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Full Length Research Paper

Bioprotection mechanisms of pea plant by *Rhizobium leguminosarum* against *Orobanche crenata*

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Broomrapes are achlorophyl holoparasites of many important economic dicotyledonous crops. As weeds, they significantly decrease crop yields. *Orobanche crenata* is an important constraint to Mediterranean area pea (*Pisum sativum* L.) cultivation, as no resistant variety is available. Our previous researches showed that several *Rhizobium leguminosarum* strains decrease damages caused by *O. crenata* in pea. The aim of this work was to characterize the resistance induced by *R. leguminosarum* against broomrape. Higher concentrations of phenolic compounds and lignin were observed in pea roots inoculated with Rhizobia, in comparison to non inoculated. Polyphenol oxidase and peroxidase activities were significantly higher in inoculated plants as compared to non-inoculated ones. These results suggest that the mechanisms of induced resistance by Rhizobia against *O. crenata* involve an elevated induction of the phenylpropanoid pathway, conferring mechanical and chemical barriers confronting the invading parasite.

Key words: Broomrape, rhizobium, biocontrol, defense related enzymes, PPO, POX, phenols, lignin.

INTRODUCTION

Orobanche spp. (broomrapes) are root parasitic angiosperms lacking chlorophyll and are totally dependent on their host for the supply of nutrients and organic compounds. *Orobanche crenata* threaten pulse crops since antiquity and it is a severe constraint to the cultivation of legumes (Cubero, 1994). Genetic resistance remains one of the most desirable components in an integrated control strategy.

However, resistance to *O. crenata* in legume crops such as faba bean (*Vicia faba* L.) and pea (*Pisum sativum* L.) has proved to be a polygenic character with very low heritability, making breeding for resistance a difficult task (Cubero, 1994; Rubiales et al., 2003). As a consequence, little resistance is available in commercial pea cultivars. Turning to the wild relatives for breeding is becoming necessary and somewhat an urgent matter (Rubiales, 2003; Rubiales et al., 2005). Current methods of control have had weak or limited success. The use of some selective herbicides does not decrease the yield but remains insufficient to completely control the parasite (Joel et al., 1995). Some biological means of control have been suggested with the use of virulent insects and fungal pathogens or fungal toxins (El-Kassas et al., 2005).

For a more integrated Orobanche management program, a combination of agronomic practices, chemical and biocontrol approaches would be more suitable.

In earlier studies, we showed that Rhizobium strains can be used as potential biocontrol agents for sustainable agricultural development (Arfaoui et al., 2005). Recently, it was shown that symbiosis with some *Rhizobium leguminosarum* strains could induce in pea both better development and lower susceptibility to *O. crenata* (Mabrouk et al., 2007). Induced resistance in the nodulated

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peas was characterized by a low activity of the root exudates in triggering Orobanche seed germination and by the induction of necrosis of most of the Orobanche seedlings and tubercles before and after attachment to host roots respectively. Similarly, growth promoting rhizobacteria can elicit plant defense mechanisms against fungal pathogens through a number of structural and biochemical responses. This suggests that treatment with selected endophytic bacteria could precondition plants to defend themselves against pathogen attack (Kloepper et al., 1993; Tuzun, 1995). Bioprotection is mediated by the activation of the phenylpropanoid pathway controlling the production of diverse resistance-related compounds, including phenolics (lignin), suberin and (iso-) flavonoids (Nicholson and Hammerschmidt, 1992).

Several defence responses activated in plants against micro-organisms have also been identified as induced in response to the process of colonization by parasitic plants. These responses include increased levels of phenolics and peroxidase activity (Goldwasser et al., 1999; Perez-de-Luque et al., 2005a), induction of phytoalexins (Serghini et al., 2001), lignification (Goldwasser et al., 1999; Perez-de-Luque et al., 2005b). A later resistance is possible during the infection process through gum accumulation in host xylem vessels, limiting water and nutrient fluxes towards the parasite and leading to necrosis of established tubercles (Labrousse et al., 2004; Perez de Luque et al., 2006b).

Among the *R. Leguminosarum* strains tested, the P.SOM strain was the most efficient in both promoting pea growth and eliciting resistance to *O. crenata* (Mabrouk et al., 2007). In the present study, we focus on the elucidation of some of the biochemical processes involved in pea resistance induced by Rhizobium strain P.SOM against *O. crenata*.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The *R. leguminosarum* strain P.SOM was initially isolated from pea roots in soil of Orobanche-free crops in Morocco and kindly provided by INRAT(Institut National de La Recherche Agronomique de Tunisie). The strain was grown in the laboratory at 28°C on a yeast extract mannitol medium containing 0.08% yeast extract (w/v) and 1% mannitol (w/v). For further root inoculations, the bacterial suspensions were prepared in distilled water, with several washes to remove traces of growth medium.

Seed sterilization, preconditioning and germination

Pea (*P. sativum* L., cv. 'Douce de Provence') seeds were surface-sterilized with 3.6% calcium hypochlorite for 5 min and rinsed five times with sterile distilled water. Seeds were sown in vials containing glass beads (2 mm-diameter) moistened with sterile distilled water (El Halmouch et al., 2006). Germination occurred following 7 to 10 days in adequately watered vials. Seeds of *O. crenata* Forsk were collected in infested pea fields from Ariana (Tunisia) in 2001. Once cleaned, the seeds were sterilized in 3.6% calcium hypochlorite solution and rinsed five times with sterile distilled water. Orobanche seeds were preconditioned at 25°C for 7days on glass fibre filter paper moistened with 5 ml sterile distilled water in a 90 mm Petri dish.

Hydroponic co-culture of pea, rhizobium and orobanche

Co-cultures were performed according to Labrousse et al. (2004). Briefly pea seedlings (7 to 10 day old) were transferred to Petri dishes (120 x 120 x 17 mm, Greiner). Three perforations were made, in the two opposite borders of the Petri dish, one to hold the pea shoot, out of the dish and the others to allow plant root feeding in the culture medium. Roots were spread between the dish cover and a fibre filter paper (MN 85/90, 12.5 cm diameter, Macherey–Nagel). A one cm thick rock-wool layer (Master from Grodan) was placed on the other side of the glass fibre filter paper. Petri dishes were closed, stored vertically in a sterile polypropylene tray containing sterile solution of Coïc neutrophile nutrient solution (Coïc and Lesaint, 1975) and the whole was covered with aluminium foil and maintained at 21°C with 100 µmol m-2 s-1 PAR under a 16 h photoperiod. When 15-day-old pea plants displayed well-developed roots on the glass fibre filter paper, 3 ml of P.SOM bacteria suspension (107 rhizobia ml-1) were added along the root. In addition, preconditioned seeds of *O. crenata* (100 seeds) were placed regularly at 1 to 2 mm from roots. Simultaneously three separate controls were performed, including both P.SOM and Orobanche-free peas, only P.SOM-inoculated peas and only Orobanche-infected peas. Regardless of the treatment, pea roots were sampled 7, 14, 21, 28 and 35 days after adding P.SOM inoculum and/or Orobanche seeds, then frozen in liquid nitrogen and stored at -80°C until enzyme assays, biochemical and molecular analyses.

Enzyme essays

Frozen root tissue (0.5 g fresh weight (FW)) was homogenized in liquid nitrogen by using a pestle and mortar. The powder was extracted with 2 ml of potassium-phosphate buffer 0.1 M (pH 7) in the presence of polyvinylpyrrolidone (0.1 %, w/v). The homogenate was centrifuged 20 min (8000 x g, 4°C). Proteins were quantified in the supernatant (crude extract) using Bradford assay against bovine serum albumin (Sigma) as a standard (Bradford, 1976).

Polyphenol oxidase (PPO) activity was measured spectrophotometrically at 25°C using catechol as a substrate (Chen et al., 2000). Initially, 200 µl of crude extract was mixed with 700 µl of phosphate buffer 0.1 M pH 7, and reaction was started by adding 100 µl 0.2 M catechol. The enzyme activity was calculated from the initial rate of the A420 increase and expressed as OD min-1 mg protein-1.

Peroxidase activity was assayed according to the methods described by Anderson et al., (1995) and Lin and Kao (1999). Soluble peroxidase activity was assayed spectrophotometrically at 470 nm in a reaction medium containing 9 mM gaiacol, 1 mM hydrogen peroxide and crude enzyme extract. Peroxidase activity was estimated at 30°C and expressed as U mg-1 protein (U: µmole tetragaïacol produced per min).

Total soluble phenolics contents

Frozen root tissue (0.5 g FW) was homogenised in liquid nitrogen by using a pestle and mortar and extracted three times with 5 ml MeOH/water (80/20 v/v) at 4°C under continuous stirring. The combined homogenates were centrifuged (4000 x g, 10 min). Total soluble phenolics content was spectrophotometrically estimated (A760) from the supernatant using the Folin-Ciocalteu Reagent

Table 1. Responses of *O. crenata* seeds and tubercles on roots of pea plants growing in Petri dish assays and inoculated or not with Rhizobium strain P.SOM.

	Necrotic germinated seeds (% of total germinated seeds)	Total tubercle number/plant	Necrotic tubercles (% of total tubercle number)
Infected pea	5.51 ± 2.37	14.5 ± 1.32	0
Infected pea + P.SOM	83.72 ± 5.71	3.00 ± 0.40	58.33 ± 9.42

(Waterman and Mole, 1994). The reaction mixture containing 790 µl of distilled water, 10 µl of sample and 50 µl of Folin-Ciocalteu reagent. After 1 min, 150 µl of aqueous sodium carbonate (20%) was added and the mixture was vortexed for 30 min. The total phenol concentration was calculated from the calibration curve, using catechin as standard, and the results were expressed as µg of catechin equivalents per g FW.

Extraction and determination of lignin

Lignin was determined according to Cahill and McComb (1992). The pellets remaining from the phenolic tests were washed twice with distilled water and centrifuged at 14000 x g for 20 min. Five millilitres of methanol was added and centrifuged as above. Solid residues were dried on aluminium foil at 37°C for 48 h and their dry weight determined. Samples were then suspended in test tubes containing a mixture of 0.5 ml thioglycolic acid and 5 ml of 2 N HCl, sealed and placed in a 95°C water bath for 4 h. The supernatants were removed and the remaining pellets washed twice with 2 ml distilled water and centrifuged at 13000 x g for 15 min. The pellets were suspended in 5 ml 0.5 N NaOH overnight at 4°C, centrifuged and washed twice with 2 ml distilled water. The washing water was combined with the NaOH supernatant and titrated with 1 ml concentrated HCl and placed in 4°C for 4 h. After centrifugation, the precipitate was washed twice with 2 ml 0.1 N HCl and dissolved in 3 ml 0.5 N NaOH. The lignin content of the samples was determined by measuring the absorbance of their lignin-like thioglycolic acid (LTGA) derivatives at 280 nm.

Statistical analysis

Enzyme assays and biochemical determinations were performed with six separate replicates per treatment. Consequently, the data are means ± confidence interval ($n = 6$, $\propto = p = 0.05$). Statistical analysis (ANOVA) was performed with SPSS 12.0 for Windows. For each interaction, gene expression analyses was performed on three separate RNA extracts.

RESULTS

Kinetics of pea nodulation and infestation

Regardless of the presence of Orobanche, pre-nodules were formed at day 21 after inoculation (DAI) and nodules became evident at 28 DAI. Orobanche seed germinated rapidly 7 DAI, germination was reduced by a factor of 3 in plants inoculated with Rhizobia. A strong decrease in the number of Orobanche seedlings that succeeded in attaching pea roots and then in developing tubercles was observed when peas were inoculated with the P.SOM strain. These data confirmed the observations previously reported by Mabrouk et al. (2007) though co-cultures were performed differently. A major part of germinated Orobanche seeds (83%) and high percentage of the established tubercles (58%) on rhizobia inoculated pea became necrotic after 35 DAI (Table 1).

Impact of inoculation with *R. leguminosarum* and infection by *O. crenata* on PPO and Pox activities in pea roots

PPO activity increased gradually from 7 to 35 DAI when peas were inoculated singly by *R. leguminosarum* (Figure 1A). A similar pattern was observed in roots inoculated by the bacteria and concomitantly infected by the parasitic weed. On the other hand, plants exhibited a basal level of PPO activity when they were either healthy or only infected by Orobanche. Consequently, infection by *R. leguminosarum* triggered in pea a four-fold increase in PPO activity 35 DAI, regardless of the presence of Orobanche.

Soluble peroxidase activity remained unchanged during 45 DAI at a low value in healthy pea roots. Infection by the pathogen did not affect significantly the peroxidase activity in roots of either inoculated or non-inoculated peas (Figure 1B). Low and relatively constant activity occurred in healthy and infected peas with no inoculation, regardless of the presence of Orobanche. From 10 DAI, P.SOM-related activities were significantly higher ($P < 0.05$) than those of the other treatments. In contrast, pea inoculation with the isolate P.SOM induced an obvious 4-fold rise in soluble peroxidase activity of infested peas, which rapidly reached a maximal value at 10 DAI when neither parasite attachment to pea roots nor nodule formation was observed. Nodules were observed on pea roots from 19 DAI with P.SOM.

Impact of rhizobium and orobanche on the phenylpropanoid/isoflavonoid pathways in peas

Total soluble phenolics accumulated from 28 to 35 DAI in the bacterized pea (Figure 2A). Similar pattern was observed when the bacterized pea was concomitantly infected by Orobanche. On the other hand, healthy pea and plants only infected by Orobanche exhibited low relative amounts of total soluble phenolics. Only negligible

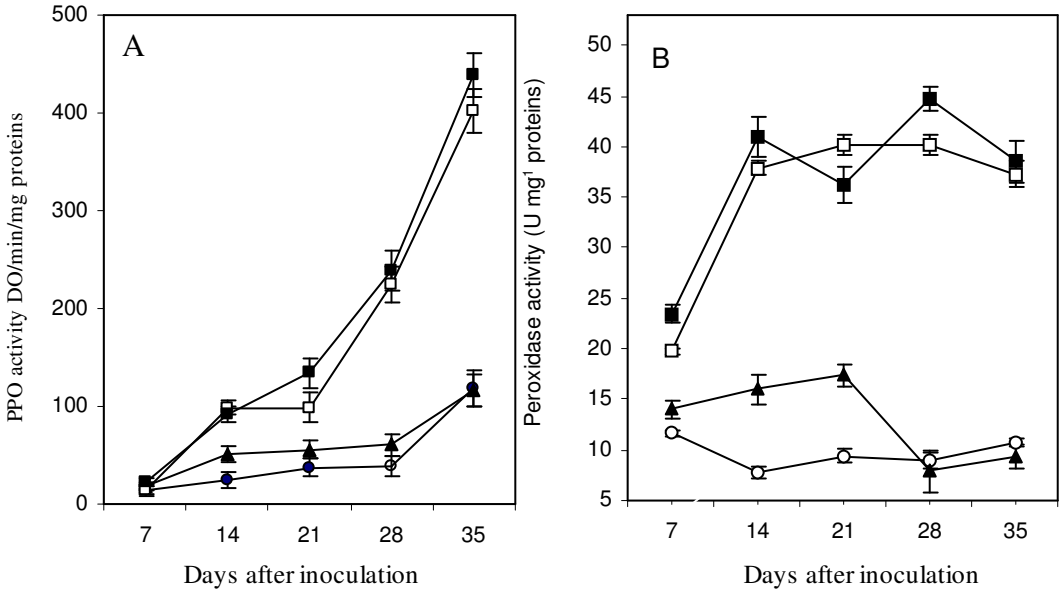

Figure 1. Changes in PPO (A), and Pox (B) activities in pea roots following inoculation with *Rhizobium Leguminosarum* (strain P.SOM) and infection by *Orobanche crenata*. Activities were measured at 7, 14, 21, 28 and 35 DAI in pea inoculated with *R. Leguminosarum* and concomitantly infected by *O. crenata* (□). Controls were performed with healthy pea (O), pea singly inoculated by *R. Leguminosarum* (■) or infected by *O. crenata* (▲).

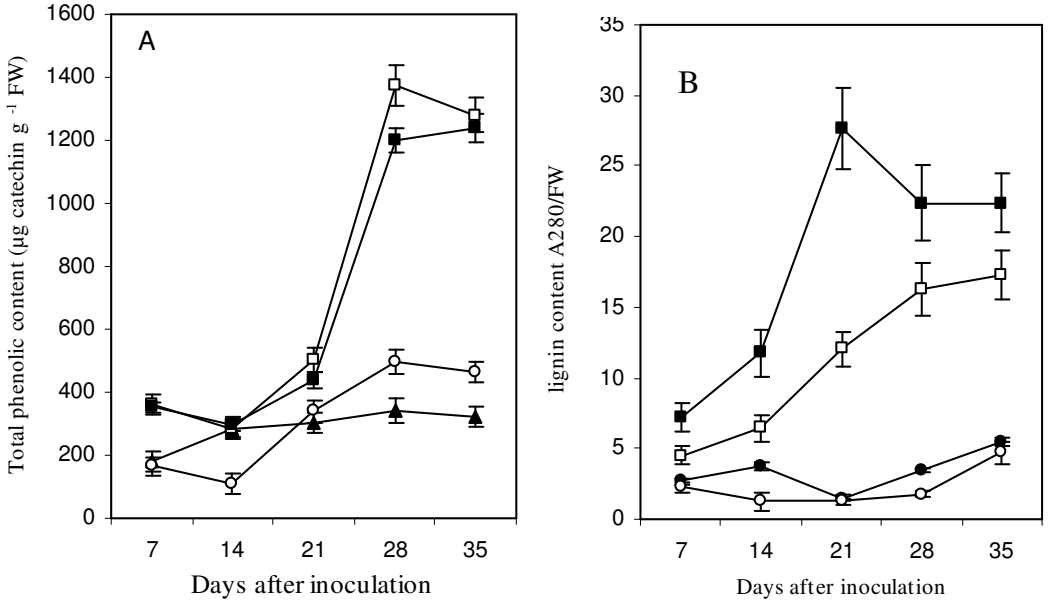

Figure 2. Changes in total phenolic (A) and lignin (B) contents in pea roots following inoculation with *Rhizobium Leguminosarum* (strain P.SOM) and infection by *Orobanche crenata*. Activities were measured at 7, 14, 21, 28 and 35 DAI in pea inoculated with *R. Leguminosarum* and concomitantly infected by *O. crenata* (□). Controls were performed with healthy pea (O), pea singly inoculated by *R. Leguminosarum* (■) or infected by *O. crenata* (▲).

quantities of lignin were measured in roots of healthy pea or pea infested with Orobanche (Figure 2B). Inoculation with Rhizobium strain P.SOM induced an accumulation of lignin in the roots, which increased gradually reaching

higher values at 28 to 35 DAI.

DISCUSSION

Non-pathogenic rhizobacteria can induce systemic resistance in plants against fungi, bacteria and viruses that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Induced systemic resistance (ISR) of plants against pathogens has been intensively investigated with respect to the underlying signaling pathways, as well as to its potential use in plant protection (Tripathi et al., 2006). The present study, reports some of the first data concerning mechanisms of the nodulating-rhizobacteria-induced resistance of pea to the root-parasitic weed *O. crenata* Forsk. Local and systemic properties of the induced resistance were not investigated at this time but the findings identified an activation of several related-resistance processes in roots upon inoculation.

Akimova et al. (2002) demonstrated that pea responded to Rhizobium inoculation by enhancing peroxidase activity. In this work, P.SOM-inoculated peas displayed an enhanced peroxidase activity, in addition to a constantly high PPO activity in comparison with non-inoculated peas. This was observed from 7 DAI when both nodulation and broomrape attachment did not occur. Consequently, we hypothesize that these enzymes are involved in pea resistance, as a result of induction by Rhizobium during early and later stages of infection to counter infestation by broomrape. Oxidases such as PPO and peroxidase are known to be involved in the cellwall reinforcement discussed above, especially during plant responses to pathogens (Hammerschmidt et al., 1982). For example, peroxidases can polymerise polysaccharides and polyphenols to produce stable vascular occluding gels (Crews et al., 2003). Consequently, both enzymes could be implicated in broomrape avoidance of inoculated pea by preventing parasite penetration of the host root or by lowering nutrient fluxes toward the parasite when connection succeeds. Several studies have reported that plants resistant to Orobanche, including some pea varieties, displayed high peroxidase amount and activity (Antonova and Ter Borg, 1996; Perez-de-Luque et al., 2005a; 2006a). Expression of peroxidase encoding genes was enhanced in *Arabidopsis thaliana* following *O. ramosa* infection (Vieira et al., 2003).

Phenolics accumulation close to the site of penetration and lignification of endoderm and pericycle could be observed in plants showing high resistance to Orobanche (Perez et al., 2006a). While a cytological approach was not performed in the present study, it could be demonstrated that P.SOM inoculation induced enrichment in total soluble phenolics of pea roots (Figure 2). There are other evidences, that Rhizobacteria can trigger accumulation of soluble phenolics (mainly gallic, chlorogenic and cinnamic acids), especially in pea, leading to a better plant performance upon infection by pathogens (Singh et al., 2002; Mishra et al., 2006). Although, these data are consistent with the hypothesis that phenolics contribute to Orobanche necrosis on bacterized roots, involvement of such a mechanism in Rhizobacteria-induced resistance in pea needs additional studies.

As shown by the significant decrease in the number of attached tubercles on pea roots and the induced necrosis of the attached tubercles, the defense elicited by the bacteria is strongly efficient. Moreover, as shown by Mabrouk et al. (2007) using pot experiments, resistance was kept during the development period of the nodulated pea. Nevertheless, additional experiments are useful to estimate the efficiency of the bioprotection of pea in Orobanche-infested field conditions before the implementation of a biocontrol strategy to reduce Orobanche in pea and other legumes fields, using some nodulating Rhizobium strains.

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