

Plant defence reactions against fusarium wilt in chickpea induced by incompatible race 0 of *Fusarium oxysporum* f.sp. *ciceris* and nonhost isolates of *F. oxysporum*

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Germinated seeds of ‘kabuli’ chickpea cv. ICCV 4 were inoculated with a conidial suspension of the incompatible race 0 of *Fusarium oxysporum* f.sp. *ciceris* (Foc) or of nonhost *F. oxysporum* resistance ‘inducers’, and 3 days later were challenged by root dip with a conidial suspension of highly virulent Foc race 5. Prior inoculation with inducers delayed the onset of symptoms and/or significantly reduced the final amount of fusarium wilt caused by race 5. However, the extent of disease suppression varied with the nature of the inducing agent; the nonhost isolates of *F. oxysporum* were more effective at disease suppression than the incompatible Foc race 0. Inoculation with the inducers gave rise to synthesis of maackiain and medicarpin phytoalexins in inoculated seedlings; these did not accumulate in plant tissues but were released into the inoculum suspension. Inoculation with inducers also resulted in accumulation of chitinase, β -1,3-glucanase and peroxidase activities in plant roots. These defence-related responses were induced more consistently and intensely by nonhost isolates of *F. oxysporum* than by incompatible Foc race 0. The phytoalexins and, to a lesser extent, the antifungal hydrolases, were also induced after challenge inoculation with Foc race 5. However, in this case the defence responses were induced in both preinduced and noninduced plants infected by the pathogen. It is concluded that the suppression of fusarium wilt in this study possibly involved an inhibitory effect on the pathogen of preinduced plant defences, rather than an increase in the expression of defence mechanisms of preinduced plants following a subsequent challenge inoculation.

Keywords: biological control, chitinase, *Cicer arietinum*, β -1,3-glucanase, peroxidase, phytoalexins

Introduction

Chickpea (*Cicer arietinum*) is one of the most important food legumes grown worldwide, especially in dry areas of the Indian subcontinent (Saxena, 1990). In the European Union, chickpea production is concentrated in the Mediterranean Basin, with Spain being the principal producer. Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceris* (Foc), is a major constraint to chickpea production worldwide (Jalali & Chand, 1992). Annual chickpea yield losses from fusarium wilt vary from 10 to 15% (Traper-Casas & Jiménez-Díaz, 1985; Jalali & Chand, 1992), but the disease can completely destroy the crop under specific conditions (Halila & Strange, 1996).

The most effective and practical method for management of the disease worldwide is the use of resistant

cultivars (Jiménez-Díaz *et al.*, 1991; Jalali & Chand, 1992; Kraft *et al.*, 1994). However, the effectiveness of host resistance is curtailed by the occurrence of pathogenic races in Foc. Seven Foc races (0–6) have been identified (Haware & Nene, 1982; Jiménez-Díaz *et al.*, 1993). Races 1–4 were first described in India (Haware & Nene, 1982). Later, race 0 was reported in California (USA), Israel, Lebanon, Spain, Syria, Tunisia and Turkey; and races 1 and 6 were identified in California, Israel, Morocco and Spain. Race 5, the most virulent of the races occurring in Spain, also occurs in California (Jiménez-Díaz *et al.*, 1993; Halila & Strange, 1996; R.M. Jiménez-Díaz, unpublished results).

The use of resistant cultivars for management of fusarium wilt in chickpea may be enhanced by means of biological control using either bacterial or fungal antagonists. Biological control by nonhost *F. oxysporum* isolates (Ogawa & Komada, 1985; Paulitz *et al.*, 1987; Mandeel & Baker, 1991; Alabouvette *et al.*, 1993; Hervás *et al.*, 1995; Larkin *et al.*, 1996; Fuchs *et al.*, 1997; Hervás *et al.*, 1997) and incompatible races of the same *forma*

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specialis (Biles & Martyn, 1989; Martyn *et al.*, 1991; Hervás *et al.*, 1995) is a promising strategy for management of fusarium wilt diseases. Hervás *et al.* (1995) showed that prior inoculation of germinated chickpea seeds with either incompatible Foc races or nonhost *F. oxysporum* isolates can suppress fusarium wilt caused by the highly virulent Foc race 5. Further studies (Hervás *et al.*, 1997; Hervás *et al.*, 1998) supported the potential of the nonhost *F. oxysporum* isolate Fo 90105 as a biocontrol agent against fusarium wilt of chickpea.

Different mechanisms may be involved in the biological control of fusarium wilt diseases by nonhost *F. oxysporum* isolates. These mechanisms include saprophytic competition for nutrients; parasitic competition for infection sites; and induced or enhanced resistance within the host (Schneider, 1984; Alabouvette, 1986; Matta, 1989; Mandeel & Baker, 1991; Fuchs *et al.*, 1997). These mechanisms are not necessarily exclusive of one another, and several mechanisms may be responsible for disease suppression by many biocontrol agents (Mandel & Baker, 1991). In previous studies it was shown that certain plant defence responses, namely phytoalexin synthesis and accumulation of chitinase and β -1,3-glucanase activities, may be involved in the nonhost resistance of chickpea against nonhost *F. oxysporum* isolates (Armero *et al.*, 1993; Cabello, 1994; Armero, 1996). More recently, Stevenson *et al.* (1997) concluded that chickpea phytoalexins (the pterocarpan maackiain and medicarpin) are fundamental components of the resistance mechanism of this plant to fusarium wilt.

The objective of this research was to determine the roles of phytoalexin synthesis and accumulation of several defence-related enzymes, such as chitinase, β -1,3-glucanase and peroxidase, early in the interaction of chickpea with nonhost *F. oxysporum* isolates and with the incompatible Foc race 0 which leads to biological control of fusarium wilt in the Foc race 0-resistant cv. ICCV4.

Materials and methods

Fungal isolates

Fusarium oxysporum f.sp. *ciceris* isolates Foc 7802 and Foc 8012 (representative of Foc races 0 and 5, respectively), and nonhost *F. oxysporum* isolates Fo 9009 and Fo 90105, were used in this study. Isolates Foc 7802 and Foc 8012 were obtained from infected chickpeas in southern Spain, and have been used previously (Trapero-Casas & Jiménez-Díaz, 1985; Jiménez-Díaz *et al.*, 1991; Jiménez-Díaz *et al.*, 1993; Hervás *et al.*, 1995; Hervás *et al.*, 1997). *Fusarium oxysporum* isolates Fo 9009 and Fo 90105 were originally isolated from roots of healthy chickpeas grown in a naturally infested field at Santaella (Córdoba, southern Spain). These isolates were shown to be effective in protecting chickpeas from disease caused by Foc race 5 (Hervás *et al.*, 1995; Hervás *et al.*, 1997). Monoconidial fungal isolates were stored and cultured as indicated previously (Hervás *et al.*, 1995; Hervás *et al.*, 1997).

Suppression of fusarium wilt by nonhost *F. oxysporum* and incompatible *F. oxysporum* f.sp. *ciceris* race 0

A study was conducted to determine the efficacy of *F. oxysporum* isolates Fo 9009 and Fo 90105, and Foc race 0 isolate Foc 7802, to suppress fusarium wilt of chickpea cv. ICCV4 caused by Foc race 5. ICCV4 is a 'kabuli' chickpea (ram-head-shaped, beige seed) susceptible to Foc race 5, but completely resistant to Foc races 0 and 1 (Kumar *et al.*, 1985; Jiménez-Díaz *et al.*, 1993). 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 = 1–2 cm), were placed in a conidial suspension in sterile water (5×10^6 conidia mL⁻¹) of inducing inoculum (either Foc 7802, Fo 9009 or Fo 90105 isolates) or sterile distilled water (control), and incubated in the dark on an orbital shaker at 50 r.p.m., 25°C for 16 h. Inoculated and control seeds were sown in sterile sand in trays (60 × 40 × 10 cm; one tray per inducing treatment), and seedlings were incubated in a growth chamber adjusted to 25°C, 60–90% relative humidity and a 14 h photoperiod of fluorescent light at 360 μ E m⁻² s⁻¹ for 3 days. Inducer-inoculated seedlings were removed from the tray, selected for uniformity of root length, washed free of sand under tap water, and challenge-inoculated with Foc 5 isolate Foc 8012 by root dipping in a conidial suspension in sterile water (10^5 conidia mL⁻¹) for 16 h, as indicated above for inducer inoculation. Nonchallenged control seedlings were dipped in sterile water. The following treatments (inducer inoculation/challenge inoculation) were included: (i) water/water; (ii) water/Foc race 5; (iii) Foc race 0/water; (iv) Foc race 0/Foc race 5; (v) Fo 9009/water; (vi) Fo 9009/Foc race 5; (vii) Fo 90105/water; (viii) Fo 90105/Foc race 5.

After challenge inoculation, seedlings were transplanted into 15 cm diameter clay pots (four plants per pot) filled with an autoclaved soil mixture (clay loam/peat, 2 : 1, v/v). Plants were incubated in a growth chamber adjusted to give the same conditions as described above, which are optimal for disease development during the time required (3 days for plant sampling for biochemical analysis, 50 days for disease assessment; Landa *et al.*, 2001). Plants were watered as needed and, when applicable, fertilized weekly with 100 mL Hoagland's nutrient solution (Hoagland & Arnon, 1950). Just before challenge inoculations, isolations were made from a sample of 10–25 inducer-inoculated seedlings to determine the ability of inducing inocula to infect and colonize 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 V-8 juice-oxgall-PCNB agar (VOPA; Bouhot & Rouxel, 1971), and incubated at 25°C with a 12 h photoperiod of fluorescent and near-UV light at 36 μ E m⁻² s⁻¹ for 3–5 days. Inoculum suspensions collected at the end of both inducer and challenge inoculations were analysed for phytoalexin excretion. Also, seedlings were sampled at several time points (between 0 and 72 h) following the inducer and

challenger inoculations, and root tissues were analysed for accumulation of enzyme activities. There were four replicated pots in a randomized complete block design, each treatment consisting of 16 plants (four pots and four plants per pot). The experiment was repeated twice.

Disease assessment and data analyses

In all experiments, disease incidence (percentage) and severity were assessed at 2-day intervals. Severity of symptoms on individual plants were rated on a scale from 0–4 according to the percentage of foliage with yellowing or necrosis in acropetal progression: 0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, 4 = dead plant, as used previously (Hervás *et al.*, 1995; Hervás *et al.*, 1997). On termination of the experiments, isolations were made from stem segments of symptomless plants to determine the occurrence of vascular infections. Stem segments were cut into 5 mm long pieces, surface disinfested in 1% NaOCl for 90 s, plated on VOPA, and incubated as indicated above. Incidence and severity data (0–4 scale) within a pot were used to calculate a disease intensity index (DII) by the equation:

$$DII = [(\sum S_i \times N_i) / (4 \times N_t)] \times 100$$

where S_i = symptom severity; N_i = number of plants with S_i symptom severity; and N_t = total number of plants (Hervás *et al.*, 1997). Thus DII expresses the mean value of disease intensity at a given moment as a proportion of the maximum possible intensity. Disease progress curves were obtained from the accumulated DII over time in days from the date of challenge inoculation. Percentage values were transformed into $\arcsin (Y/100)^{1/2}$ for ANOVA. Data were subjected to ANOVA using STATISTIX (NA Analytical Software, Roseville, MN, USA). Treatment means were compared using Fisher's protected least significance test (LSD) at $P = 0.05$. There was no variance heterogeneity among the three repeated experiments according to Barlett's test for equal variances. Therefore results from the third experiment, which is representative of all three, are presented in this paper.

Extraction and assay of enzyme activities

Soluble and ionically bound enzymes were extracted from root tissues by homogenization in a 1 : 4 (w/v) proportion in Tris-HCl buffer (25 mM, pH 7.5), containing 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride and 10% (w/w) polyvinyl pyrrolidone. Homogenates were centrifuged at 20 000 g, 4°C for 15 min. Streptomycin sulphate was added to the supernatant to a final concentration of 15 g L⁻¹ and the precipitate was removed by centrifugation at 20 000 g for 15 min. Solid ammonium sulphate was added to the supernatant to 80% saturation and proteins were precipitated at 4°C for 60 min. The precipitate was collected by centrifugation (20 000 g, 15 min), redissolved in extraction buffer without NaCl (0.25 vol of initial tissue fresh weight), and dialysed overnight against this buffer. The dialysed solution was used as crude enzyme preparation.

The above protocol of ammonium sulphate precipitation, precipitate redissolution and dialysis was adopted to concentrate the samples and, especially, to eliminate reducing substances that might interfere with the β -1,3-glucanase assay (Cabello *et al.*, 1994). Under the extraction conditions with a high-saline buffer (0.5 M NaCl), 80% ammonium sulphate precipitation produced nearly complete recovery of both chitinase and peroxidase activities and some improvement in their specific activities.

Chitinase and β -1,3-glucanase activities were determined as described by Cabello *et al.* (1994). In brief, chitinase activity was assayed using insoluble chitin (Sigma, St Louis, MO, USA) as substrate, and quantification by the method of Reissig *et al.* (1955), of *N*-acetylglucosamine formed after degradation of the soluble enzyme products with cytohellicase (Sigma). Chitinase activity was expressed as pkat mg⁻¹ protein, with 1 pkat defined as the enzyme activity catalysing the formation of 1 pmol *N*-acetylglucosamine equivalents in 1 s under assay conditions. β -1,3-Glucanase activity was assayed using soluble laminarin (Sigma) as substrate, and quantification by the Nelson-Somogyi method (Marais *et al.*, 1986) of reducing groups formed by enzyme action. β -1,3-Glucanase activity was expressed as nkat mg⁻¹ protein, with 1 nkat defined as the enzyme activity catalysing the formation of 1 nmol glucose equivalents in 1 s under assay conditions. Peroxidase activity was assayed with guaiacol (Merck, Darmstadt, Germany) as substrate. The standard assay contained 0.25 mL enzyme extract, 0.5 mL K-phosphate buffer (0.2 M, pH 6.5), 0.25 mL H₂O₂ (0.88 M in water) and 0.5 mL guaiacol (0.1 M in water). The reaction was initiated by adding the enzyme extract and was followed at A₄₇₀. A reaction mixture without enzyme was used as a blank. Peroxidase activity was expressed as enzyme units (EU) mg⁻¹ protein, with 1 EU defined as the enzyme activity producing a change of absorbance of 0.01 in 1 min under assay conditions.

Enzyme activities were also analysed by native polyacrylamide gel electrophoresis (PAGE), performed anodally or cathodally with a Mini-Protean II electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Anodic PAGE was used for the separation of peroxidase isoenzymes. It was carried out according to the method of Davis (1964) with 5% (w/v) stacking and 10% resolving polyacrylamide gels, buffered with 1.5 M Tris-HCl (pH 8.8), and at a constant current of 20 mA. Cathodic PAGE was used for the separation of chitinase and β -1,3-glucanase isoenzymes. It was carried out according to the method of Reisfeld *et al.* (1962) with 5% stacking and 12.5% resolving gels, buffered with 100 mM sodium acetate buffer (pH 4.3), and at a constant current of 30 mA. Equal amounts of protein (18–20 μ g) were charged in each gel lane. Following electrophoretic separation, gels were stained according to Seever *et al.* (1971) for peroxidase activity, Trudel & Asselin (1989) for chitinase activity, and Pan *et al.* (1989) for β -1,3-glucanase activity.

Protein contents of enzyme extracts were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Extraction and determination of phytoalexins and related isoflavones

The effects of inducing inoculation and challenge inoculation on phytoalexin (medicarpin and maackiain) and related isoflavones (biochanin A and formononetin) synthesis in chickpea were investigated by analysing the levels of such compounds released into the inoculum suspension at the end of the respective 16 h inoculation periods. These media were filtered, adjusted to pH 3 with HCl, and extracted twice with 0.5 vol cold (-20°C) ethyl acetate. The extracts were dried over anhydrous sodium sulphate, evaporated to dryness at 40°C under reduced pressure, redissolved in a fixed volume of 96% (v/v) ethanol/water, and filtered through a $0.45\ \mu\text{m}$ filter (Millex-HV, Millipore, Bedford, MA, USA). The isoflavonoids analysis was carried out by HPLC as described by Köster *et al.* (1983a); Köster *et al.* (1983b). A Beckman System Gold HPLC system (Beckman, Palo Alto, CA, USA) equipped with a reversed-phase column ($250 \times 4\ \text{mm}$, LiChrospher 100 RP-18 $5\ \mu\text{m}$, Merck) preceded by a similar guard column was used. The injected volume was $20\ \mu\text{L}$ and elution was made at $0.8\ \text{mL min}^{-1}$ with an A (acetonitrile)/B ($15\ \text{g L}^{-1}$ acetic acid in water) gradient, as described below. The column was equilibrated with 20% A in B (v/v), and the percentage of A in B was linearly increased from 20% to 45% (v/v) over 20 min and maintained isocratically for 8 min before returning and re-equilibrating at the initial conditions of 20% A. Eluting compounds were monitored with a Beckman 168 Diode Array Detector set at 260 and 290 nm. Under the above conditions, typical elution times for the isoflavones formononetin and biochanin A and the pterocarpan phytoalexins maackiain and medicarpin were 20.3, 27.3, 22.5 and 23.9 min, respectively. As all these compounds have been widely reported as constituents of normal or elicited chickpea tissues and cell cultures, they were easily identified from their HPLC retention times and UV spectra (Jaques *et al.*, 1987; Kessmann *et al.*, 1988). Amounts of isoflavonoid compounds found in the samples were expressed as areas of integrated UV signals measured at 290 nm (maackiain and medicarpin), or 260 nm (biochanin A and formononetin) per g plant fresh weight ($\text{IA g}^{-1}\ \text{FW}$). Because of the structural resemblances between biochanin A (B) and formononetin (F), and maackiain and medicarpin, the molar extinction coefficients of each pair of compounds are very similar; hence similar contents in terms of IA units reflect similar contents in terms of moles or grams (thus 1 IA unit at 260 nm was found equivalent to about $4.4\ \text{nmol}$ or $1.28\ \mu\text{g}$ B, and $5\ \text{nmol}$ or $1.34\ \mu\text{g}$ F).

Results

Suppression of fusarium wilt by nonhost *F. oxysporum* and incompatible *F. oxysporum* f.sp. *ciceris* race 0

Prior inoculation of germinated seeds with either nonhost *F. oxysporum* isolates Fo 9009 and Fo 90105 or Foc race 0 isolate Foc 7802, influenced the development of

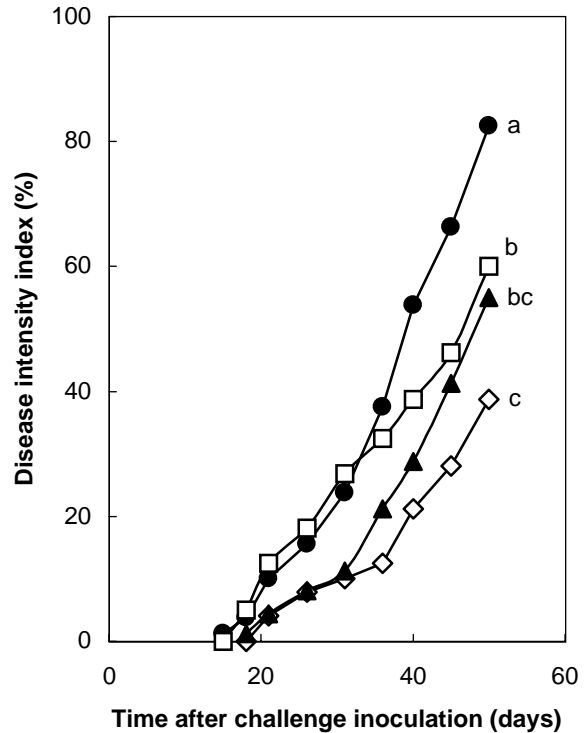


Figure 1 Development of fusarium wilt in preinduced chickpea cv. ICCV4 seedlings after challenge inoculation with *Fusarium oxysporum* f.sp. *ciceris* race 5 (Foc race 5). Seedlings were treated with water (control, ●) or preinoculated in a conidial suspension of incompatible *F. oxysporum* f.sp. *ciceris* race 0 (□) or nonhost *F. oxysporum* isolate Fo 9009 (◇) or Fo 90105 (▲) and 3 days later challenged with Foc race 5. Values followed by the same letter are not significantly different at $P < 0.05$.

fusarium wilt in ICCV4 chickpea caused by Foc race 5 (Fig. 1). There were no symptoms in the aerial plant parts of inducer-inoculated controls. However, compared with water-inoculated controls, chickpea seedlings inoculated with isolates Fo 9009, Fo 90105 or Foc 7802 developed a characteristic brown discoloration at surfaces of roots, cotyledonary nodes and cotyledons. These discolorations were more extensive and intense in seedlings inoculated with the nonhost *F. oxysporum* isolates than in those inoculated with Foc race 0 (not shown). Isolations from seedlings inoculated with isolates Fo 9009, Fo 90105 or Foc 7802 prior to challenge inoculation with Foc race 5 recovered each of the fungi from cotyledons and older cortical root tissues, but never from the root apex. On termination of the experiments, *F. oxysporum* was isolated from tissues of the hypocotyl and root cortex, but never from vascular tissues.

Noninduced, challenge-inoculated plants did not develop brown discoloration of roots and cotyledons. In these plants, symptoms characteristic of wilting caused by Foc race 5 developed 15 days after challenge inoculation. Severe disease developed thereafter, reaching a final DII higher than 80% (Fig. 1). Prior inoculation with nonhost isolates *F. oxysporum* Fo 9009 and Fo 90105 or with Foc

race 0 isolate 7802 did not modify the disease syndrome caused by Foc race 5 in cv. ICCV4. However, isolates Fo 9009 and Fo 90105 delayed the onset of symptoms by 3–7 days and significantly ($P < 0.05$) reduced the final DII by 24–40% compared with noninduced, challenge-inoculated controls (Fig. 1). Prior inoculation with Foc 7802 did not influence the onset of symptoms, but significantly ($P < 0.05$) reduced final DII by 20% compared with non-induced controls (Fig. 1). Taken together, the influence of inducer treatments on development of fusarium wilt indicated the following order of decreasing efficacy of disease suppression: Fo 9009 > Fo 90105 > Foc race 0.

Phytoalexin production after inducing inoculation and challenge inoculation

Chickpea seedlings treated with pathogens or elicitors experience induction of phytoalexin and related isoflavonoid synthesis, which can be demonstrated from the release of such compounds into the liquid treatment carrier rather than from their accumulation in the plant root tissue (Armero *et al.*, 1994; Armero *et al.*, 2001). Consequently, in this study the levels of phytoalexins and related isoflavones were analysed in inducer and challenge inoculum suspensions at the end of the 16 h inoculation periods. Compared with the water-treated control,

inoculation of germinated ICCV 4 seeds with nonhost *F. oxysporum* Fo 9009 or Fo 90105 significantly increased the production of medicarpin and maackiain phytoalexins. Conversely, prior inoculation with incompatible Foc race 0 isolate 7802 significantly increased the level of maackiain only, although this increase was about half of that attained with the other inducers (Table 1). Variation of levels of the constitutive isoflavones formononetin (a precursor of medicarpin and maackiain) and biochanin A was more variable, as compared with the control. While isolates Fo 9009 and Foc 7802 increased the amount of formononetin, isolate Fo 90105 reduced that of both formononetin and biochanin A, although in some cases the differences were not statistically significant (Table 1). As chickpea isoflavones can undergo peroxidative polymerization reactions (Barz *et al.*, 1988; Barz *et al.*, 1990), the lower amounts of these compounds found in the Fo 90105 inoculum medium could be a consequence of the higher peroxidative activity developed in this interaction than in the remaining interactions (see below).

Subsequent inoculation of induced seedlings with the challenger gave rise to accumulation of very high levels of medicarpin and maackiain phytoalexins in the inoculum suspensions (Table 2, treatments 3–8). In particular, the accumulation of maackiain was unexpected as it attained levels comparable to that of medicarpin despite being a

Table 1 Production of phytoalexins and related constitutive isoflavones by chickpea cv. ICCV4 seedlings after inoculation with inducing agents

Compound	Control	Foc race 0	Fo 9009	Fo 90105
Medicarpin	24.7 ± 10.3a	38.9 ± 11.2a	86.7 ± 20.9b	81.3 ± 29.5b
Maackiain	0.6 ± 0.2a	2.6 ± 1.3b	6.0 ± 1.4c	6.0 ± 1.5c
Formononetin	48.0 ± 21.2ab	91.7 ± 54.1c	86.2 ± 69.6bc	21.5 ± 10.2a
Biochanin A	27.5 ± 10.1a	27.5 ± 9.5a	30.0 ± 18.7a	10.1 ± 5.4b

Values are means ± SD of three replicates and are expressed in IA per g fresh plant weight. Values within a row followed by the same letter are not significantly different at $P < 0.05$. Seedlings were inoculated for 16 h in a conidial suspension of either ICCV4-incompatible *Fusarium oxysporum* f.sp. *ciceris* (Foc) race 0 or nonhost *F. oxysporum* isolates Fo 9009 and 90105. The isoflavonoids released into the inoculum suspension after inoculation was completed were extracted and analysed by HPLC.

Table 2 Production of phytoalexins by inducer-inoculated chickpea cv. ICCV4 seedlings after challenge inoculation

Treatments (Preinoculation/challenge inoculation)	Medicarpin	Maackiain
1 (Water/water)	62.4 ± 4.0a	11.1 ± 4.2a
2 (Water/Foc race 5)	213.2 ± 49.9df	145.4 ± 15.8cd
3 (Foc race 0/water)	99.6 ± 42.7ab	78.2 ± 23.9b
4 (Foc race 0/Foc race 5)	120.1 ± 71.1bc	87.5 ± 29.1bc
5 (Fo 9009/water)	191.0 ± 32.2cd	185.0 ± 26.0f
6 (Fo 9009/Foc race 5)	234.5 ± 35.6df	228.2 ± 24.7g
7 (Fo 90105/water)	236.0 ± 82.5df	181.6 ± 23.5df
8 (Fo 90105/Foc race 5)	255.2 ± 66.0f	262.9 ± 74.3g

Values are means ± SD of three replicates and are expressed in IA per g fresh plant weight. Values within a column followed by the same letter are not significantly different at $P < 0.05$. Seedlings were preinoculated for 16 h with either ICCV4-incompatible *F. oxysporum* f.sp. *ciceris* (Foc) race 0 or nonhost *F. oxysporum* isolates Fo 9009 and 90105, and 3 days later they were challenge-inoculated in a conidial suspension of the pathogenic *F. oxysporum* f.sp. *ciceris* (Foc) race 5 for 16 h. Phytoalexins released into the challenge-inoculum suspension after inoculation was completed were extracted and analysed by HPLC.

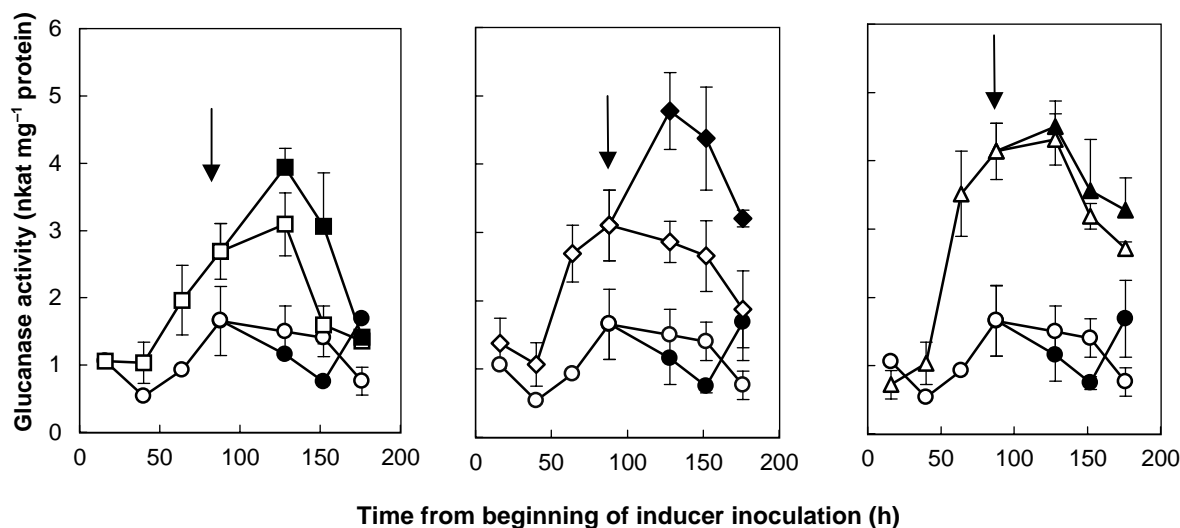


Figure 2 Changes in β -1,3-glucanase activity in roots of chickpea cv. ICCV4 seedlings after inducer and challenge inoculations. Seedlings were inoculated in a conidial suspension of an inducer [incompatible *Fusarium oxysporum* f.sp. *ciceris* race 0 (Foc race 0) or nonhost *F. oxysporum* isolate Fo 9009 or Fo 90105] for 16 h, and 72 h later (arrow) they were challenge-inoculated with *F. oxysporum* f.sp. *ciceris* race 5 (Foc race 5) for 16 h. Treatments (inducing inoculation/challenge inoculation) are: water/water (○), water/Foc race 5 (●), Foc race 0/water (□), Foc race 0/Foc race 5 (■), Fo 9009/water (◇), Fo 9009/Foc race 5 (◆), Fo 90105/water (△), and Fo 90105/Foc-5 (▲). Values are means \pm SD of three replicates. Vertical bars indicate SD when larger than the graphical symbol.

secondary phytoalexin in chickpea. The levels of accumulated phytoalexins varied among treatments, being influenced by prior inducer inoculation rather than by subsequent challenge inoculation with Foc race 5. Thus, higher values of phytoalexin accumulation tended to occur for Fo 9009- and Fo 90105-induced plants (treatments 5–8, Table 2) rather than for Foc race 0-induced plants (treatments 3 and 4, Table 2), irrespective of whether or not the induced plants were challenge-inoculated with Foc race 5. In fact, except for the low increase in maackiain accumulation for Fo 9009/Foc race 5 (treatment 6) and Fo 90105/Foc race 5 (treatment 8), as compared with their nonchallenged control treatments 5 and 7, respectively (Table 2), the overall phytoalexin levels released by induced seedlings after challenge inoculation (treatments 4, 6 and 8, Table 2) did not differ from those released by their respective nonchallenged controls (treatments 3, 5 and 7, respectively, Table 2). Conversely, inoculation of noninduced seedlings with Foc race 5 resulted in a dramatic increase in the amount of phytoalexins released into the inoculum suspension (compare treatments 2 and 1 in Table 2).

The amounts of constitutive isoflavones released into the challenge-inoculum medium showed little variation among treatments (formononetin) or varied inconsistently (biochanin A) in a manner that could not be related either to the inducing agents or to the conditions of challenge inoculation (data not shown).

Accumulation of antifungal hydrolases and peroxidase activities after inducing and challenge inoculations

The levels of extractable β -1,3-glucanase, chitinase and peroxidase activities were analysed in root tissues at

different times after inducer and challenge inoculations. Concerning the 72 h postinduction period studied, the prior inoculation of germinated ICCV 4 seedlings with isolate Fo 9009, Fo 90105 or Foc 7802 significantly increased the extractable levels of the three enzyme activities studied, compared with those in noninduced plants (Figs 2–4). The time course of the response varied for the different enzyme activities. Similarly, the maximal induction levels differed for the various inducer agents. β -1,3-glucanase activity increased during the entire 72 h post-induction period, reaching maximal values at the end of it (Fig. 2). Conversely, chitinase (Fig. 3) and peroxidase (Fig. 4) activities attained their maximal levels 24 and 48 h, respectively, after inoculations with the inducers were complete, and declined thereafter. In terms of maximal levels of induced enzyme activities, inducers were placed in the following order of decreasing induction: Fo 90105 > Fo 9009 > Foc race 0, although differences between Fo 9009 and Foc 7802 were not statistically significant. Electrophoretic analyses of enzyme extracts from root samples of inducer-inoculated seedlings showed that two basic isoforms of β -1,3-glucanase (Fig. 5a), one basic isoform of chitinase (not shown), and three acidic isoforms of peroxidase (Fig. 5b) were induced after inoculation with isolates Fo 9009, Fo 90105 and Foc 7802. Among the induced isoforms, the β -1,3-glucanase isoform (Fig. 5a) and the two peroxidase isoforms (Fig. 5b) that migrated most quickly, and the chitinase isoform, occurred in minute amounts, if any, in control, noninduced root extracts.

When induced seedlings were challenge-inoculated for 16 h and then analysed for enzyme activities after 24, 48 and 72 h (points 128, 152 and 176 h, respectively, on the x axes of Figs 2–4), there was no modification in the

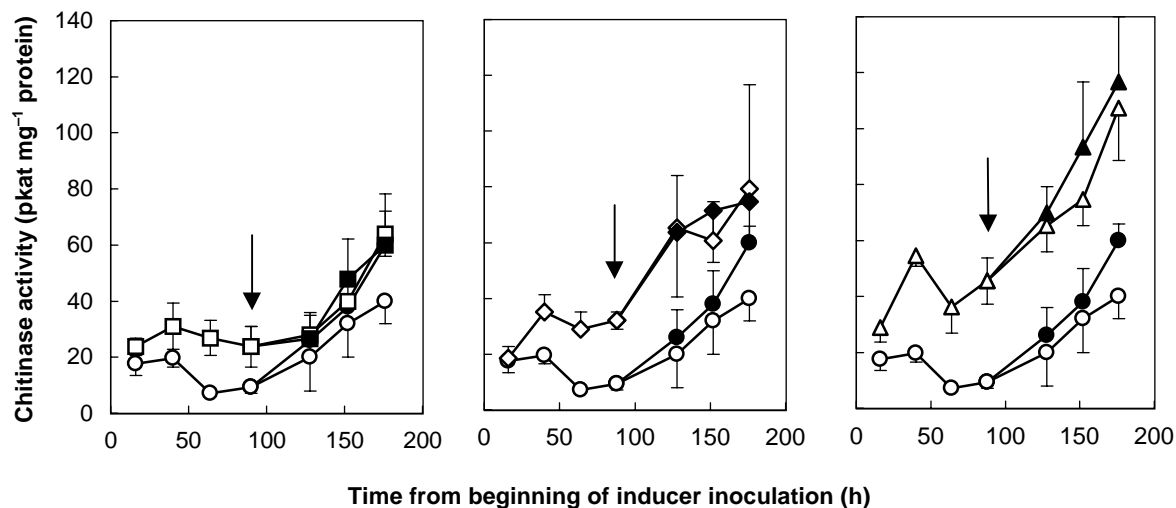


Figure 3 Changes in chitinase activity in roots of chickpea cv. ICCV4 seedlings after inducer and challenge inoculations. Seedlings were inoculated in a conidial suspension of an inducer [incompatible *Fusarium oxysporum* f.sp. *ciceris* race 0 (Foc race 0) or nonhost *F. oxysporum* isolate Fo 9009 or Fo 90105] for 16 h, and 72 h later (arrow) they were challenge-inoculated with *F. oxysporum* f.sp. *ciceris* race 5 (Foc race 5) for 16 h. Treatments (inducing inoculation/challenge inoculation) are: water/water (○), water/Foc race 5 (●), Foc race 0/water (□), Foc race 0/Foc race 5 (■), Fo 9009/water (◇), Fo 9009/Foc race 5 (◆), Fo 90105/water (△), and Fo 90105/Foc-5 (▲). Values are means \pm SD of three replicates. Vertical bars indicate SD when larger than the graphical symbol.

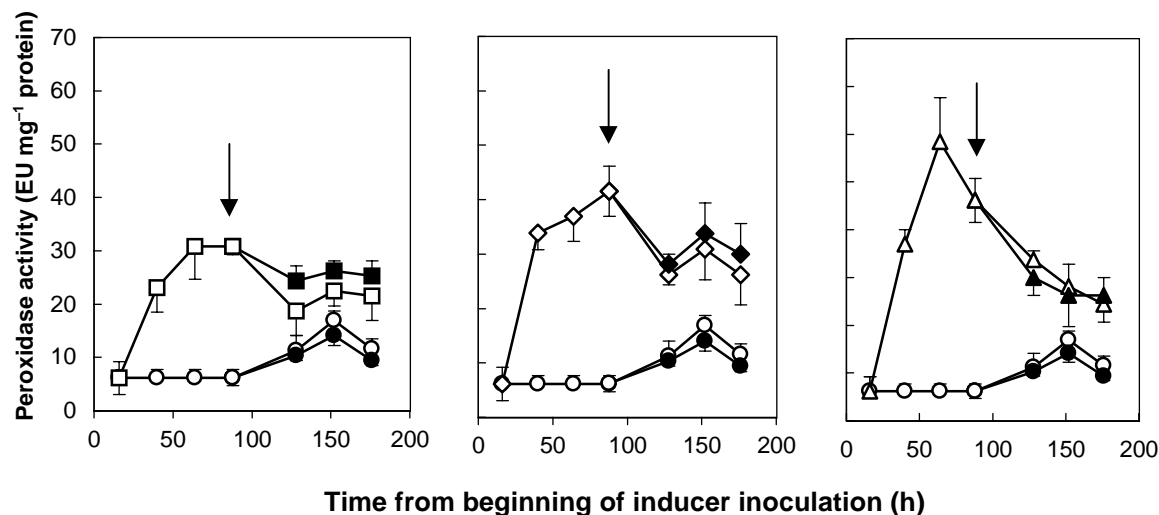


Figure 4 Changes in peroxidase activity in roots of chickpea cv. ICCV4 after inducer and challenge inoculations. Seedlings were inoculated in a conidial suspension of an inducer [incompatible *Fusarium oxysporum* f.sp. *ciceris* race 0 (Foc race 0) or nonhost *F. oxysporum* isolate Fo 9009 or Fo 90105] for 16 h, and 72 h later (arrow) they were challenge inoculated with *F. oxysporum* f.sp. *ciceris* race 5 (Foc race 5) for 16 h. Treatments (inducing inoculation/challenge inoculation) are: water/water (○), water/Foc race 5 (●), Foc race 0/water (□), Foc race 0/Foc race 5 (■), Fo 9009/water (◇), Fo 9009/Foc race 5 (◆), Fo 90105/water (△), and Fo 90105/Foc-5 (▲). Values are means \pm SD of three replicates. Vertical bars indicate SD when larger than the graphical symbol.

pattern of enzyme activities compared with nonchallenged control seedlings. β -1,3-Glucanase (Fig. 2) and peroxidase (Fig. 4) activities decreased, the first after an initial increase, whereas chitinase activity (Fig. 3) continuously increased during the 72 h period after challenge inoculation. In fact, only β -1,3-glucanase activity, at certain times after challenge inoculation, was enhanced compared with nonchallenged controls (Fig. 2). All increases in enzyme activities resulting after challenge inoculation could be

assigned to the same enzymic isoform(s) accumulated after the inducing inoculation (data not shown).

Discussion

Previous studies by Hervás *et al.* (1995) showed that prior inoculation of germinated chickpea seeds with conidial suspensions of either nonhost isolates of *F. oxysporum* or incompatible races of *F. oxysporum* f.sp. *ciceris*

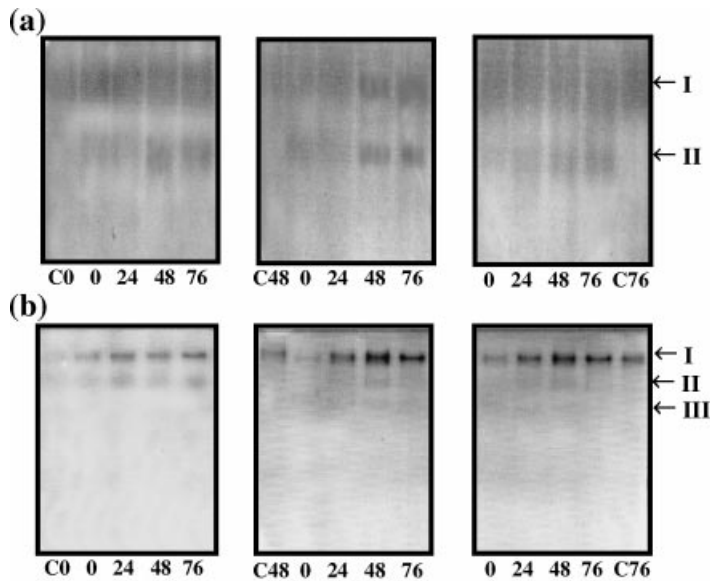


Figure 5 Native PAGE analysis for β -1,3-glucanase (a) and peroxidase (b) activities in roots of chickpea cv. ICCV4 seedlings after inducer inoculation. Seedlings were inoculated in a conidial suspension of an inducer [incompatible *Fusarium oxysporum* f.sp. *ciceris* race 0 (Foc race 0) or nonhost *F. oxysporum* isolate Fo 9009 or Fo 90105] for 16 h. Activities were analysed at four times (between 0 and 72 h) after the end of inducer inoculation with Foc race 0 (left-hand panels), Fo 9009 (central panels), and Fo 90105 (right-hand panels). Lanes C0, C48 and C72 correspond to control samples at 0, 48 and 72 h, respectively, after the end of mock-inoculation with water. The same quantity of protein (18–20 μ g) was loaded in all cases.

suppressed fusarium wilt caused by the highly virulent Foc race 5 in chickpea cvs JG 62 ('desi' type: small, angular, coloured seeds) and ICCV 4. Further studies (Hervás *et al.*, 1997; Hervás *et al.*, 1998) supported the potential of nonhost *F. oxysporum* isolate Fo 90105 as a biocontrol agent of fusarium wilt of chickpea. The present work focused mainly on the role that phytoalexin synthesis and accumulation of activities of antifungal hydrolases and peroxidase might have in the suppression of fusarium wilt in chickpea.

The results of this work agree with previous studies (Hervás *et al.*, 1995) in that nonhost *F. oxysporum* isolates Fo 9009 and Fo 90105 are effective in suppressing fusarium wilt in ICCV 4 to a degree (up to 40%); and also that nonhost *F. oxysporum* isolates are more effective at disease suppression than the ICCV 4-incompatible Foc race 0. Chickpea seedlings inoculated with these biocontrol agents developed a brown discoloration on surfaces of roots, cotyledonary nodes and cotyledons, the intensity of which varied with the biocontrol agent and was correlated with the degree of fusarium wilt suppression conferred by them. As the intensity of discoloration might be quantitatively related to the level of plant defence reactions against inducing microbial agents, it may be suggested that induction of defence responses and fusarium wilt suppression in chickpea are correlated. This situation seems similar to the generally accepted relationship between hypersensitive necrosis and systemic acquired resistance (SAR) (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Keller *et al.*, 1996), although recent results indicate that a hypersensitive reaction is not always connected with SAR (Jones & Dangl, 1996). Nevertheless, Stevenson *et al.* (1997) showed that resistant chickpea cultivars inoculated with incompatible Foc races did not develop hypersensitive reactions or gross structural changes, such as lignification in the vicinity of the invading hyphae, suggesting that the host resistance was dependent upon

chemical rather than physical mechanisms (Stevenson *et al.*, 1997).

Although direct experimental evidence is lacking, the more plausible interpretation from a mechanistic viewpoint for the phenomenon of induced resistance is that the defence reactions developed by plants in response to a previous infection or related process may cause local or systemic protection against a later infection. It is also possible that, after a first inoculation, the plant becomes sensitized in such a manner that its defence response to a second attack is produced earlier and/or more intensively. In its attempt to link a set of defence reactions with induced resistance to fusarium wilt in chickpea, this study has followed up the development of such reactions, namely the production of phytoalexins and accumulation of activities of antifungal hydrolases and peroxidase, both after seedling inoculation with the inducing agents and the subsequent challenge inoculation with the pathogen.

In this study, as in a previous work (Armero, 1996), phytoalexins produced in response to infection of chickpea roots were released rather than accumulated in root tissues. It has been suggested that phytoalexin synthesis occurs only in a small population of root cells (probably in root cortical cells), such that at the whole-root level their concentration remains below the analytical detection limit. This does not happen in the rhizosphere where, by means of their continued release from root cells, the phytoalexins may attain high transient concentrations (Armero, 1996; Armero *et al.*, 2001). Because of the high levels of phytoalexins found in the inoculum suspensions at the end of the 16 h inoculation periods (Table 1), it was concluded that infection by the inducer agents promotes the synthesis of phytoalexins. This response, as well as the brown discoloration, was much greater for nonhost *F. oxysporum* isolates Fo 9009 and 90105 than for the incompatible Foc race 0, which is in agreement with previous results (Armero *et al.*, 1993). Control plants also

released appreciable amounts of phytoalexins, probably as a consequence of stress conditions imposed by the plants being maintained in water for a long time. This was especially so for the main chickpea phytoalexin, medicarpin, of which the control level did not significantly differ from that in the Foc race 0 inoculation treatment. Thus phytoalexin production appears to be associated with the plant resistance response to the various inducers, especially with nonhost resistance to nonhost *F. oxysporum* isolates (Armero *et al.*, 1993). On the other hand, the apparent correlation that seemed to exist between the levels of phytoalexin production and disease suppression (both higher for nonhost isolates Fo 9009 and 90105 than for Foc race 0) suggests a mechanistic relationship of this defence response in the suppression of fusarium wilt. Conversely, the constitutive isoflavones biochanin A and formononetin did not appear to play any roles in the above processes. Stevenson *et al.* (1997) showed that phytoalexin (medicarpin and maackiain) concentrations in roots of four chickpea cultivars increased in the presence of Foc races 1 and 2. Both pre- and postinduction concentrations of phytoalexins were significantly greater in wilt-resistant cultivars, indicating an association between phytoalexin induction and host resistance. Also, medicarpin and maackiain showed antifungal activity to *F. oxysporum* f.sp. *ciceris*, indicating that they may be potential components of the resistance mechanism of chickpeas to fusarium wilt. ED₅₀ values for spore germination were found to be about 80 and 160 $\mu\text{g mL}^{-1}$ of medicarpin and maackiain, respectively, whereas both phytoalexins caused 50% inhibition of germ-tube growth at concentrations as low as 15 $\mu\text{g mL}^{-1}$ (Stevenson *et al.*, 1997). These figures are clearly higher than those found in the inoculating media at the end of the inducer-inoculation period in the present study, which according to data in Table 1 would be within the range of 5–15 $\mu\text{g mL}^{-1}$ for medicarpin and 0.1–1 $\mu\text{g mL}^{-1}$ for maackiain. However, the lower values found in these inoculating media do not necessarily mean that inhibitory concentrations of phytoalexins, especially medicarpin, would not be reached in localized sites within root tissues following inducer inoculation.

Phytoalexins were also produced after challenge inoculation with Foc race 5. This induction was especially notable for the secondary phytoalexin, maackiain, whose concentration became as high as that of medicarpin (Table 2). The highest phytoalexin levels were attained by plants that exhibited the highest suppression of fusarium wilt, namely plants that had been induced with nonhost isolates Fo 90105 and Fo 9009 prior to challenge inoculation. However, phytoalexin levels in such plants (treatments 6 and 8, Table 2) were only slightly different from those produced in the nonchallenged controls (treatments 5 and 7, Table 2) as well as in noninduced, challenger-inoculated plants (treatment 2, Table 2). Induction of phytoalexin synthesis by challenge inoculation was observed after inoculation of noninduced plants with Foc race 5 (compare treatments 2 and 1, Table 2), but not when induced plants were subsequently challenged with the pathogen (compare treatments 4, 6 and 8 with their

respective control treatments 3, 5 and 7, Table 2). Thus, in contrast to the situation found after inducer inoculation, the levels of phytoalexins accumulated after challenge inoculation could not be easily connected with subsequent disease suppression. Rather, these levels of phytoalexin seem to be a consequence of both the previous inducing treatment (highest levels in plants pre-inoculated with nonhost isolates Fo 9009 and Fo 90105) – a factor that may be related to disease suppression – and the degree of colonization of the nonprotected, susceptible plant by the challenger (high levels in treatment 2) – a factor related to the lack of disease suppression. In any case, the mechanistic role of the phytoalexin response in induced resistance to fusarium wilt in chickpea does not appear to relate to a plant-sensitization process provoked by the inducing agents that enhances phytoalexin production after plant-challenger interaction. Rather, it seems to be a consequence of the response developed in the plant by the prior interaction with the inducing agent.

Accumulation of β -1,3-glucanases and chitinases is one of the best characterized plant defence responses (Boller, 1987; Bol *et al.*, 1990; Linthorst, 1991). Both antifungal hydrolases are induced in coordination with other pathogenesis-related proteins in typical systemic acquired resistance responses (Bol *et al.*, 1990; Ryals *et al.*, 1996). On the other hand, peroxidases implicated in several plant defence mechanisms, such as lignin synthesis, oxidative cross-linking of different plant cell wall components, or generation of oxygen reactive species (Mehdy, 1994; Lamb & Dixon, 1997), have also been associated with induced resistance responses (Hammerschmidt *et al.*, 1982; Smith *et al.*, 1991; Rasmussen *et al.*, 1995). The results presented here indicate that all these three enzyme activities increased in chickpea root tissue after inoculation with the inducing agents (Figs 2–4). The induction of the two hydrolases was not completely coordinated, as both activities showed a different pattern of variation during the 72 h postinduction period prior to challenge inoculation (Figs 2 and 3). Similarly to the situation described for phytoalexins, induced enzymatic levels were, in general, higher for the most efficient biocontrol agents (isolates Fo 90105 and Fo 9009) than for the less efficient treatment with Foc race 0. This suggests that antifungal hydrolases and peroxidase may also be implicated in the induced resistance response. In line with this hypothesis, the 3-day time interval between inducing and challenge inoculations, determined by Hervás *et al.* (1995) as optimum for disease suppression, coincides fairly well with the time of maximal joint accumulation of the enzyme activities studied. A similar correlation between time of maximal accumulation of β -1,3-glucanases and chitinases and maximum development of induced resistance was found for other systems (Enkerli *et al.*, 1993). The advantage of coinciding high joint levels of β -1,3-glucanase and chitinase seems clear because of their synergistic antifungal action (Boller, 1993). Similarly to the phytoalexin response, the activities of hydrolases and peroxidase found after challenge inoculation with Foc race 5 could not be correlated with the degree of fusarium wilt suppression (Figs 2–4).

In summary, the results indicate that biocontrol agents induce the expression of plant defence responses, but do not increase the plant's potential for responding to a subsequent challenge inoculation. Overall, the best biocontrol agents (nonhost *F. oxysporum* isolates Fo 90105 and Fo 9009) were those that induced higher levels of plant defence responses; and the optimal time between inducing and challenge inoculations was that for which a maximum level of joint expression of defence reactions was attained. Prior inoculation of germinated seedlings with these agents, followed by subsequent inoculation with the pathogen, resulted in delayed disease development and reduced disease incidence. These effects can be associated with a reduction in efficiency of the initial pathogen inoculum for causing disease. Therefore, the expressed defences before challenge inoculation seem to interfere with such inoculum, and thus to contribute, among other possible mechanisms, to induced resistance involved in fusarium wilt suppression in chickpea.

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