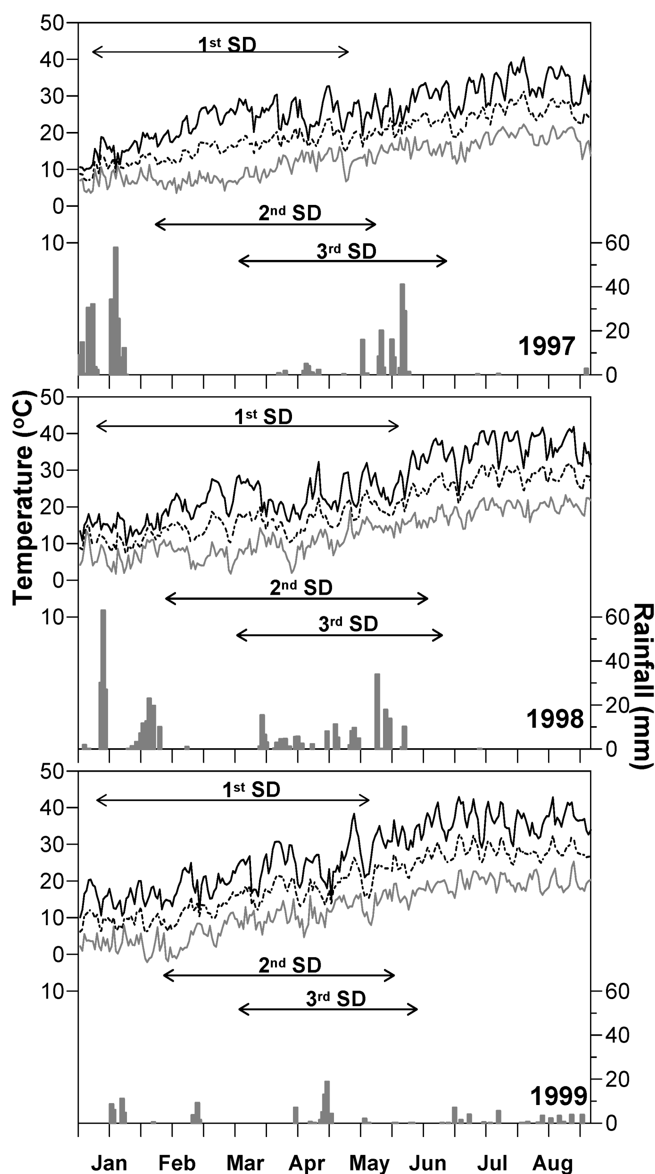

Fig. 2. Daily high (upper solid line), low (lower solid gray line), and average (middle dashed line) temperatures and daily rainfall (vertical bars) from January to August 1997, 1998, and 1999 from the experiment at the "Alameda del Obispo" Research Station, Córdoba, Spain. Horizontal lines represent the time period from sowing to the last disease assessment date in each sowing date.

DISCUSSION

Plant disease management is a component of agricultural production systems. Modification in crop management practices to improve yield or reduce inputs may influence the effectiveness of disease control measures (41). Thus, it is essential that disease management strategies work in conjunction with all components of the cropping system.

Our research addresses the efficacy of crop management practices, such as sowing date, in combination with disease control measures, such as the use of partially resistant chickpea genotypes and treatments with biocontrol agents, for the integrated management of Fusarium wilt in chickpeas. Traditionally, chickpeas in the Mediterranean region are sown in spring time, which

subjects the crop to heat and water stresses, often resulting in low yields. In contrast, a winter sowing strategy of chickpeas developed by the International Center for Agricultural Research in the Dry Areas in cooperation with ICRISAT enables matching crop growth with more appropriate environmental conditions. This prolongs the reproductive phase and period of seed build-up and results in increased yield (43). Chickpea winter sowing also contributes to control of Fusarium wilt and consequently to increased yield (19,36,37). However, the efficacy of winter sowing as a management practice for Fusarium wilt is influenced by several factors in the pathosystem, and may be significantly reduced or lost if practiced with a highly susceptible chickpea cultivar and/or in soils where a highly virulent race of the pathogen is prevalent (36,37).

In the present study, we confirmed that sowing date was the factor with the greatest effect on Fusarium wilt and yield of chickpea, as reported by Navas-Cortés et al. (36,37), and showed that under the Mediterranean environment prevailing in Andalucía, southern Spain, advancing chickpea sowing from early spring to winter significantly delays the onset, reduces the final

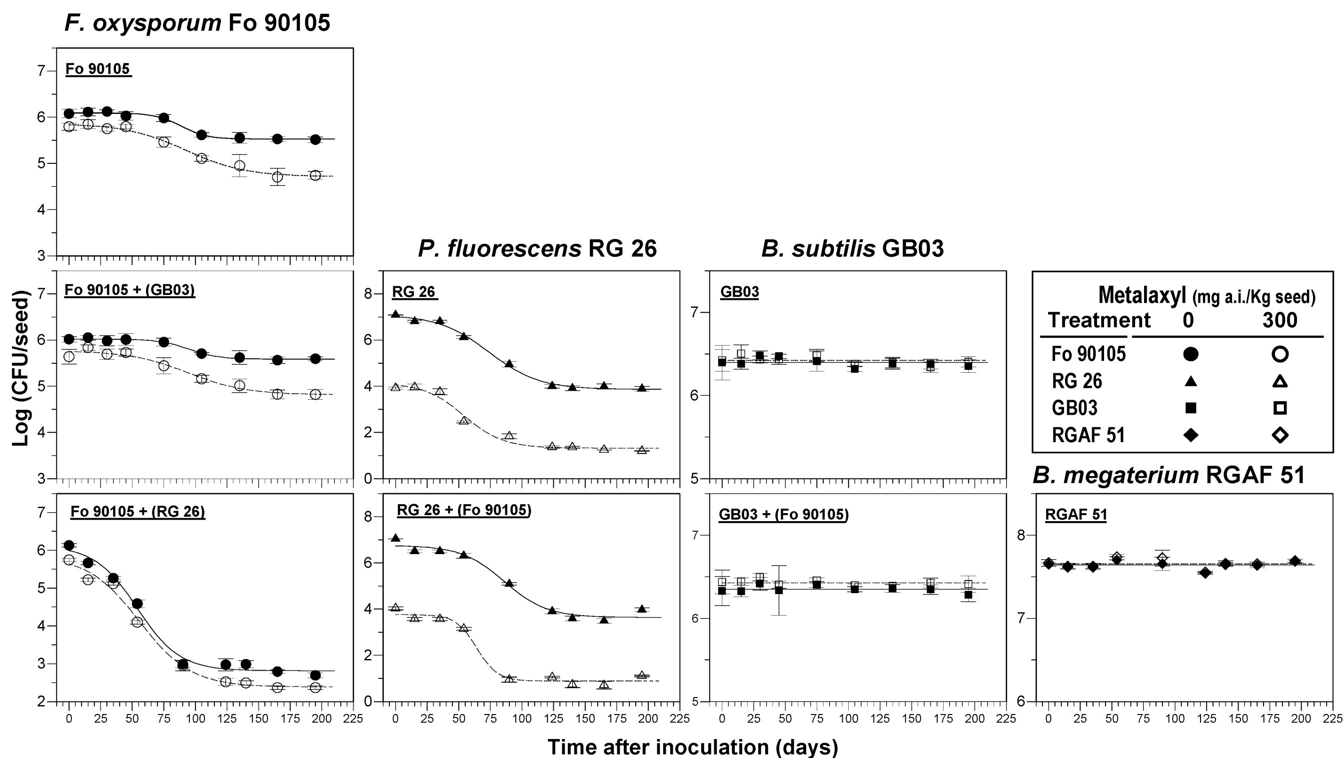

Fig. 3. Population dynamics of nonpathogenic (NP) *Fusarium oxysporum* Fo 90105, *Bacillus megaterium* RGAF 51, *B. subtilis* GB03, and *Pseudomonas fluorescens* RG 26 on chickpea seeds treated with a single organism or with a combination of them (in brackets) and with metalaxyl at a rate of 0 or 300 mg a.i. per kg of seed over time. Values (log[CFU/seed]) are the mean population densities ± standard errors. The solid line represents the predicted curves calculated by the negative asymptotic model (NP *F. oxysporum* Fo 90105 and *P. fluorescens* RG 26) or the mean value (*B. subtilis* GB03 and *B. megaterium* RGAF 51).

TABLE 6. Estimated parameters of a negative asymptotic model fitted by nonlinear regression analyses to the number of days that inocula of nonpathogenic (NP) *Fusarium oxysporum* Fo 90105 and *Pseudomonas fluorescens* RG 26 remained viable on chickpea seeds, when applied alone or in combination with *Bacillus subtilis* GB03 or *P. fluorescens* RG 26 and metalaxyl and stored at 4°C for 200 days

	Metalaxyl (0 mg a.i./kg of seed)						Metalaxyl (300 mg a.i./kg of seed)					
	Parameter estimates and statistics[b]						Parameter estimates and statistics[b]					
Biocontrol agent[a]	I_i (SE)	I_f (SE)	b (SE)	r (SE)	R^2	MSE	I_i (SE)	I_f (SE)	b (SE)	r (SE)	R^2	MSE
NP *F. oxysporum* Fo 90105												
Fo 90105	6.091*	0.563	8.586*	–0.097*	0.991	0.0055	5.860	1.145*	3.894	–0.042	0.989	0.0200
	(0.017)	(0.017)	(1.774)	(0.017)			(0.061)	(0.102)	(0.964)	(0.010)		
Fo 90105 plus GB03	6.020*	0.432	8.095*	–0.085	0.990	0.0034	5.766	0.949*	4.111	–0.044	0.975	0.0327
	(0.014)	(0.023)	(1.902)	(0.019)			(0.072)	(0.124)	(1.525)	(0.016)		
Fo 90105 plus RG 26	6.114*	3.299	3.234	–0.060	0.991	0.1413	5.860	3.474	2.706	–0.049	0.993	0.1070
	(0.223)	(0.264)	(0.856)	(0.014)			(0.243)	(0.289)	(0.651)	(0.010)		
P. fluorescens RG 26												
RG 26	7.092*	3.227	3.712	–0.050	0.995	0.0768	4.142	2.820	3.450	–0.064	0.979	0.2502
	(0.133)	(0.178)	(0.634)	(0.008)			(0.276)	(0.327)	(1.393)	(0.024)		
RG 26 plus Fo 90105	6.755*	3.111	5.100	–0.059	0.980	0.3314	3.763	2.874	7.821	–0.122	0.985	0.2523
	(0.200)	(0.303)	(1.911)	(0.021)			(0.145)	(0.191)	(3.419)	(0.060)		

[a] Seed treatment with biocontrol agents alone or in combination was performed as described in the text.
[b] Metalaxyl (Apron 20, Syngenta Agro, Madrid, Spain). I_i, I_f, b, and r = parameter estimates of negative asymptotic model. I_i is the initial inoculum density, I_f is the difference between I_i and the final inoculum density on seeds, b is a parameter related to the time when there is a decrease in the inoculum viability, and r is the rate of loss of viability. SE = standard error, R^2 = coefficient of determination, and MSE = final mean square error. Underlined values of a parameter in a column are significantly different ($P < 0.05$) from the corresponding parameter obtained among biocontrol agents at the same metalaxyl concentration. * Indicates values of a parameter in a row significantly different ($P < 0.05$) between metalaxyl concentrations. The standard errors of the parameters obtained from regression analyses were used to compare the effect of metalaxyl or co-inoculation on viability of a biocontrol microorganism.

amount of disease caused by highly virulent *F. oxysporum* f. sp. *ciceris* race 5, and increases chickpea yields.

Under our experimental conditions, the degree of control of Fusarium wilt conferred by winter sowing was influenced by the level of susceptibility in the chickpea genotypes sown and seed and soil treatment with biocontrol agents. However, the complex interactions found across the experiment made it difficult to quantify the contribution of each of the management practices to disease suppression. Principal component analysis of data enabled an overall assessment of the global effect of sowing date, chickpea genotypes, and biocontrol agents in Fusarium wilt management, as found in other studies (28,36). In the present study, the use of chickpea genotypes with no resistance or a moderate level of resistance annulled the benefits of disease suppression and SY increase provided by winter sowing. This could be due to compensating interactions among factors in the study. Even though conditions in winter sowing were not favorable for Fusarium wilt development, lack of resistance or a low degree of resistance in the plant resulted in high disease intensity (32,35).

Chickpea lines CA-252 and CA-255 used in our study were selected based on high yield, large seed size, tall plant type, and partial disease resistance against Fusarium wilt and Ascochyta blight (38; R. M. Jiménez-Díaz, *unpublished data*). Winter sowing exposes chickpeas to environmental conditions highly conducive for Ascochyta blight (13,39); therefore, an appropriate level of resistance to blight in chickpea cultivars is also a prerequisite for winter sowing as a management practice for Fusarium wilt (43). Overall, development of Fusarium wilt was slowed and there was lower disease intensity in partially resistant CA-255 and CA-252 compared with that in cvs. ICCV-4 and PV-61. This emphasizes the usefulness of using chickpea genotypes with a moderate level of disease resistance when implementing Fusarium wilt management by means of winter sowing (38).

The amount of Fusarium wilt varied considerably among the 3 years of the study, but more importantly among sowing dates within each year. Based on regression analyses, disease appeared earlier and progressed faster as temperature increased and rainfall decreased. Temperature was the primary determinant of the time to disease onset. On the other hand, once disease started to develop, rainfall was the more important factor driving further disease progression. These results are in agreement with observations in India (11) and California (48) where annual variation in the severity of Fusarium wilt was attributed to differences in temperature and soil moisture. In laboratory studies, severity of Fusarium wilt was positively correlated with increasing soil temperature and inoculum density of the pathogen (3,32,35). A temperature range of 20 to 30°C favored chickpea wilt, with the optimum at 24.8 to 28.5°C (44). This optimum range is close to temperatures usually prevailing during May and June in Andalucía, when wilt reached the highest absolute disease intensity values for the different treatment combinations in the experiment.

Root pathogens causing systemic infections such as formae speciales of *F. oxysporum* are difficult to manage with biocontrol agents. Although biocontrol agents can reduce the incidence and severity of Fusarium wilt diseases (1,2,8,17,32,33,40,47), most of the disease suppression appears to be temporary (49). This probably occurs because of motile infection courts that characterize those diseases. New root tips, the primary site for infection by the pathogen, continually develop as long as the root grows, thus increasing the likelihood of successful infections when conditions are optimal for disease. Essentially, the plant is susceptible throughout most of the crop season (32,42). Nevertheless, biocontrol agents can be useful in delaying disease onset and development (17,32,42), and reduction of disease incidence and severity early in the season may have a significant impact on yield. For Fusarium wilt of chickpeas, the extent of crop loss due to disease depends on the time at which wilting occurs (14,37), and total yield loss can result if wilt occurs before pod development (2). Consequently, a delay in disease onset could have a positive effect on SY. With few exceptions in our study, the main effect of biocontrol agents was a delay of disease onset and development, which resulted in lower final disease severity values and higher SY compared with that of the untreated, infected control.

In a number of studies, it has been shown that effective disease suppression by a biocontrol agent can be obtained only under low to moderate disease pressure or environmental conditions moderately favorable for disease development (4,8,32). For instance, *P. putida* WCS358 and *P. fluorescens* WCS374, NP *F. oxysporum* Fo47, and NP *F. oxysporum* Fo 90105 reduced Fusarium wilt of radish, tomato, and chickpea, respectively, only if disease incidence or pathogen inoculum density was low (8,17,18,40). Similarly, suppression of Fusarium wilt of chickpeas by *P. fluorescens* RG 26 in a previous study decreased as conditions became more favorable for disease development (i.e., optimal temperature for disease development and high inoculum density of the pathogen) (32). In contrast, in the present study, the environmental conditions determined by the year of experiment or sowing date that apparently were less favorable for disease to develop reduced the efficacy of biocontrol agents on disease control and increased chickpea SY. The biocontrol treatments were less effective in 1997, the year with lowest disease intensity and higher yields. Conversely, in 1998 and 1999, there was a trend for disease intensity to increase and chickpea yields to decrease in the untreated control. Treatments with biocontrol agents were effective in suppressing disease intensity and progression in 1998, and in delaying epidemic onset and increasing yields in 1999. Efficacy of biocontrol agents also was affected by sowing date. A small effect in disease suppression occurred in January sowings, when conditions were less favorable for Fusarium wilt development; whereas a maximum increase in chickpea SY by biocontrol agents occurred in February sowings, even with a moderate reduction in disease intensity. For sowings in March, when environment was most conducive to disease, the biocontrol agents provided relatively significant disease suppression and delayed the onset of disease as well as increased SLE. It might be possible that environmental conditions less suitable for Fusarium wilt also interfered with activities of the biocontrol agents related to biological control, and/or that the lesser level of disease occurring in 1997, and particularly in January sowings, made it difficult to detect a significant suppression of disease. Nevertheless, although treatments with biocontrol agents provided a moderate level of Fusarium wilt suppression, a significant increase in chickpea SY was obtained under environmental conditions moderately conducive for disease development, indicating a potential benefit of biocontrol agents as a component for integrated Fusarium wilt management.

Improvement in the efficacy of biological control and overcoming inconsistencies in the performance of individual biocontrol agents could be achieved by combining biocontrol agents or combining biocontrol and chemical treatments (33). However, those approaches require compatibility between biocontrol agents and between biocontrol agents and chemicals. In southern Spain, where chickpeas are traditionally sown in spring, fungicides are not normally applied to seed. However, the cooler and wetter weather occurring when chickpea sowing is advanced from spring to winter favors Pythium seed rot and preemergence damping-off, making fungicide seed treatment necessary (26). In our study, we treated seeds with metalaxyl because of its efficacy against Pythium seed rot of chickpea under field conditions (26). Based on our results, metalaxyl influenced survival of the biocontrol agents on seeds differentially. Metalaxyl had no effect on *B. megaterium* RGAF 51 and *B. subtilis* GB03 during storage, but reduced viability of NP *F. oxysporum* Fo 90105 to some extent immediately after application and during storage. Metalaxyl was even more deleterious to *P. fluorescens* RG 26. Fungicides evaluated with biocontrol agents as seed treatments have had varying effects on the biocontrol agents (50). In general, *Bacillus* spp. are least

affected by fungicide treatments probably because bacterial endospores are used as inoculum (34,50). However, even though metalaxyl affected viability of *P. fluorescens* RG 26 and NP *F. oxysporum* Fo 90105 in our study, treatments with these organisms either alone or in combination were among the most effective ones suppressing wilt and increasing chickpea SYs. The biocontrol agents in the study were applied jointly to seed and soil in the furrow, which may have enabled the microorganisms to colonize the chickpea rhizosphere effectively. Combining seed and soil treatment was shown previously to be more efficient for colonization of the chickpea rhizosphere and suppression of Fusarium wilt (31). Effectiveness of *P. fluorescens* Pf1 in suppressing chickpea wilt and increasing SY was enhanced when seed treatment with Pf1 was followed by application of the bacterium to soil (47).

Several authors have addressed the use of mixtures of NP *F. oxysporum* and rhizobacteria (especially fluorescent pseudomonads) for enhancing efficacy in the biological control of Fusarium wilts with variable success (1,17,18,33). In our study, combining NP *F. oxysporum* 90105 with either *B. subtilis* GB03 or *P. fluorescens* RG 26 was more effective at suppressing Fusarium wilt than single applications of NP *F. oxysporum* 90105, but was equally as effective as each bacterium alone. Even though use of mixtures of biocontrol agents did not provide a significant improvement in disease suppression over that obtained with each of the single components in a mixture, combining different biocontrol agents may still be advantageous for other reasons, including suppression of other pathogens (33).

Nevertheless, use of mixtures of biological agents requires consideration of the compatibility between agents when applied together; for example, as to whether or not each of the components in the mixture has tolerance to secondary metabolites produced by the other (30). In a previous study, growth of *Bacillus* spp. was inhibited by fusaric acid produced by pathogenic and NP *F. oxysporum* isolates, whereas that of *P. fluorescens* RG 26 was not affected although siderophore production by this bacterium was inhibited by the toxic metabolite (30). On the other hand, growth of NP *F. oxysporum* 90105 was inhibited by antifungal metabolites (including siderophores) produced by *P. fluorescens* RG 26 (30). These results may explain why mixing NP *F. oxysporum* 90105 and *P. fluorescens* RG 26 inocula for seed treatment resulted in a loss of viability of each of the agents on treated seeds which was greater compared with that of each microorganism when used alone.

In summary, we conclude that management of Fusarium wilt of chickpea in sustainable cropping systems of Mediterranean-type environments should be based on strategies that integrate several control measures; and in that approach sowing date and host resistance are two key measures for a rational management of the disease. Chickpea genotypes should be adapted to winter sowings, have an appropriate level of disease resistance, and a high potential for economic return. Unfortunately, cultivars with high levels of Fusarium wilt resistance are not widely planted because of low economic profitability. Lack of a sufficient level of disease resistance can be compensated to some extent by the use of biocontrol treatments, such as those tested in this study, to maximize SYs. By following this strategy, chickpeas would maintain their critical role in Mediterranean farming systems as a major source of protein and as a contributing factor in agriculture sustainability through improvement of soil fertility.

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