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Effect of *Rhizobium* isolates on isoflavonoid levels in chickpea plants infected with *Fusarium oxysporum* f. sp. *ciceris*

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Summary. The aim of the present studies was to determine the effect of two biocontrol agents, belonging to the genus *Rhizobium*, PchDMS and Pch43, on the accumulation of soluble phenolic compounds, and particularly constitutive isoflavonoids, in chickpea roots infected with *Fusarium oxysporum* f. sp. *ciceris* (Foc), the causal agent of Fusarium wilt of chickpea. Pretreatment of roots with the bacterial isolates before challenge with Foc significantly increased levels of soluble phenolic compounds in both the susceptible ILC482 and the moderately resistant INRAT87/1 chickpea cultivars. High performance liquid chromatography analysis revealed the isoflavones biochanin A and formononetin in the chickpea roots, in both the free and the glycosidically bound forms. Bacterization of the roots with *Rhizobium* isolates before challenge with Foc increased levels of these isoflavones in plant roots. The antifungal activity of crude phenolics extracted from the chickpea roots was tested *in vitro* on PDA amended with various concentrations of these extracts and inoculated with Foc. Crude phenolics significantly reduced fungal growth and caused considerable morphological changes in the mycelium, including marked cellular disorganization.

Key words: Fusarium wilt, biocontrol, phenolics, disease resistance.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc), is the most destructive fungal disease of chickpea (*Cicer arietinum* L.). The fungus invades the vascular tissues and causes severe wilting by blocking xylem transport and impeding water flow (Trapero-Casas and Jiménez-Diaz, 1985; Beckman, 1987; Halila and Strange, 1996). Biological control of this disease is ecologically preferable to the use of synthetic fungicides.

Recently several studies have reported the importance of strains of non-pathogenic plant growth-promoting rhizobacteria (PGPR) in enhancing plant resistance (Kloepper, 1993; Martin and Loper, 1998; Silva *et al*., 2004). Among the most extensively studied PGPR are some *Pseudomonas* spp. that cause resistance to disease in plants (Alström, 1995; Saikia *et al*., 2003; Singh *et al*., 2003). Other

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examples of PGPR are some *Rhizobium* spp. which have recently been shown to induce a defence response in chickpea infected with *Fusarium oxysporum* f. sp. *ciceris*. A previous study by our team found that treatment of germinated seeds with *Rhizobium* induced the expression of compounds involved in plant defence such as peroxidases and polyphenoloxidases and increased levels of phenolic compounds (Arfaoui *et al*., 2005).

Many defence mechanisms are induced by PGPR in plants challenged by pathogens. Such mechanisms cause biochemical changes in the plant, including the synthesis of pathogenesis-related proteins (Carr and Klessig, 1989; Stein *et al*., 1993; Cachinero *et al.,* 2002) and the biosynthesis of antimicrobial low-molecular-weight secondary metabolites such as the phytoalexins (Métraux and Raskin, 1993; Bennet and Wallsgrove, 1994; Kuc, 1995; Saunders *et al*., 2004). The pterocarpan phytoalexins, medicarpin, and maackiain are synthesized by chickpea plants (Köster *et al.,* 1983; Jaques *et al.,* 1987; Armero *et al*., 2001). The role of these phytoalexins, and of the related isoflavonoids biochanin A and formononetin, in the defence response of chickpea was extensively investigated by Barz and his group in Germany, who studied the induction of such defence responses in cell-suspension cultures treated with elicitors (Kessman and Barz, 1987; Barz and Mackenbrock, 1994). These workers found that the constitutive isoflavones biochanin A and formononetin were implicated in increasing the resistance of chickpea to various pathogens. Formononetin is also reported to be the central intermediate in the biosynthesis of the phytoalexins, medicarpin and maackiain (Dewick, 1975).

The objectives of the present study were to determine the extent to which these two isoflavonoids following the activity of the two *Rhizobium* isolates Pch43 and PchDMS, are involved in the biocontrol of Fusarium wilt, and to examine the effect of crude phenolic extracts on fungal mycelial growth (Curir *et al*., 2003; Olivia *et al*., 2003).

Materials and methods

Plant material

Seeds of the two chickpea (*Cicer arietinum* L.) cultivars ILC482 and INRAT87/1, respectively susceptible and moderately resistant to *Fusarium oxysporum* f. sp. *ciceris* race 0, were used in the experiment.

Fungal isolate

Fusarium oxysporum f. sp. *ciceris* (Foc) race 0 was originally isolated from the roots of infected chickpea grown in a naturally infected field at Oued Béja in northern Tunisia (Halila and Strange, 1996). Monoconidial Foc cultures were stored in sterile sand tubes at 4°C. Active cultures were obtained from small aliquots of a sand culture plated on potato dextrose agar (PDA). Cultures were incubated at 25°C for 8 days with a 12 h photoperiod of fluorescent and near-ultraviolet light.

Bacterial isolates

The *Rhizobium* isolates were obtained from the nodules of chickpea as described in Beck *et al*. (1993). Bacterial cells were stored on yeast extract mannitol agar at 4°C (Vincent, 1970). The *Rhizobium* isolates PchDMS and Pch43 were selected by a nodulation test (Beck *et al*., 1993).

Bacterization and inoculation of seedlings

Chickpea seeds were surface-disinfected in 2% NaOCl for 3 min, washed three times in sterile distilled water, and germinated on autoclaved layers of paper towels in a wet chamber at 25°C for 7 days. Germinated seeds, selected for uniformity (length of radicle), were placed in a *Rhizobium* suspension (cfu=10^8 ml^{-1}) or in sterile distilled water (control) and incubated at 25°C for 24 h. Both preinoculated and control seedlings were challenged with Foc race 0 isolate by dipping the roots in a conidial suspension (10^5 conidia ml^{-1}) in sterile water for 24 h. Unchallenged control seedlings were dipped in sterile water. The following treatments were tested:

(i) water + water; (ii) water + Foc; (iii) *Rhizobium* isolate PchDMS + water; (iv) *Rhizobium* isolate Pch43 + water; (v) PchDMS + Foc and (vi) Pch43 + Foc.

After challenge-inoculation, seedlings were transplanted to a growth chamber and incubated for 6 weeks at 25°C, 60–90% relative humidity and a 14 h photoperiod.

Extraction of phenolics

Phenolics from the different treatments were

extracted three times from frozen roots with 80% aq. MeOH at 4°C under continuous stirring. The homogenate was centrifuged at 7000× *g* for 3 min and the supernatants were stored at -20°C until they were analysed by spectrophotometry and HPLC.

Total phenol concentration

The concentration of total phenolics (mg eq catechin g^{-1} f wt),was estimated with the Folin Ciocalteu reagent and the optical density was determined at 760 nm.

HPLC analysis

Phenolic extracts were also analysed by HPLC using a Waters 600E HPLC apparatus (Paris, France) equipped with a Waters 990 photodiode array detector and Millipore software for data analysis. An effective gradient of acetonitrile-o-phosphoric acidified bidistilled water (pH 2.6) was used with an Interchrom C18, 5 ×m reversed phase analytical column (4.6×150, 5 μm, Machery-Nagel, Düren, Germany). Three wavelengths (280, 320 and 350 nm) were used during elution. Phenolics were identified on the basis of their retention times and their spectra as compared with standards. For quantitative measurements, the amounts of isoflavonoids found in the root samples were expressed as areas of integrated UV-signals measured at 280 nm per gram initial plant fresh weight (IA g^{-1} f wt).

Acid hydrolysis

Phenolic extracts were treated with HCl (12 N), at 100°C for 1 h. Hydrolysis products were extracted with diethyl ether, evaporated to dryness and dissolved in methanol for HPLC analysis.

Microbial bioassay

Crude phenolics extracted from the roots of chickpea cultivars ILC482 and INRAT78/1 infected with Foc were tested for their effect on Foc growth by culturing the pathogen on PDA amended or not amended (control) with different concentrations of phenolic extract (0, 100 and 500 μg eq catechin ml^{-1}). Radial growth was measured after 5 days of incubation at 25°C and the mycelium inspected under the light microscope.

Statistical analysis

Each treatment was carried out in triplicate and each experiment was repeated twice. The total phenolic content was measured and the HPLC analysis carried out twice for each sample. All the data were submitted to Anova using Sigma Stat. Statistical Software (SPSS, version 10), followed by Duncan's multiple range test (P=0.05).

Results

Phenolic analysis

In the control roots of the susceptible cultivar ILC482, the level of total soluble phenolic compounds increased very slowly, reaching 2 mg g^{-1} f wt after 20 days (Fig. 1a-b). These levels did not change when the roots were bacterized. In response to Foc infection, the total phenolic compounds started to increase 5 days after infection. By the 10th day they reached 2 mg g^{-1} f wt and thereafter increased steadily to 3 mg g^{-1} f wt 20 days after infection (Fig. 1a-b).

Treatment of the roots with both bacterial isolates, Pch43 and PchDMS, before inoculation with Foc increased total phenolic levels (Fig. 1a-b). The highest phenol levels were recorded 20 days after inoculation, when they reached 4 mg g^{-1} f wt (Fig. 1a) and 5 mg g^{-1} f wt (Fig. 1b) in roots pre-treated with isolates Pch43 and PchDMS respectively.

With the moderately resistant cultivar INRAT87/1, the highest level of soluble phenolic compounds was found with treatment PchDMS + Foc (Fig. 1d) 20 days after inoculation when the phenolic level reached 3.97 mg g^{-1} f wt, about 30% higher than the concentration recorded at the same time in the roots infected with Foc alone.

The more accurate HPLC measurements revealed a marked increase in the levels of several isoflavones (Fig. 2). The most important of these compounds were those corresponding to peaks 7 and 9, which were identified as formononetin and biochanin A. Chromatography and spectral analyses of the hydrolysis products showed that peaks 1 and 3 were the glycoside conjugate forms of formononetin, and peaks 4 and 5 were the glycoside conjugate forms of biochanin A.

HPLC profiles for control and inoculated roots revealed the isoflavones biochanin A and formononetin, both in their free forms (peaks 9 and 7) and in their glycosidically bound forms (peaks 4 and 5 for biochanin A and peaks 1 and 3 for formononetin).

The level of biochanin A in its two forms was more than four times as high as that of formonon-

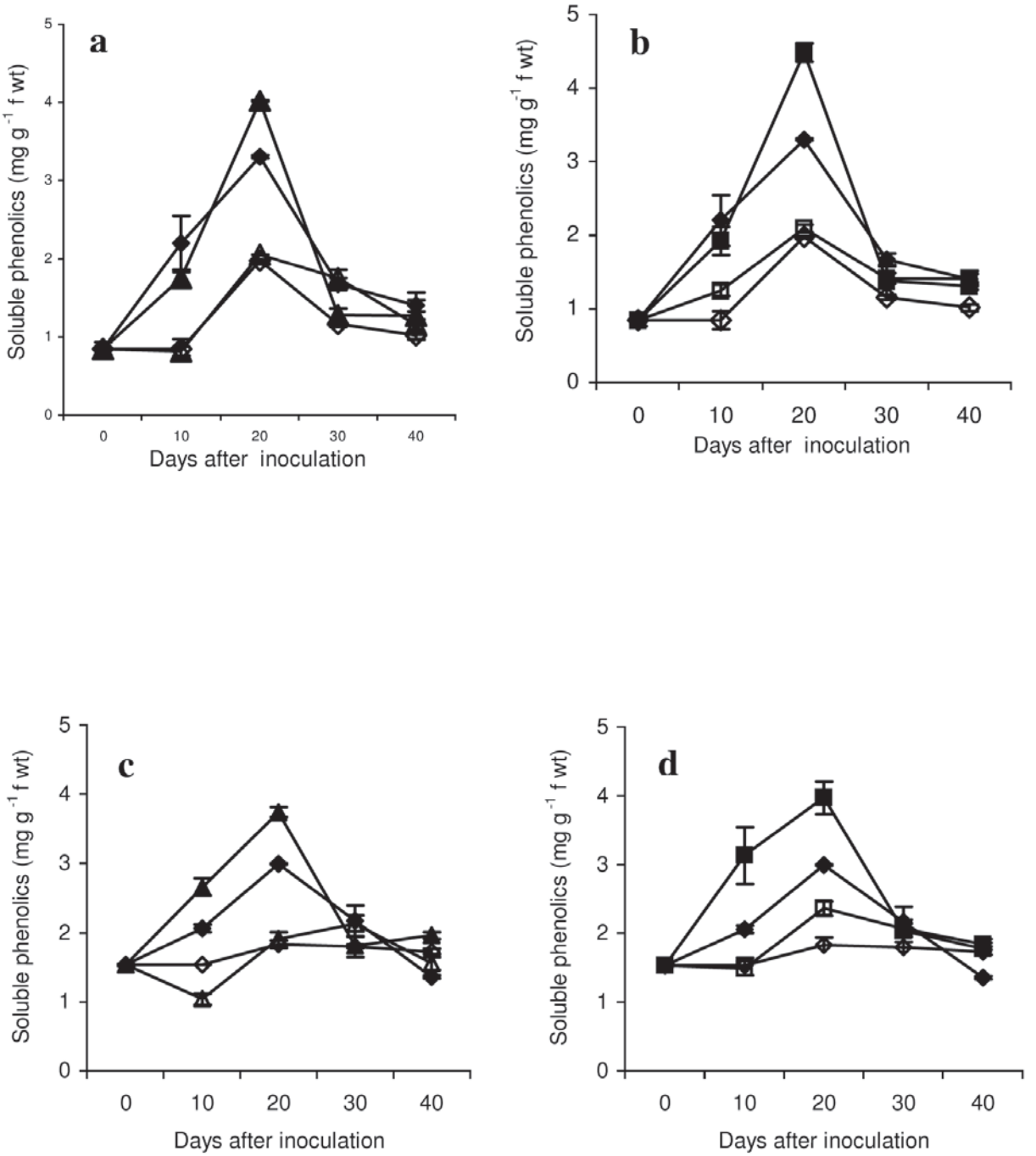

Fig. 1. Time-course diagram of soluble phenolic levels in the roots of the susceptible chickpea cv. ILC482 (a and b) and the moderately resistant cv. INRAT87/1 (c and d) subjected to various treatments (-◇- water + water; -□- *Rhizobium* isolate PchDMS + water; -△- *Rhizobium* isolate Pch43 + water; -◆- Water + Foc; -■- PchDMS + Foc; -▲- Pch43 + Foc).

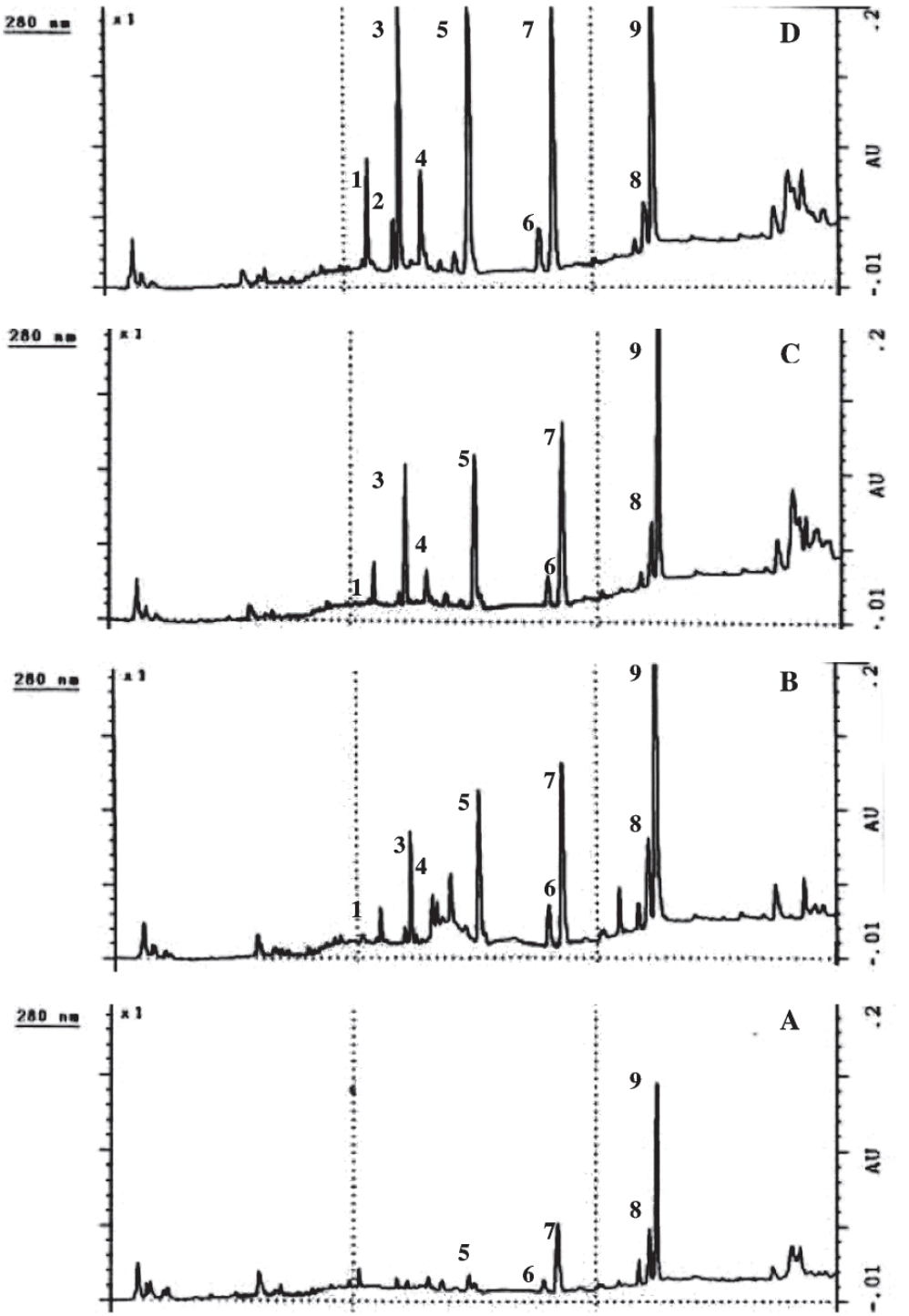

Fig. 2. HPLC profiles of phenolic extracts at 280 nm from the roots of chickpea cv. ILC482 subjected to various treatments. A, control; B, roots bacterized with *Rhizobium* isolate PchDMS; C, roots inoculated with Foc; D, roots bacterized with *Rhizobium* isolate PchDMS and inoculated with Foc. Peaks: 7, formononetin; 1 and 3, formononetin glycoside conjugates; 9, biochanin A; 4 and 5, biochanin glycoside conjugates; 2, 6 and 8, non-identified compounds.

etin (Fig. 3 and 4). In addition, the free forms of these isoflavones exhibited much higher levels than the glycosidically bound forms (Fig. 3 and 4). Taking both forms together, biochanin A and formononetin levels varied with the treatments as compared with the control.

Formononetin (Fig. 3) was detected in the root extracts of both cultivars and its concentration increased to reach a maximum after 10 days of inoculation with Foc, after which it declined. Bacterization of the roots with either PchDMS or Pch43 before inoculation with Foc increased total for-

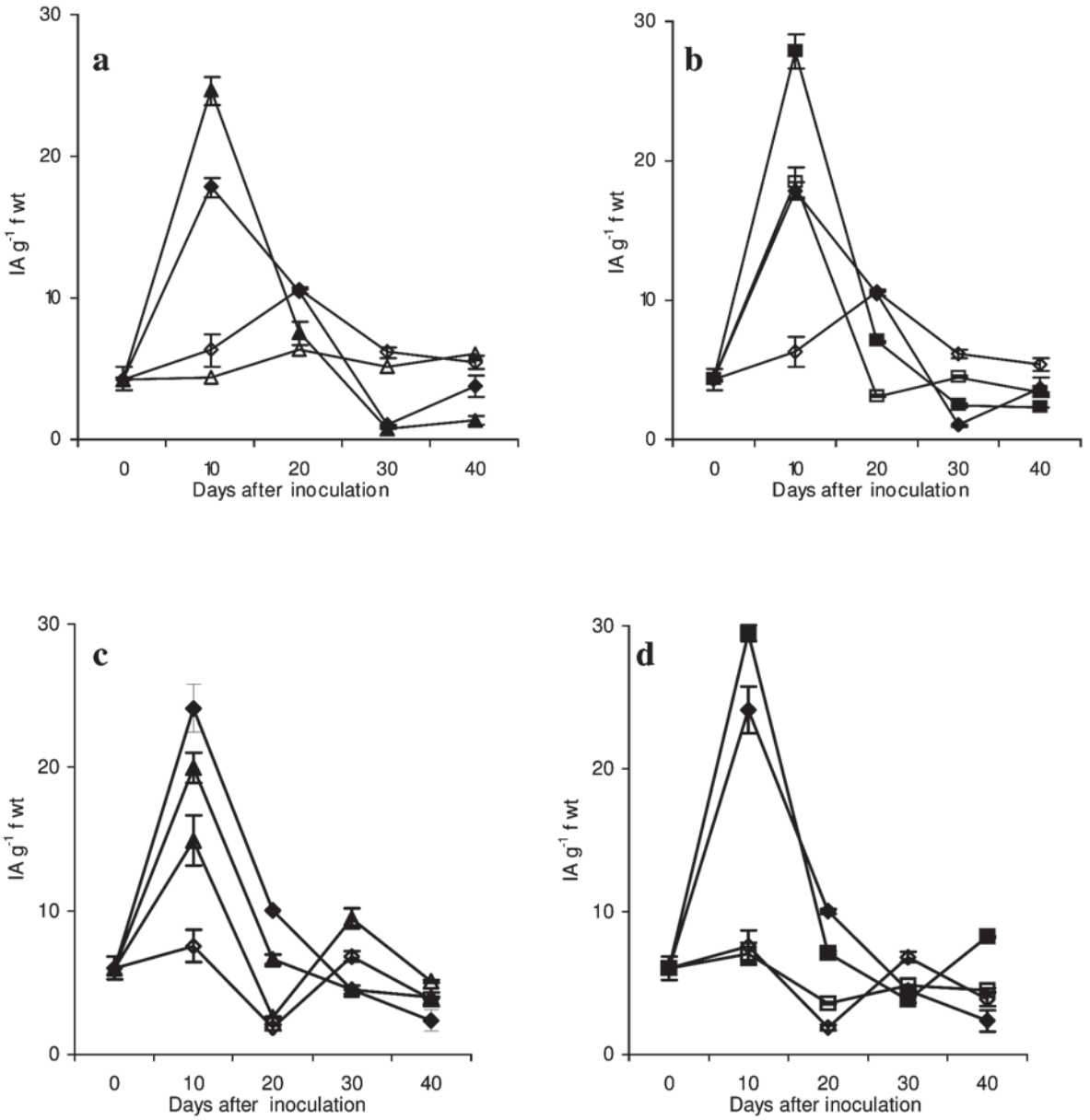

Fig. 3. Time-course diagram of formononetin levels in the roots of the susceptible chickpea cv. ILC482 (a and b) and the moderately resistant cv. INRAT87/1 (c and d) subjected to various treatments (- ◇ - water + water; - □ - *Rhizobium* isolate PchDMS + water; - △ - *Rhizobium* isolate Pch43 + water; - ◆ - Water + Foc; - ■ - PchDMS + Foc; - ▲ - Pch43 + Foc).

mononetin levels. This increase was more than 50% and 35% in the susceptible cv. ILC482 treated with PchDMS/Foc and Pch43/Foc respectively, as compared with the unbacterized control (water + Foc) (Fig. 3a-b).

Biochanin A levels peaked 20 days after inoculation (Fig. 4). The bacterial isolates brought about a considerable increase in the total content of biochanin A in the susceptible cultivar ILC482 (Fig. 4a-b). Biochanin A levels in cv. ILC482 increased by more than 140% after inoculation with PchDMS/Foc and by more than 115% after inoculation with

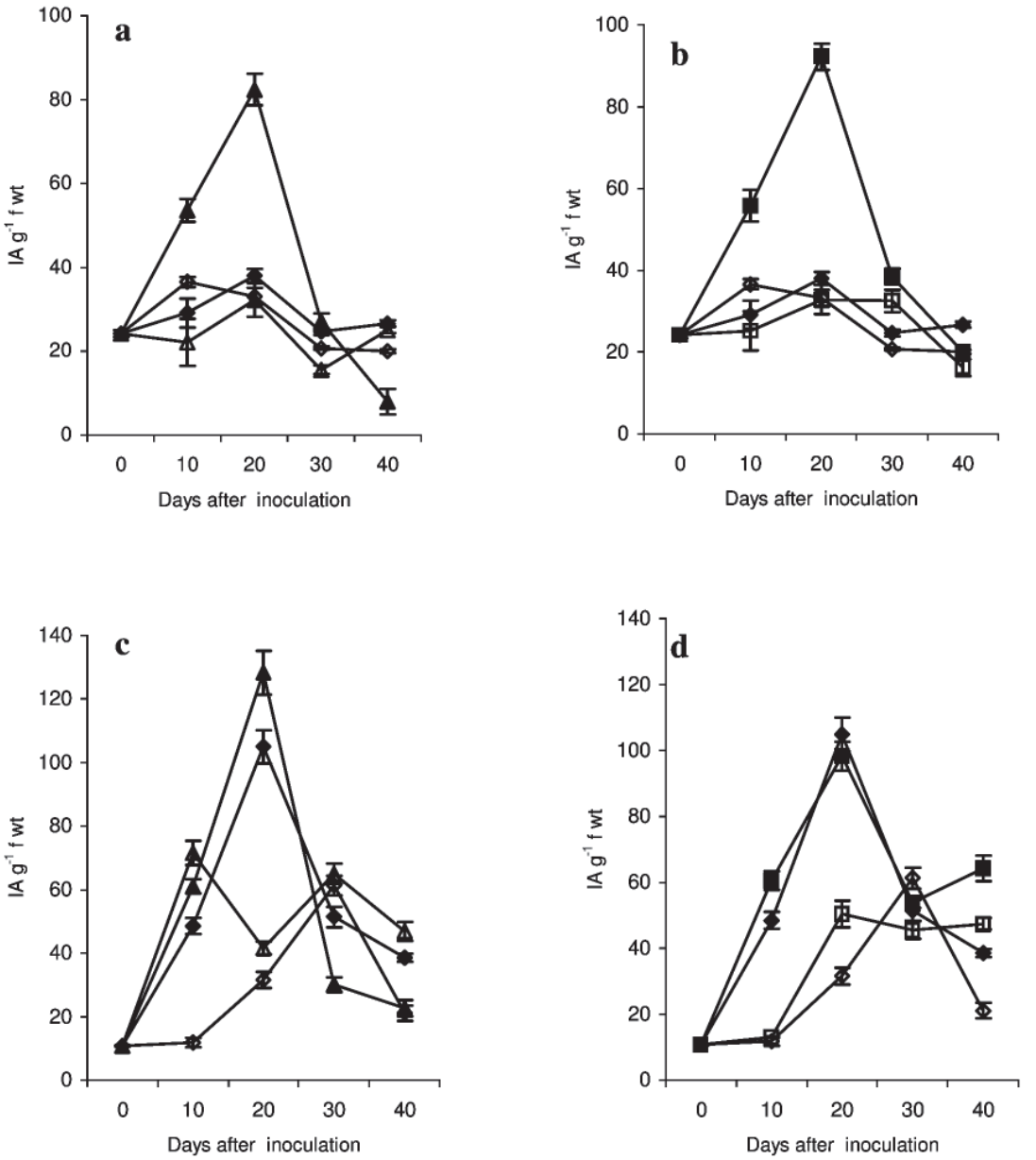

Fig. 4. Time-course diagram of biochanin A levels in the roots of the susceptible chickpea cv. ILC482 (a and b) and the moderately resistant cv. INRAT87/1 (c and d) subjected to various treatments - ◇ - water + water; - □ - *Rhizobium* isolate PchDMS + water; - △ - *Rhizobium* isolate Pch43 + water; - ◆ - Water + Foc; - ■ - PchDMS + Foc; - ▲ - Pch43 + Foc).

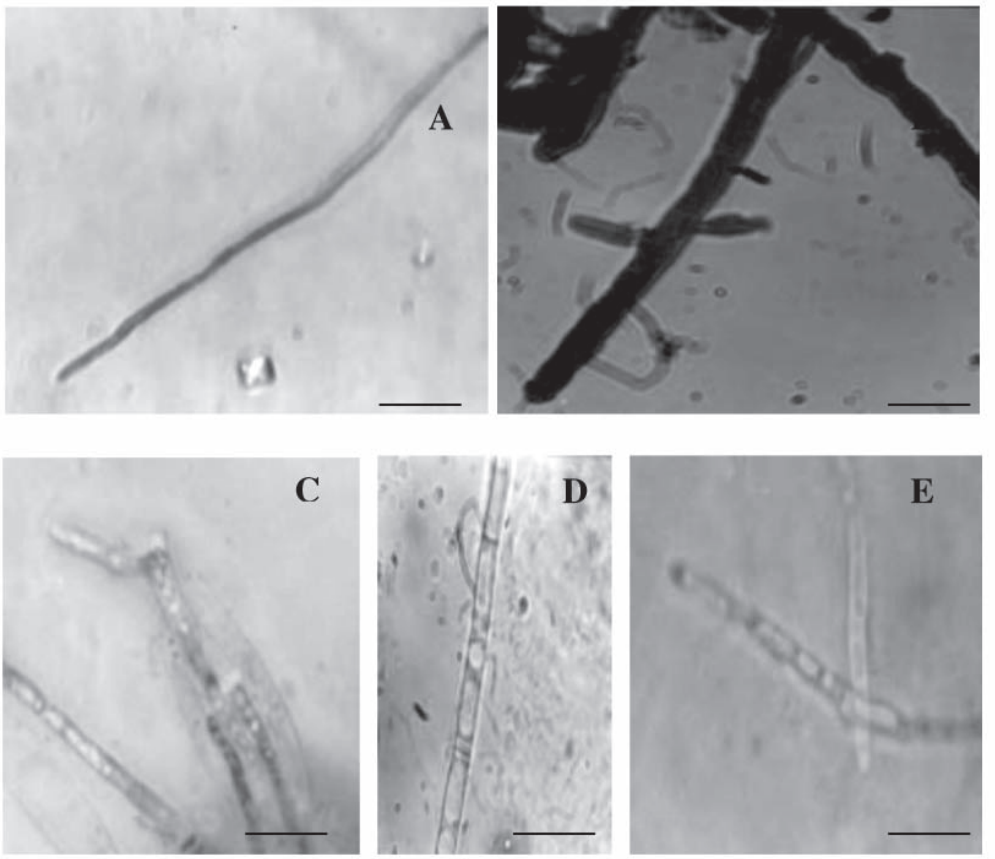

Fig. 5. Microscope photographs of *Fusarium oxysporum* f. sp. *ciceris* hyphae cultured on PDA amended with crude phenolic extract (500 μg eq catechin ml^{-1}). A and B, control (respectively young and old healthy mycelium); C, presence of vesicles; D, vacuolation; E, emptied mycelium. Bar=60μm.

Pch43/Foc as compared with the inoculated control (Fig. 4).

By contrast, in the moderately resistant INRAT87/1 cultivar, neither bacterial isolate caused considerable changes in formononetin or biochanin A levels compared with the inoculated control (Fig. 4C-D).

Effect of phenolic crude extracts on the growth and the morphology of Foc

Crude phenolics extracted from the infected roots strongly inhibited radial growth of Foc. The degree of inhibition depended on the concentration of the extracts. At the greatest extract concentration (500 μg ml^{-1}), growth inhibition exceeded 60% after five days of incubation.

Inspection under the light microscope revealed that at a concentration of 500 μg ml^{-1}, crude phenolic extracts caused marked changes in Foc morphology as compared to healthy mycelium (Fig. 5A-B), including morphological alterations, the formation of abundant vesicles in the densely stained remaining portions of the cytoplasm (Fig. 5C), and large vacuoles within the hyphal cells (Fig. 5D). At more advanced stages the phenolic extracts seemed to deplete the fungal cells of their contents (Fig. 5E).

Discussion

Rhizobium isolates are already in use as biological agents to control a variety of soil-borne plant pathogens (Ehteshamul-Haque and Ghaffar, 1993; Peoples *et al*., 1995). These bacteria promote plant growth (Siddiqui-Zaki and Singh, 2004) and also induce plant defence reactions. Nevertheless, the mechanism whereby plant disease resistance is

induced remains poorly understood. The present study was an attempt to shed some light on this question.

Isoflavonoids are synthesized as part of the phenylpropanoid pathway (Dixon *et al*., 2002). Their role in boosting plant resistance is well established for the leguminosae family (Dixon *et al*., 1983; Dakora and Philips, 1996). Phenolic compounds well documented in chickpea include the isoflavones formononetin and biochanin A, the isoflavanones homoferreirin and cicerin, and the pterocarpans medicarpin and maackiain (Barz and Well, 1992). The isoflavones formononetin and biochanin A were constitutively synthesized in chickpea cells and stored as glycoside conjugates in the vacuole (Mackenbrock *et al*., 1992). When chickpea roots were infected with Foc, the levels of these compounds rose substantially through the conversion of glycoside conjugates to aglycons, which themselves were later converted to pterocarpans. Protection by these biocontrol agents was generally associated with an increase in the mRNA of the defence gene, the phenylpropanoid pathway gene encoding phenylalanine ammonia lyase (PAL) and by higher levels of secondary metabolites of a phenolic nature (Yedidia *et al*., 2003). The findings of the present study were consistent with those other studies. Pretreatment of chickpea seedlings with the *Rhizobium* isolates before challenge with Foc significantly increased levels of total phenolics and of the constitutive isoflavonoids formononetin and biochanin A. These increases were particularly evident after pretreatment with *Rhizobium* isolate PchDMS, which was also found to be the most effective against Fusarium wilt in an earlier study (Arfaoui *et al*., 2005).

Various studies reported the importance of the phytoalexins medicarpin and maackiain in the overall defence response of chickpea (Stevenson *et al*., 1997). Peroxidases and hydrolases, particularly chitinases and glucanases, also play a major role in the defence mechanisms of this plant, and recently Cachinero *et al*. (2003) provided evidence that pretreatment of chickpea seedlings with a nonpathogenic *Fusarium* isolate increased the activity of those enzymes.

Similarly, Singh *et al*. (2003) reported that treatment of chickpea seeds with *Pseudomonas* spp. increased the synthesis of cinnamic, ferulic and chlorogenic acid, all of which have antifungal effects against *Sclerotium rolfsii*. In our experiments, the addition of crude phenolic extracts to the culture medium inhibited mycelial growth of Foc. This antimicrobial activity of the phenolic extracts may be due to compounds such as medicarpin and maackiain, which were found to be fungitoxic to Foc, and which inhibited spore germination and germtube growth (Stevenson *et al*., 1997). The toxicity of chickpea isoflavonoids has been reported in many studies. For instance, Monique *et al*. (2001) found that maackiain and judaicin played a role in lowering the susceptibility of chickpea to *Heliocoverpa amigera* larvae. Wang *et al*. (1998), reported that the isoflavones formononetin, genistein, biochanin A and their corresponding glycosides were a deterrent to the red-legged earth mites (*Halotydeus destructor*). In other pathosystems, treatment of date palm roots with antagonists of *Fusarium oxysporum* f. sp. *albedinis* or with chitosan resulted in higher levels of several non-constitutive hydroxycinnamic acid derivatives (El Hassni *et al.,* 2004). These hydroxycinnamic acid derivatives were known to be toxic to this form of *F. oxysporum*, inhibiting the germination of its conidia and the growth of its germ tubes (El Hadrami *et al*., 1997; Ramos *et al*., 1997).

Light microscope inspection of fungal hyphae exposed to crude phenolic extracts at 500 $\times$g ml^{-1} revealed considerable morphological changes, with marked cellular disruption. Similar results have been reported with pathogens treated with antagonists (Benhamou *et al*., 1999; Ait Barka *et al*., 2002), fungicides, or chitosan (Roberston and Fuller, 1990; El Hassni *et al*. 2004).

The results provide evidence that *Rhizobium* isolates raise levels of soluble phenolics and of the constitutive isoflavonoids formononetin and biochanin A in chickpea plants. These induced compounds showed significant antifungal activity and toxicity towards Foc, indicating that they had a role in the resistance of chickpea to *Fusarium* wilt.

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