Induced Resistance Against Fusarium Wilt of Chickpea by Nonpathogenic Races of *Fusarium oxysporum* f. sp. *ciceris* and Nonpathogenic Isolates of *F. oxysporum*

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ABSTRACT

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Germinated seeds of chickpea cultivars JG 62 and ICCV 4 were inoculated with a conidial suspension of nonpathogenic races 0 and 1 of *Fusarium oxysporum* f. sp. *ciceris* or isolates of *F. oxysporum* (inducers), then challenged by root dip in a conidial suspension of *F. o.* f. sp. *ciceris* race 5. Prior inoculation with the inducers significantly reduced disease incidence and severity caused by race 5. However, the extent of protection decreased when the inoculum concentration of the challenger was close to that of the inducing agent, and it varied with the nature of inducing agent and genotype of the host. Also, resistance induced by race 0 in JG 62 was annulled when more than 3 days elapsed between inoculations with the nonpathogen and the challenger.

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the most important soilborne diseases of chickpea (*Cicer arietinum* L.) throughout the world, particularly in the Indian subcontinent and the Mediterranean region (10,24). Yield losses up to 10% have been attributed to Fusarium wilt in India (26) and Spain (29), and up to 40% in Tunisia (3).

The most practical and economical method for control of Fusarium wilt of chickpea worldwide is the use of resistant cultivars (10,14,15,24), the effectiveness of which is threatened by the occurrence of races of the pathogen. Seven races, designated 0 to 6, of *F. o.* f. sp. *ciceris* have been identified. Races 1 to 4 were first identified in India (11). Later, three additional races, 0, 5, and 6, and race 1 were reported from Spain (13,16). Race 0 occurs in California, Spain, and Tunisia; races 1 and 6 were identified in California, Morocco, and Spain; and race 5 was found in California and Spain only (13; R. M. Jiménez-Díaz, *unpublished*). Race 0, the least virulent of the seven races, is not pathogenic to desi cultivar JG 62 (small, angular, colored seeds), which is susceptible to all other known *F. o.* f. sp. *ciceris* races (11,13,16). Race 5, the most virulent of those occurring in California and Spain, is pathogenic to desi cultivars JG 74 and CPS 1, which are resistant to race 1 (11,13,16). Kabuli chickpeas (large, ram-head-shaped, beige seeds), the most economically important and widely grown in Spain, vary in susceptibility to race 0 but are very susceptible to all other races of the pathogen (15). Thus, new strategies are needed for the control of Fusarium wilt in kabuli chickpeas.

An alternative and attractive control method for Fusarium wilt of chickpea might be the biological control strategy of induced resistance. Induced resistance, which is a common response of plants to nonpathogenic bacteria, fungi, and viruses, is the phenomenon whereby once a plant has been infected by a microbial agent, it acquires enhanced resistance to subsequent infections by the same or different agents (17). Enhancement of resistance is accomplished by the inoculation of a plant with an inducer agent prior to, or concomitant with, a second ("challenge") inoculation with a pathogen. Several diseases caused by formae speciales of *F. oxysporum* have been controlled, with varying degrees of success, by preinoculation of the plant with pathogenic or nonpathogenic fungi of genera different from *Fusarium* (6,9), *Fusarium* species such as *F. solani* (Mart.) Sacc. (19), nonpathogenic formae speciales of *F. oxysporum* (1,6, 7,31) or nonpathogenic races of the same forma specialis (1,21), nonpathogenic *F. oxysporum* isolates (20,25), bacteria (8,30), and viruses (9). Results of most studies have shown that effective inducers of resistance are often closely related physiologically and taxonomically to the challenger isolate. Davis (6) reported that different formae speciales of *F. oxysporum* were more effective in inducing resistance to a given host's pathogenic forma specialis than were other root pathogens (*Verticillium albo-atrum* Reinke & Berthier and *Rhizoctonia solani* Kühn) or nonpathogens (*Penicillium notatum* Westling and *Neurospora crassa* Shear & Dodge). Biles and Martyn (1) and Martyn et al. (21) found that nonpathogenic races of *F. o.* f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Hans. (races 0 or 1) were better inducers of resistance on watermelon than the related forma specialis *F. o.* f. sp. *cucumerinum* J.H. Owen. Similar results were reported by Mas et al. (23) with Fusarium wilt of muskmelon. Apart from the inducer agent, induced resistance in Fusarium wilt diseases depends on the concentration of and the method of applying the inducing inoculum, the plant growth medium, the plant age at the time of induction, and the time between induction and challenge inoculations (1,7,9,25, 31).

The objective of this research was to determine the ability of two nonpathogenic races of *F. o.* f. sp. *ciceris* (races 0 and 1) and two nonpathogenic isolates of *F. oxysporum* (Fo 9009 and Fo 90105) to induce resistance against *F. o.* f. sp. *ciceris* race 5 in race 0- and race 1-resistant chickpea cultivars.

MATERIALS AND METHODS

Fungal isolates and inoculum production. Monoconidial isolates of *F. o.* f. sp. *ciceris* (Foc), isolates Fo 7802, Fo 7989, and Fo 8012 (representative of races 0 [Foc-0], 1 [Foc-1], and 5 [Foc-5], respectively) and of nonpathogenic *F. oxysporum,* isolates Fo 9009 and Fo 90105, were used in this study. Isolates Fo 7802 and Fo 8012 were obtained from infected chickpeas in different locations in southern Spain and have been used in previous studies (14,16,29). Isolate Fo 7989 was kindly provided by Y. L. Nene, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. Fo 9009 and Fo 90105 were originally isolated from the rhizosphere of healthy chickpeas grown in an infested site at Santaella, Córdoba, southern Spain. This site has been used repeatedly for resistance screening of chickpea germ plasm (14; A. R. Alcalá-Jiménez and R. M. Jiménez-Díaz, *unpublished*).

All isolates were stored in sterile soil tubes at 4°C. Active cultures were obtained from small aliquots of a soil culture

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plated on potato-dextrose agar (PDA) and incubated at 25°C with a 12-h photoperiod of fluorescent and near-UV light at 36 $\mu E \cdot m^{-2} \cdot s^{-1}$. Inoculum was increased in potato-dextrose broth (PDB) incubated on a rotatory shaker at 120 rpm under the same conditions as PDA cultures for 7 days. Then liquid cultures, containing mostly microconidia, were filtered through eight layers of sterile cheesecloth to remove mycelial mats. Microconidia left in the filtrate were pelleted by centrifugation (10,000 rpm, 10 min) and washed three times with sterile distilled water to remove traces of nutrients. Inoculum concentration was adjusted as needed using a hemacytometer.

Plant growth and inoculation. Chickpea cultivars JG 62 (desi type) and ICCV 4 (kabuli type) were used. JG 62 and ICCV 4 are resistant to Foc-0 and to Foc-0 and Foc-1, respectively (13,16,18). Seeds of these cultivars were kindly provided by H. A. van Rheenen, ICRISAT, Hyderabad, India. Seeds were surface-disinfested in 2% NaOCl for 3 min, washed three times in sterile distilled water, and germinated on autoclaved layers of paper towels in moist chambers at 25°C for 30 h. Germinated seeds, selected for uniformity (length of radicle = 1 to 2 cm), were placed in a microconidial suspension of inducing inoculum (either Foc-0, Foc-1, Fo 9009, or Fo 90105) or sterile distilled water (control) and incubated at 25°C in the darkness on a rotatory shaker at 50 rpm for 16 h. Inoculated seeds were sown in sterile sand in trays (60 × 40 × 10 cm; one tray per inducing treatment), and seedlings were grown in a growth chamber adjusted to 25°C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ for the appropriate time.

Inducer-inoculated seedlings were removed from the trays, selected for uniformity of root length, washed free of sand without intentional wounding under running tap water, and challenge inoculated with Foc-5. For inoculation, seedlings were placed in a microconidial suspension in 6-cm-diameter cylindrical glass bottles and incubated at 25°C with a 14-h photoperiod of fluorescent light at 360 $\mu E \cdot m^{-2} \cdot s^{-1}$ on a rotatory shaker at 50 rpm for 16 h. Control seedlings were placed in bottles filled with sterile distilled water. Inoculated and control seedlings were transplanted into 15-cm-diameter clay pots (four plants per pot) filled with an autoclaved soil mixture (clay loam/sand/peat, 1:1:1, vol/vol/vol). Just before challenge inoculations, isolations were made from a sample of 10 to 25 inducer-inoculated seedlings to determine the ability of inducing inocula to infect and colonize JG 62 and ICCV 4 chickpeas. Hypocotyl and root tissues were cut into 5-mm-long pieces and, together with cotyledons, surface-disinfested in 0.4% NaOCl for 1 min, plated on V8 juice-oxgall-PCNB agar (VOPA), a Fusarium-selective medium (2), and incubated for 3 to 5 days. Plants were grown in a growth chamber adjusted as above for 41 to 45 days. Plants were fertilized weekly with 100 ml of Hoagland's nutrient solution (12).

Induced resistance in cultivar JG 62. Two experiments were conducted to determine the ability of Foc-0 (nonpathogenic to JG 62) and of isolates Fo 9009 and Fo 90105 to induce resistance against Foc-5 in susceptible JG 62 chickpeas. In one experiment, germinated seeds were inoculated with 5×10^6 conidia per ml of Foc-0; and after 3, 5, or 7 days of incubation (time after inducing inoculation) they were challenge inoculated with either 2×10^4 (low dose = L) or 2×10^6 (high dose = H) conidia per ml of Foc-5. Thus, there were six treatments for each time period as follows (inducing inoculation/challenge inoculation): (i) water/ water; (ii) Foc-0/water; (iii) water/L Foc-5; (iv) water/H Foc-5; (v) Foc-0/L Foc-5; and (vi) Foc-0/H Foc-5. There were five replicated pots for each treatment–time period combination. A split-plot experiment design was used in which time periods between inducing and challenge inoculations were main plots, and treatments were subplots.

In another experiment, germinated seeds were inoculated with 5×10^6 conidia per ml of either Fo 9009 or Fo 90105, and 3 days later they were challenge inoculated with 5×10^4 (L) or 5×10^6 (H) conidia per ml of Foc-5. Thus, the following treatments were included: (i) water/water; (ii) Fo 9009/water; (iii) Fo 90105/water; (iv) water/L Foc-5; (v) water/H Foc-5; (vi) Fo 9009/L Foc-5; (vii) Fo 9009/H Foc-5; (viii) Fo 90105/L Foc-5; and (ix) Fo 90105/H Foc-5. A randomized complete block design with four replicates was used. The two experiments were repeated once.

Induced resistance in cultivar ICCV 4. Two experiments (I, II) were carried out to determine the ability of Foc-0 and Foc-1 (both nonpathogenic to ICCV 4) and of isolates Fo 9009 and Fo 90105 to induce resistance against Foc-5 in the susceptible ICCV 4. For experiment I, germinated seeds were inoculated with 5×10^6 conidia per ml of either Foc-0, Foc-1, Fo 9009, or Fo 90105; and 3 days later they were challenge inoculated with 2×10^4 or 2×10^6 conidia per ml of Foc-5. Experiment II was carried out using 2×10^7 conidia per ml of the inducing inocula and 1×10^6 or 1×10^7 conidia per ml for the challenge inoculation. For the two experiments, the additional treatments were included, as before: (i) water/water; (ii) inducer inocula/water; and (iii) water/challenger inoculum. Experiments consisted of a randomized complete block design with four replicated pots for each treatment, and experiments were repeated once.

Disease assessment and data analyses. Disease reactions were assessed by the incidence (percentage) and severity of symptoms. Each plant was assessed for symptom severity at 2-day intervals using a 0 to 4 rating scale according to percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Upon termination of experiments, isolations on VOPA were made from stem segments of symptomless plants to determine the occurrence of vascular infections. Procedures for isolations were described previously for inducer-inoculated seedlings.

All experiments were repeated once, and similarity among experiments tested by preliminary analyses of variance using experimental runs as blocks allowed combining data for analyses of variance and linear regression. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analysis of variance. Data were analyzed using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference test (LSD) at $P = 0.05$. Disease incidence values over time were transformed by the monomolecular transformation $\ln[1/(1 - Y)]$. Incidence values of 0 or 100% were not used in these transformations. Then linear regression analyses were performed with transformed data. Coefficient of determination (R^2), coefficient of determination adjusted for degrees of freedom (Ra^2), and pattern of residuals plotted against expected values were used to indicate appropriateness of the model to describe the data (4,5). Slope values of linear regressions over time were compared by t test at $P = 0.05$ (27).

RESULTS

Resistance in cultivar JG 62 induced by *F. o.* f. sp. *ciceris* race 0. Both the inducer–challenge treatments and time intervals between inducing and challenge inoculations influenced the development of disease caused by Foc-5 in JG 62. There were no symptoms in control plants. Plants inoculated only with Foc-0 were free of symptoms, and upon termination of experiments the fungus was never isolated from vascular tissues but was isolated from the cortex of hypocotyl and roots. Furthermore, isolations from inoculated seedlings prior to challenge inoculations recovered the fungus from cotyledons and the cortical tissue of older portions of the root, but not from the root apex (data not shown).

Noninduced, challenge-inoculated plants showed wilt symptoms characteristic of those induced by Foc-5 in susceptible cultivars (16,29). Disease development in these plants was not significantly influenced by the time interval between the water treatment and challenge inoculation. However, the rate of increase of disease

incidence over time (Fig. 1) was significantly higher ($P < 0.05$) with 2×10^6 than with 2×10^4 conidia per ml. There were no significant differences ($P = 0.05$) in the final disease incidence or severity (Table 1). In addition, when plants were inoculated with 2×10^4 conidia per ml, a larger percentage of plants was symptomless but systemically infected or noninfected than when 2×10^6 conidia per ml were used (Table 1).

Prior inoculation with Foc-0 did not modify the disease syndrome caused by Foc-5 in susceptible cultivars. However, when seedlings inoculated with Foc-0 were inoculated 3 days later with 2×10^4 conidia per ml of Foc-5, the onset of symptoms was delayed by about 10 days (not shown) and the rate of increase of disease incidence was significantly lower ($P < 0.05$) than in noninduced controls (Fig. 1). As a result, the final mean disease severity in Foc-0-induced plants was significantly ($P < 0.05$) lower than in noninduced ones (Table 1), and the final disease incidence was significantly reduced ($P < 0.05$) by some 43% compared to noninduced challenge-inoculated controls (Table 1). Of the 67.5% Foc-0-induced plants that remained symptomless by the end of the experiments, a significant 42.5% ($P < 0.05$) were not systemically infected by Foc-5 (Table 1). When the challenge inoculum concentration was increased from 2×10^4 to 2×10^6 conidia per ml, the onset of symptoms was delayed by about 7 days with respect to noninduced controls (not shown), but afterward disease incidence in Foc-0-induced plants increased over time at a rate significantly higher ($P < 0.05$) than that in noninduced plants (Fig. 1). Consequently, there were no significant differences ($P < 0.05$) in the final disease incidence or severity between the two treatments (Table 1), and only 5% of plants remained symptomless and not systemically infected (Table 1). The increase in the time interval between inducing and challenge inoculations from 3 to 5 or 7 days annulled all effects described, and there were no significant differences ($P = 0.05$) between treatments except for the lower inoculum density of Foc-5 at 7 days between inoculations. For this treatment, the onset of symptoms was delayed by about 3 days (not shown); afterward the disease incidence in induced plants increased at a significantly higher rate ($P < 0.05$) than in noninduced plants (Fig. 1).

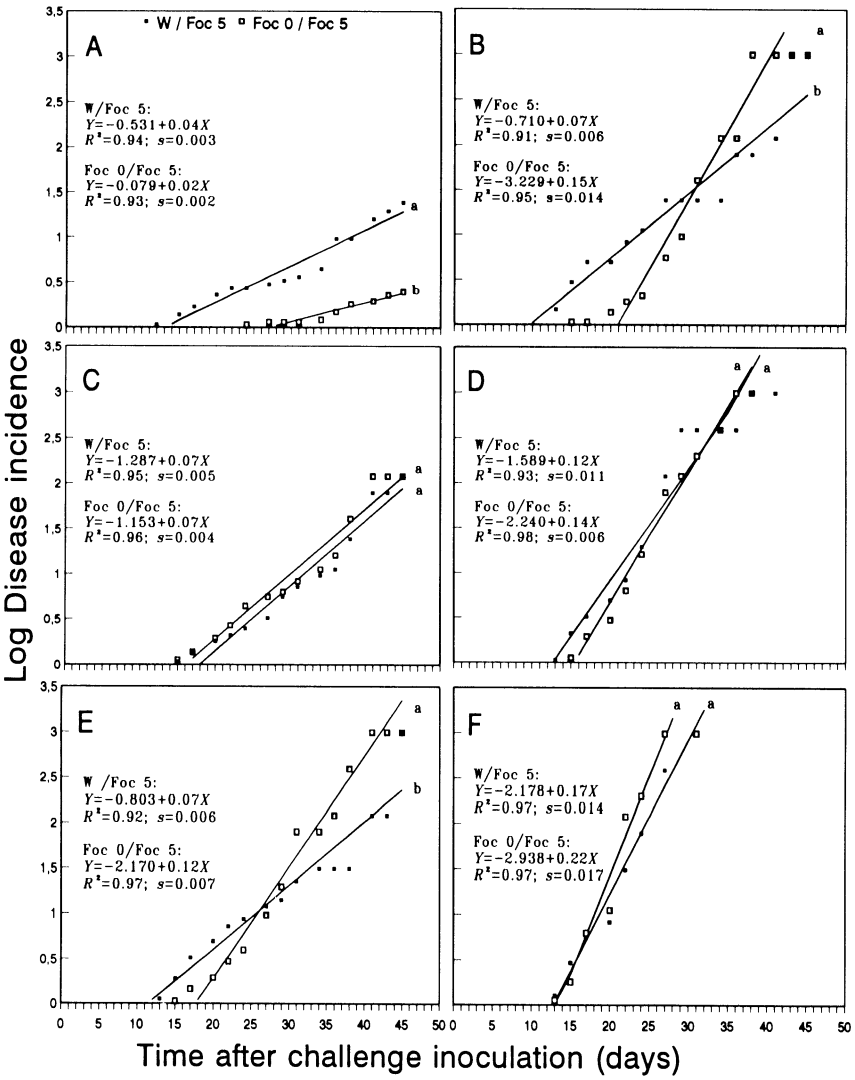

Fig. 1. Linear regression analysis of increase in disease incidence over time (Y) in chickpea cultivar JG 62 chickpeas inoculated with different inoculum concentrations of *Fusarium oxysporum* f. sp. *ciceris* (Foc) race 5 following a prior inoculation with 5×10^6 conidia per ml of nonpathogenic *F. o.* f. sp. *ciceris* race 0. **(A)**, **(C)**, and **(E)** seedlings were inoculated with 2×10^4 conidia per ml of Foc-race 5 at 3 **(A)**, 5 **(C)**, and 7 **(E)** days after inoculation with Foc-race 0. **(B)**, **(D)**, and **(F)** seedlings were inoculated with 2×10^6 conidia per ml of Foc-race 5 at 3 **(B)**, 5 **(D)**, or 7 **(F)** days after inoculation with Foc-race 0. Disease incidence over time was transformed by $\text{Ln}[1/(1 - Y)]$ for regression analysis. Slopes of lines with different letters are significantly different according to Fisher's protected LSD ($P < 0.05$).

Resistance in cultivar JG 62 induced by nonpathogenic isolates Fo 9009 and Fo 90105. No symptoms developed in inducer-inoculated controls. Isolations from seedlings inoculated with isolates Fo 9009 or Fo 90105 prior to challenge inoculation with Foc-5 recovered the fungus from cotyledons and older cortical root tissues but not from the root apex. Upon termination of experiments, *F. oxysporum* was isolated from the hypocotyl and root cortex but never from vascular tissues. Plants that were only challenge inoculated showed wilt symptoms characteristic of infections by Foc-5 in susceptible plants. The rate of increase of disease incidence in these plants was significantly higher ($P < 0.05$) when they were inoculated with 5×10^6 conidia per ml than with 5×10^4 conidia per ml (Fig. 2). However, there were no significant differences ($P = 0.05$) either in final disease incidence or in final disease severity (Table 2).

The effect of prior inoculation with nonpathogenic isolates of *F. oxysporum* on disease development in plants challenged with Foc-5 varied with the isolate and challenger inoculum concentration. Compared to plants in noninduced challenge-inoculated controls, prior inoculation with Fo 9009 significantly ($P < 0.05$) reduced the rate of increase of disease incidence over time (Fig. 2) and final disease incidence and severity (Table 2) in plants challenged with 5×10^4 conidia per ml of Foc race 5. At the end of the experiments, most symptomless plants were systemically infected with Foc-5 (Table 2). When inoculum concentration of the challenger was increased to 5×10^6 conidia per ml, disease incidence increased over time at a rate slightly lower ($P < 0.05$) than in noninduced challenge-inoculated controls, but higher than with 5×10^4 conidia per ml (Fig. 2). There were no significant differences ($P = 0.05$) in final disease incidence or severity between the two inoculum concentrations used. Prior inoculation with isolate Fo 90105 had no significant ($P = 0.05$) effect on disease development in plants challenged with Foc race 5 (Fig. 2, Table 2).

Resistance in cultivar ICCV 4 induced by nonpathogenic *F. o.* f. sp. *ciceris* races 0 and 1 and *F. oxysporum* isolates Fo 9009 and Fo 90105. There were no symptoms in inducer-inoculated controls. Isolations from seedlings inoculated with Foc-0, Foc 1, Fo 9009, or Fo 90105 prior to challenge inoculation recovered the fungus from cotyledons and older cortical root tissues but not from the root apex. Upon termination of the experiments, *F. oxysporum* was isolated from hypocotyl and root cortex tissues but never from vascular tissues. Noninduced seedlings that were challenge inoculated with 2×10^6 conidia per ml of Foc 5 showed wilt symptoms similar to those induced by Foc-5 in moderately susceptible cultivars (16). Similar seedlings inoculated with 2×10^4 conidia per ml of Foc-5 (experiment I) developed mild symptoms, and data from this challenge inoculum concentration are not presented. Prior inoculation with either Foc-0, Foc-1, Fo 9009, or Fo 90105 did not modify the disease syndrome caused by Foc-5 in noninduced, challenge-inoculated plants.

In experiment I, prior inoculation of seedlings with 5×10^6 conidia per ml of nonpathogenic *F. o.* f. sp. *ciceris* races 0 and 1 or *F. oxysporum* isolates did not significantly ($P = 0.05$) modify the rate of increase of disease incidence over time, as compared to that in noninduced challenge-inoculated controls (Fig. 3, Table 3). Preinoculation of seedlings with Foc-0 or with isolates Fo 9009 and Fo 90105 delayed the onset of symptoms by 3, 4, and 6 days, respectively, compared to noninduced controls (Fig. 3). However, Fo 90105 significantly ($P < 0.05$) reduced final disease incidence and severity (Table 4). Of the 40.6 and 46. 9% Fo 9009- and Fo 90105-induced plants, respectively, that remained symptomless by the end of the experiments, 15.6% were not systemically infected by Foc-5. Wilt symptoms appeared 3 days earlier in Foc-1-induced plants (Fig. 3), but the rate of increase of disease incidence over time was not sig-

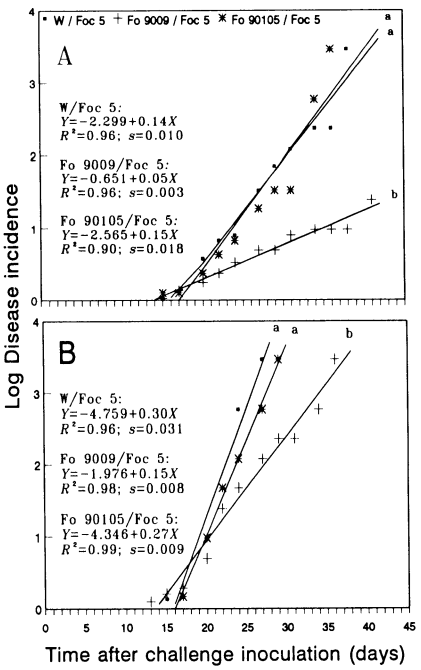

Fig. 2. Linear regression analysis of increase in disease incidence over time (Y) in chickpea cultivar JG 62 inoculated with different inoculum concentrations of *Fusarium oxysporum* f. sp. *ciceris* (Foc) race 5 following a prior inoculation with 5×10^6 conidia per ml of nonpathogenic *F. oxysporum* isolates Fo 9009 and Fo 90105. Seedlings were inoculated with **(A)** 5×10^4 or **(B)** 5×10^6 conidia per ml of Foc-race 5 at 3 days after inoculation with nonpathogenic *F. oxysporum* isolates. Disease incidence over time was transformed by $\text{Ln}[1/(1 - Y)]$ for regression analysis. Slopes of lines with different letters are significantly different according to Fisher's protected LSD ($P < 0.05$).

Table 1. Effect of prior inoculation of chickpea cultivar JG 62 with nonpathogenic *Fusarium oxysporum* f. sp. *ciceris* (Foc) race 0 on disease development after challenge inoculation with *F. o.* f. sp. *ciceris* race 5

Race 5 (conidia/ml)	Days after race 0 inoc.	Treatments (Prior inoc./ challenge inoc.)[x]	Incidence (%)[y]			Disease severity
				Symptomless		
			Affected	Infected	Noninfected	
2×10^4	3	Water/Foc-race 5	75.0[z] a	15.0	10.0 a	2.9 a
		Foc-race 0/Foc-race 5	32.5 bA	25.0	42.5 bA	1.1 bA
	5	Water/Foc-race 5	87.5	5.0	7.5	3.3
		Foc-race 0/Foc-race 5	87.5 B	5.0	7.5 B	3.4 B
	7	Water/Foc-race 5	95.0	2.5	2.5	3.6
		Foc-race 0/Foc-race 5	95.0 B	2.5	2.5 B	3.8 B
2×10^6	3	Water/Foc-race 5	95.0	2.5	2.5	3.7
		Foc-race 0/Foc-race 5	95.0	0.0	5.0	3.8
	5	Water/Foc-race 5	97.5	0.0	2.5	3.9
		Foc-race 0/Foc-race 5	95.0	0.0	5.0	3.8
	7	Water/Foc-race 5	100	0.0	0.0	4.0
		Foc-race 0/Foc-race 5	95.0	0.0	5.0	3.8

[x] Germinated seeds were inoculated with 5×10^6 conidia of Foc-race 0 per ml, and 3, 5, or 7 days later they were challenged with the indicated inoculum concentration of Foc-race 5.

[y] Determined 45 days after challenge inoculation. Isolations were done from all symptomless plants after that time to determine the occurrence of vascular infection. Disease severity was assessed on a 0 to 4 rating scale according to the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant).

[z] Data are the average of two experiments with five replicated pots, each with four plants. Control treatments (water/water; and Foc-race 0/water) were free from symptoms and vascular infections (not shown) and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. Means in a column followed by different letters are significantly different according to Fisher's protected LSD ($P = 0.05$). Comparison between control and inducer treatments within a time period of challenge inoculation is indicated with small letters. Comparison between time elapsed from inducing to challenge inoculations is indicated with capital letters. Only significant differences are indicated.

Table 2. Effect of prior inoculation of chickpea cultivar JG 62 with nonpathogenic *Fusarium oxysporum* isolates Fo 9009 and Fo 90105 on disease development after challenge inoculation with *F. o.* f. sp. *ciceris* (Foc) race 5

Race 5 (conidia/ml)	Treatments (Prior inoc./ challenge inoc.)[x]	Incidence (%)[y]			Disease severity
			Symptomless		
		Affected	Infected	Noninfected	
5×10^4	Water/Foc-race 5	96.9[z] a	3.1	0.0	3.7 a
	Fo 9009/Foc-race 5	75.0 b	18.8	6.2	2.8 bA
	Fo 90105/Foc-race 5	100 a	0.0	0.0	3.8 a
5×10^6	Water/Foc-race 5	100	0.0	0.0	4.0
	Fo 9009/Foc-race 5	96.9	0.0	3.1	3.8 B
	Fo 90105/Foc-race 5	100	0.0	0.0	4.0

[x] Germinated seeds were inoculated with 5×10^6 conidia of Fo 9009 or Fo 90105 per ml, and 3 days later they were challenged with the indicated inoculum of Foc-race 5.

[y] Determined 40 days after challenge inoculation. Isolations were done from all symptomless plants after that time to determine the occurrence of vascular infection. Disease severity was assessed on a 0 to 4 rating scale according to the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant).

[z] Data are the average of two experiments with four replicated pots, each with four plants. Control treatments (water/water, Fo 9009/water, and Fo 90105/water) were free from symptoms and vascular infections (not shown) and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. Means in a column followed by the same letter are not significantly different according to Fisher's protected LSD ($P = 0.05$). Comparison between control and inducer treatments within an inoculum concentration of the challenger is indicated with small letters. For an inducer treatment, comparison between different inoculum concentrations of the challenger is indicated with capital letters. Only significant differences are indicated.

nificantly ($P = 0.05$) different from the noninduced, challenge-inoculated control (Fig. 3, Table 3).

When the inducing inoculum concentration was increased from 5×10^6 (experiment I) to 2×10^7 (experiment II) conidia per ml, all the inducer agents significantly ($P < 0.05$) reduced final disease incidence and severity compared to that in the noninduced, challenge-inoculated control (Fig. 3, Table 4). This effect was detected in plants challenge inoculated with both 1×10^6 and 1×10^7 conidia per ml, although there was no significant ($P = 0.05$) difference between these two inoculum concentrations of the challenger (not shown in Table 4). Of the 45.3 and 42.2% Foc-0- and Foc-1-induced plants, respectively, that remained symptomless by the end of the experiment, 7.8 and 4.7%, respectively, were not systemically infected by Foc 5. When seedlings were induced with nonpathogenic *F. oxysporum* isolates Fo 9009 and Fo 90105, 38.5 and 58.7% of the plants, respectively, remained symptomless by the end of the experiment, with a high percentage not systemically infected with Foc-5 (Table 4). Plants induced with isolates Fo 9009 or Fo 90105 and challenge inoculated with 1×10^6 conidia per ml of Foc-5 showed a rate of increase of disease incidence significantly lower ($P < 0.05$) than noninduced plants (Fig. 3, Table 3). Wilt symptoms appeared 9 days later in the Foc-0/Foc-5 treatment than in

Table 3. Linear regression analysis of the increase in disease incidence over time (Y) in chickpea cultivar ICCV 4 inoculated with different inoculum concentrations of *Fusarium oxysporum* f. sp. *ciceris* (Foc) race 5 following prior inoculation with nonpathogenic *F. o.* f. sp. *ciceris* races 0 and 1 or *F. oxysporum* isolates Fo 9009 and Fo 90105[x]

Race 5 (conidia/ml)	Treatments (Prior inoc./ challenge inoc.)[x]	Regression equation[x]	R^2[y]	Slopes of regression equations[z]
Experiment I				
2×10^6	Water/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.39 + 0.09T$	0.84	
	Foc-race 0/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -3.43 + 0.12T$	0.84	
	Foc-race 1/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.72 + 0.12T$	0.94	
	Fo 9009/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.14 + 0.07T$	0.85	
	Fo 90105/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.28 + 0.07T$	0.92	
Experiment II				
1×10^6	Water/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -1.28 + 0.06T$	0.78	a
	Foc-race 0/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -1.49 + 0.05T$	0.93	a
	Foc-race 1/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -0.67 + 0.03T$	0.96	ab
	Fo 9009/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -0.57 + 0.02T$	0.91	b
	Fo 90105/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -0.45 + 0.02T$	0.82	b
1×10^7	Water/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.35 + 0.11T$	0.97	a
	Foc-race 0/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -1.58 + 0.06T$	0.96	b
	Foc-race 1/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.73 + 0.09T$	0.98	a
	Fo 9009/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -2.49 + 0.10T$	0.93	a
	Fo 90105/Foc-race 5	$\text{Ln}[1/(1 - Y)] = -0.75 + 0.03T$	0.90	b

[x] Germinated seeds were inoculated with 5×10^6 (experiment I) or 2×10^7 (experiment II) conidia of either Foc-race 0, Foc-race 1, Fo 9009, or Fo 90105 per ml, and 3 days later they were challenged with the indicated inoculum of Foc-race 5. Incidence (%) of affected plants (Y) was determined at 2-day intervals up to 40 days after challenge inoculation. Disease incidence over time was transformed by $\text{Ln}[1/(1 - Y)]$, and linear regression analyses were performed with transformed data. No disease developed in control treatments (water/water, Foc-race 0/water, Foc-race 1/water, Fo 9009/water and Fo 90105/water), for which data are not presented.

[y] R^2 = Coefficient of determination.

[z] For each inoculum concentration of the challenger, slopes (rate of increase of disease incidence) of regression equations with the same letter are not significantly different according to Fisher's protected LSD ($P = 0.05$). Only significant differences are indicated.

Table 4. Effect of prior inoculation of chickpea cultivar ICCV 4 with nonpathogenic *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 1 or *F. oxysporum* isolates Fo 9009 and Fo 90105 on disease development after challenge inoculation with *F. o.* f. sp. *ciceris* (Foc) race 5

Experiment	Treatments (Prior inoc./ challenge inoc.)[x]	Incidence (%)[y]			Disease severity
			Symptomless		
		Affected	Infected	Noninfected	
I	Water/Foc-race 5	82.8[z] bc	14.1	3.1 b	2.0 b
	Foc-race 0/Foc-race 5	84.4 b	15.6	0.0 b	1.9 b
	Foc-race 1/Foc-race 5	100 a	0.0	0.0 b	3.1 a
	Fo 9009/Foc-race 5	59.4 cd	25.0	15.6 a	1.4 bc
	Fo 90105/Foc-race 5	53.1 d	31.3	15.6 a	1.1 c
II	Water/Foc-race 5	86.7 a	12.5	0.8 d	2.5 a
	Foc-race 0/Foc-race 5	54.7 bc	37.5	7.8 bc	1.1 c
	Foc-race 1/Foc-race 5	57.8 bc	37.5	4.7 d	1.4 bc
	Fo 9009/Foc-race 5	61.5 b	12.8	25.7 ab	1.8 b
	Fo 90105/Foc-race 5	41.3 c	30.1	28.6 a	1.0 c

[x] Germinated seeds were inoculated with 5×10^6 (experiment I) or 2×10^7 (experiment II) conidia of either Foc-race 0, Foc-race 1, Fo 9009, or Fo 90105 per ml, and 3 days later they were challenged with 2×10^6 (experiment I) and 1×10^6 or 1×10^7 (experiment II) conidia of Foc-race 5 per ml.

[y] Determined 40 days after challenge inoculation. Isolations were done from all symptomless plants after that time to determine the occurrence of vascular infection. Disease severity was assessed on a 0 to 4 rating scale according to the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant).

[z] Data are the average of two experiments with four replicated pots, each with four plants. Control treatments (water/water, Foc-race 0/water, Foc-race 1/water, Fo 9009/water, and Fo 90105/water) were free from symptoms and vascular infections (not shown) and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. There was no significant difference between the two inoculum concentrations of the challenger in experiment II; thus the mean value of them is presented. Means in a column followed by the same letter are not significantly different according to Fisher's protected LSD ($P = 0.05$). Only significant differences are indicated.

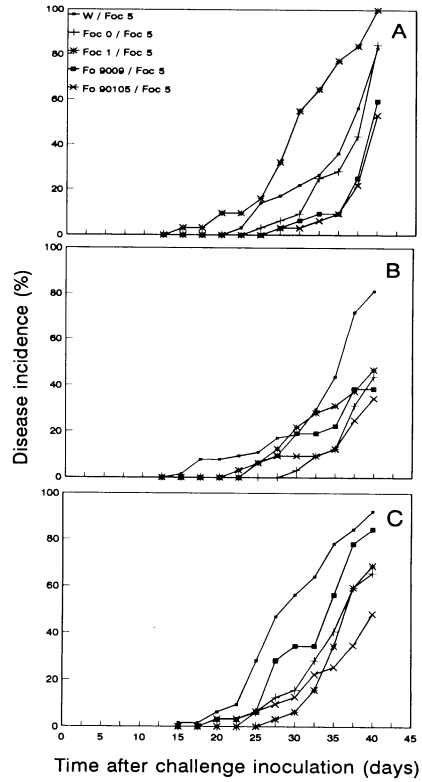

Fig. 3. Effect of prior inoculation of chickpea cultivar ICCV 4 with nonpathogenic *Fusarium oxysporum* f. sp. *ciceris* (Foc) races 0 and 1 or *F. oxysporum* isolates Fo 9009 and Fo 90105 on disease development after challenge inoculation with *F. o.* f. sp. *ciceris* race 5. **(A)** seedlings were inoculated with 2×10^6 conidia per ml of Foc-race 5 at 3 days after prior inoculation with 5×10^6 conidia per ml of any of the nonpathogenic races or isolates. Other seedlings were inoculated with **(B)** 1×10^6 or **(C)** 1×10^7 conidia per ml of Foc-race 5 3 days after prior inoculation with 2×10^7 conidia per ml of any of the nonpathogenic races or isolates. Data are the average of two experiments with four replicated pots, each with four plants.

noninduced, challenge-inoculated controls; however, neither Foc-0 nor Foc-1 significantly ($P = 0.05$) modified the rate of increase of disease incidence over time compared to noninduced, challenge-inoculated controls. When challenge inoculum concentration was increased to 1×10^7 Foc-5 conidia per ml, only Foc-0 and Fo 90105 significantly ($P < 0.05$) reduced the rate of increase of disease incidence compared to noninduced, challenge-inoculated controls. However, all the inducer agents delayed the onset of symptoms.

DISCUSSION

Prior inoculation of germinated chickpea seeds with nonpathogenic races of *F. o.* f. sp. *ciceris* or isolates of *F. oxysporum* significantly reduced disease incidence and severity after challenge inoculation with the highly virulent *F. o.* f. sp. *ciceris* race 5. Several authors have shown that the degree of protection against Fusarium wilt diseases induced by prior inoculation with pathogens or nonpathogens can be influenced by several factors (1,7,9,25,31). In our studies, the extent of protection decreased when the inoculum concentration of the challenger was similar to that of the inducing agent, and protection was annulled when the time between the subsequent inoculations was longer than 3 days. Gessler and Kuc (9) reported that several formae speciales of *F. oxysporum* induced resistance in cucumber to *F. o.* f. sp. *cucumerinum* in flask culture, and that a 3-day interval between induction and challenge was necessary for adequate protection. Resistance also was induced by foliar infection with *Colletotrichum lagenarium* (Pass.) Ell. & Halst or tobacco necrosis virus, but not with *F. o.* f. sp. *melonis* W.C. Snyder & H.N. Hans, when the interval between subsequent inoculations was increased to 7 days. Similarly, Biles and Martyn (1) found that root inoculation of watermelon cultivars with nonpathogenic races of *F. o.* f. sp. *niveum* induced a higher level of resistance to Fusarium wilt than did *F. o.* f. sp. *cucumerinum,* and although significant protection was obtained with a time interval of 24 h between induction and challenge, resistance was enhanced when 3 days elapsed between inoculations. They suggested that induced resistance to *F. o.* f. sp. *cucumerinum* was both local and systemic as well as nonspecific. Also, protection against Fusarium wilt of tomato by *F. o.* f. sp. *dianthi* (Prill. & Del.) Snyd. & Hans was only effective when the inducing inoculation took place a few days before the challenge inoculation (31) and the inoculum concentration of the inducing agent required for protection was approximately equal to or higher than that used in the challenge inoculation with the pathogen.

Our results suggest that induced resistance can be overcome by high inoculum level of the pathogen. Under simulated field conditions, Martyn et al. (21) found that nonpathogenic *F. o.* f. sp. *niveum* race 1 induced protection in watermelon against *F. o.* f. sp. *niveum* race 2 only when the pathogenic inoculum was at or near normal field conditions (750 CFU/g of soil). However, when the concentration of the challenge inoculum was increased fivefold, induced resistance was not observed except for a delay in symptom expression. Similar results were reported by Martyn and McLaughlin (22) and Sumner (28), who showed that resistance to Fusarium wilt in commercial watermelon cultivars could be overcome by logarithmic increases in inoculum concentration.

In our studies, prior inoculation of germinated seeds with either nonpathogenic *F. o.* f. sp. *ciceris* races 0 and 1 or nonpathogenic isolates of *F. oxysporum* also protected cultivar ICCV 4 against Fusarium wilt caused by *F. o.* f. sp. *ciceris* race 5. Nevertheless, the degree of protection was influenced by both the inducing and challenge inoculum densities, as well as by the inducing agent. Results suggest that in ICCV 4, resistance against *F. o.* f. sp. *ciceris* race 5 was best induced by the nonpathogenic isolate of *F. oxysporum,* Fo 90105. However, this isolate did not protect cultivar JG 62 against the same race. Thus, the magnitude of induced resistance in the *F. o.* f. sp. *ciceris/Cicer arietinum* pathosystem seems to vary with the nature of the inducing agent and the genotype of the host. Ogawa and Komada (25) demonstrated that sweet potato plants were protected against Fusarium wilt, caused by *F. o.* f. sp. *batatas* (Wr.) Snyd. & Hans, by prior inoculation with nonpathogenic *F. oxysporum* isolates, which were often found in the vessels of healthy sweet potato plants and natural soils. They reported that among 12 pathogenic and nonpathogenic Fusaria belonging to seven species, only the nonpathogenic isolates of *F. oxysporum* showed cross protection. The two nonpathogenic isolates of *F. oxysporum* used in this study were obtained from the rhizosphere of healthy chickpea plants grown in a plot with a long history of Fusarium wilt (14; A. R. Alcalá-Jiménez and R. M. Jiménez-Díaz, *unpublished*). Although these isolates can infect and colonize cotyledonary and cortical root tissues of both JG 62 and ICCV 4 chickpeas, they were never isolated from the vascular tissue of inoculated plants. Similarly, nonpathogenic *F. o.* f. sp. *ciceris* races 0 and 1 were never isolated from vascular tissue of induced plants. Therefore, our results suggest that protection was not associated with the ability of the inducer agents to infect the vascular tissue of the plant.

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