Mycobiology 32(1): 47-53 (2004)
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Induction of Defense Related Enzymes and Pathogenesis Related Proteins in *Pseudomonas fluorescens*-Treated Chickpea in Response to Infection by *Fusarium oxysporum* f. sp. *ciceri*

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(*Received October 28, 2003*)

***Pseudomonas fluorescens* 1-94 induced systemic resistance in chickpea against Fusarium wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceri* by the synthesis and accumulation of phenolic compounds, phenylalanine ammonia lyase (PAL) and pathogenesis related (PR) proteins (chitinase, β-1,3-glucanase and peroxidase). Time-course accumulation of these enzymes in chickpea plants inoculated with *P. fluorescens* was significantly (LSD, $P = 0.05$) higher than control. Maximum activities of PR-proteins were recorded at 3 days after inoculation in all induced plants; thereafter, the activity decreased progressively. Five PR peroxidases detected in induced chickpea plants. Molecular mass of these purified peroxidases was 20, 29, 43, 66 and 97 kDa. Purified peroxidases showed antifungal activity against plant pathogenic fungi.**

KEYWORDS: Defense related enzymes, *Fusarium oxysporum* f. sp. *ciceri*, Pathogenesis related proteins

Infection of plants with necrotizing pathogens induces systemic resistance to subsequent attacks by pathogens. This resistance is called as systemic acquired resistance (SAR, Ryals *et al.*, 1996). SAR is effective against a wide range of pathogens, including fungi, bacteria and viruses (Sticher *et al.*, 1997; Han *et al.*, 2000). The state of SAR is characterized by an early increase in salicylic acid (SA) levels and the activation of specific set of genes encoding pathogenesis related (PR) proteins (van Loon *et al.*, 1994). Some rhizospherical microorganisms, which do not cause necrosis, were also reported to induce systemic resistance (ISR; Pieterse *et al.*, 1996). Phenotypically, ISR resembles pathogen-induced SAR which is effective against a broad spectrum of plant pathogens (van Loon *et al.*, 1998). ISR is distinguished from the SAR by different signal pathway and resistance expression (Pieterse *et al.*, 1996). Furthermore, ISR is independent of the accumulation of SA but ethylene or jasmonic acid plays an important role for triggering ISR (van Loon *et al.*, 1998). Based on these observations, it is assumed that there are various defense mechanisms that are differently expressed depending on the treatment with the SAR-inducing factors or by the pre-inoculation with plant growth promoting rhizobacteria (PGPR).

In recent years, strains of *Pseudomonas* have been extensively used for plant growth promotion and disease control. *Pseudomonas fluorescens* has different mechanisms to reduce plant diseases such as accumulation of phenolic compound, increasing activity of phenylalanine ammonia lyase (PAL) and pathogenesis-related (PR) proteins and lysis of the fungal pathogen structure by secretion of extra-cellular lytic enzymes (Pan *et al.*, 1991; Punja and Zhang, 1993; Maurhofer *et al.*, 1994; Benhamou *et al.*, 2000; Meena *et al.*, 2000; Srivastava *et al.*, 2001). Although the function of some PR-proteins is still not known, they have been suggested to be an important component of plant defense responses to pathogens (Kim and Hwang, 1997). Production of lytic enzymes like chitinase and β-1, 3-glucanase by the several PGPR strains is considered as a major antagonistic property of these strains (Haran *et al.*, 1996; Yi and Hwang, 1996; Mathivanan *et al.*, 1998). These lytic enzymes have hydrolytic action and degrade the fungal cell wall (Haran *et al.*, 1996; Mathivanan *et al.*, 1997). Combinations of chitinase and β-1,3-glucanase inhibit growth of several pathogenic fungi. Activities of chitinase and β-1,3-glucanase are known to be induced in many plants in response to infection with fungal pathogens (Xue *et al.*, 1998).

Peroxidases are involved in the defense mechanisms of plants in response to pathogens either by their direct participation in cell wall reinforcement, or by their antioxidant role in the oxidative stress generated during plant pathogen interaction (Mehdy, 1994). It has also been implicated in phenol oxidation (Schmid and Feucht,

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1980), IAA oxidation (Beffa *et al.*, 1990), lignifications (Grisebach, 1981), plant defense (Hammerschmidt *et al.*, 1982) and regulation of plant cell elongation (Goldberg *et al.*, 1986). Increase in peroxidase has been correlated with resistance in many species including rice and wheat (Young *et al.*, 1995). In these interactions peroxidase is involved in polymerization of proteins and lignin or suberine precursors into plant cell walls (Bradley *et al.*, 1992) that could prevent pathogen penetration of cell wall or movement through vessels (Young *et al.*, 1995). Although peroxidases are highly catalytic enzyme, they have very little specificity and there exists a multitude of isozyme forms (Gasper *et al.*, 1982). Induction of peroxidases due to plant-pathogen interactions has been studied by different workers (Rasmussen *et al.*, 1995; Chittoon *et al.*, 1997; Ramanathan *et al.*, 2001). Early accumulation of peroxidase mRNA, and formation of several isoforms of peroxidases as a result of pathogen attack were also reported (Boyd *et al.*, 1994, Graham and Graham, 1991; Ye *et al.*, 1990; Thordal-Christensen *et al.*, 1992; Ray and Hammerschmidt, 1998).

We isolated several strains of *P. fluorescens* from the chickpea rhizosphere soil and assessed for their effect on charcoal rot and Fusarium wilt of chickpea (Srivastava *et al.*, 2001; Saikia *et al.*, 2003). Some of these strains showed induces systemic resistance against charcoal rot (Srivastava *et al.*, 2001) and Fusarium wilt of chickpea (Saikia *et al.*, 2003). Our previous studies indicated that shoot and root length was significantly increased in *P. fluorescens*-treated plants and the reduction in disease severity was more pronounced when chemical inducers were applied with *P. fluorescens* (Saikia *et al.*, 2003). It was exhibited that exogenously supplied salicylic acid stimulated systemic resistance against Fusarium wilt and reduced the disease severity significantly. In continuation of our previous work, present study investigates the estimation of the phenolic content, activities of phenylalanine ammonia lyase (PAL), and PR-proteins (chitinase, glucanses and peroxidses) and to analyze isozymes of peroxidase and the antifungal proprieties of peroxidases produced by induced chickpea.

Materials and Methods

Bacterial isolates and pathogen. *Pseudomonas fluorescens* 1-94 (*Pf*1-94) used in this experiment was isolated from the rhizosphere soil of chickpea of Varanasi (India) and grown on King's B (KB) medium. The isolate was grown in KB medium in 500 *ml* Erlenmeyer flasks on a rotary shaker (100 rpm) for 48 h at 28°C and cell concentration was adjusted at 1×10^8 cells ml^{-1}. The pathogen, *F. oxysporum* f. sp. *ciceri* Rs1 (*Foc*Rs1) was obtained from the Laboratory of Applied Mycology, Department of Botany, Banaras Hindu University, Varanasi, India. Chickpea cv JG-62 cultivar susceptible to *Foc*Rs1 was used in this experiment. These seeds were washed with 2% soap solution (phosphate free liquid), soaked under running water for 30 min, surface sterilized in 2% NaOCl for 10 min, and rinsed in sterile distilled water (SDW). Seeds were then allowed to germinate in glass tubes (1 seed $tube^{-1}$). The tubes were incubated in an environmental chamber under 16 h light and 8 h dark photoperiod at 28±2°C. Ten days old seedlings were transplanted to earthen pot (radius 18 cm, 3 seedlings pot^{-1}) and incubated for 30 days. After 30 days, soil was treated with either 10 *ml* of *Foc*Rs1 (10^6 conidia ml^{-1}) or *Pf*1-94 (10^8 cells ml^{-1}) alone. One day after bacterial application, one set of bacterial treated plants was challenge inoculated with 10 *ml* of *Foc*Rs1. The estimation of the phenolic content and activities of PAL, chitinase, β-1,3-glucanase and peroxidases were performed at 0, 1, 2, 3, 4, 5, 6 and 7 days.

Estimation of phenolic content. Fresh plant samples (1 g) were homogenized in 10 *ml* of 80% methanol and agitated for 15 min at 70±3°C. One *ml* of the extract was added to 5 *ml* of SDW water and 250 μl of 1N Folin-Ciocalteau reagent and the solution was kept at 25±2°C. The absorbance was measured by spectrophotometer (Thermospectronic, USA) at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in $\mu g\ g^{-1}$ fresh tissue.

Determination of PAL activity. PAL activity was measured as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson *et al.*, 1984). Plant materials (1 g) were homogenized with 5 *ml* of sodium phosphate buffer (0.1 M, pH 7) containing 0.1 g of polyvenylpolypyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 g for 30 min. The supernatant was used for enzyme activity. Extract (0.4 *ml*) was incubated with 0.5 *ml* of 0.1 M borate buffer (pH 8.8) and 0.5 *ml* of L-phenylalanine (12 mM) in the same buffer for 30 min at 30°C. In reference, 0.4 *ml* of enzyme extract was taken along with 1 *ml* borate buffer. The amount of trans-cinnamic acid synthesis was calculated by using its extinction coefficient of 9,630 $M^{-1}cm^{-1}$ as described by Dickerson *et al.* (1984). Enzyme activity was expressed on a fresh weight basis of amount of transcinnamic acid (nmol $min^{-1}g^{-1}$) synthesis.

Chitinase activity. Colloidal chitin was prepared according to the method of Berger and Reynolds (1958). One gram of chickpea plant materials was extracted with 5 *ml* of sodium citrate buffer (SCB; 0.1 M), pH 5. The homogenate was centrifuged for 20 min at 10,000 g. Supernatant was used as enzyme source. The assay mixture consisted of 10 μl of SCB (1 M, pH 4), 0.4 *ml* of

enzyme solution and 0.1 *ml* of colloidal chitin (1 mg). After 2 h of incubation at 37°C, the reaction was stopped by centrifugation. An aliquot of the supernatant (0.3 *ml*) was pipetted into a glass reagent tube containing 30 μl of potassium phosphate buffer (1 M, pH 7), and incubated with 20 μl (w/v) desalt snail gut enzyme (3%). After 1 h, the pH of the reaction mixture was brought to 9 by the addition of 70 μl of sodium borate buffer (1 M, pH 9.8). The mixture was incubated in boiling water for 3 min and then rapidly cooled in an ice water bath. Two *ml* of *p*-dimethylaminobenzaldehyde (DMAB) reagent was added and the mixture was incubated for 20 min at 37°C. The absorbance value was read at 280 nm by spectrophotometer. N-acetyl glucosamine (*GlcNac*) was used as a standard. The enzyme activity was expressed as nmol *GlcNac* $min^{-1}g^{-1}$ fresh tissues.

β-1,3-glucanase activity. Total β-1,3-glucanase activity was colorimetrically assayed by the Laminaria-Dinitrosalicyclate method (Pan *et al.*, 1991). One garm of chickpea plant was extracted with 5 *ml* SCB (0.05 M, pH 5). The extract was then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was then used as crude enzyme extract. The extract (62.5 μl) was added to laminarin (4%, 62.5 μl) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 μl of dinitrosalicylic acid reagent and heated for 5 min on boiling water bath. The resulting coloured solution was diluted with 4.5 *ml* of water, vortexed and absorbance at 500 nm was determined. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as μmol equivalent glucose release $min^{-1}g^{-1}$ fresh tissue.

Peroxidase activity. One gram of induced plant sample was homogenized with 2 *ml* of sodium phosphate buffer (0.01 M, pH 6.5) at 4°C. The homogenate was filtered through 4-layers of muslin cloth and the filtrate was centrifuged at 6,000 g at 4°C for 20 min. The supernatant was used as an enzyme source. Peroxidase activity was determined according to the procedure given by Hammerschmidt *et al.* (1982). Hundred μl of enzyme extract and 1.5 *ml* of pyrogallol (0.05 M) were taken up in a spectrophotometer sample cuvette. In reference cuvette, 100 μl of inactivated enzyme extract (by boiling) was taken along with 1.5 *ml* of pyrogallol. To initiate the reaction, 100 μl of hydrogen peroxide (1%, v/v) was added and the absorbance values were read at 420 nm. The enzyme activity was expressed as change in absorbance $min^{-1}g^{-1}$ fresh sample.

Native PAGE for isozyme analysis. Isozymes of peroxidase were analyzed by native polyacryl amide gel electrophoresis (Pan *et al.*, 1991). For native anionic PAGE, 8% polyacrylamide resolving gels (1.5 mm thick) was prepared by mixing 5.3 *ml* of 30% acrylamide (acrylamide and bis acrylamide; 30 : 0.8), 5.0 *ml* of 1.5 M Tris-HCl (pH 8.8), 0.1 *ml* of freshly prepared ammonium persulphate (10%), 9.3 *ml* of distilled water, and 10 μl of TEMED. The stacking gel contained 1.3 *ml* acrylamide (30%); 2.5 *ml* 0.5 M Tris-HCl (pH 6.8), 50 μl of 10% ammonium persulphate (freshly prepared) and 6.1 *ml* distilled sterile water. After degassing for 10 min, 10 μl TEMED was added, and the gel was poured between glass plates. The non-denaturing gel was run for 8 h at 10°C, 30 mA in a Hoefer Vertical Electrophoresis Unit. Gels were stained for peroxidase in 0.02 M acetate buffer at pH 4.5 containing 0.05% benzidine and 0.03% H_2O_2. After staining, the gel was immersed in 7% acetic acid for 3 min.

Purification of peroxidase. Peroxidase was purified by fractionating the crude enzyme extract over a sephadex G-25 column (1.5 cm × 30 cm) using 0.01 M sodium phosphate buffer (pH 6) as elution buffer (Ramanathan *et al.*, 2001). Five *ml* fractions were collected and analyzed for peroxidase activity in each fraction. Peroxidase activity was estimated through spectrophotometic method and those fractions showed peroxidase activity was pooled and applied to a DEAE-sephadex (Sigma, USA), column (1.5 cm × 30 cm) equilibrated with 0.05 M Tris-HCl (pH 8). Peroxidase was eluted from the column with a linear salt gradient of 0.2 M NaCl in a total volume of 150 *ml*. Fractions containing peroxidase activity was pooled, dialyzed four times against SDW and lyophilized. The molecular weight of purified peroxidase was determined by SDS-PAGE with protein standards of known molecular weight.

Antifungal property of the purified peroxidase. Antifungal property of the purified peroxdase was determined by agar diffusion test on PDA medium. Sterilized paper disc (5 mm dia.) containing 15 μl of purified peroxidase was placed in the centre of the Petri plates containing sterilized PDA medium. Mycelial disc (5 mm) of some actively growing pathogens, *F. oxysporum* f. sp. *ciceri*, *F. udum* and *Macrophomina pheseolina* was inoculated individually in four points of the Petri plates maintaining an equal distance from the centre. The plates were incubated for 5 days at 28±2°C and zone of inhibition was recorded.

Results

Time-course accumulation of phenolic compounds and activity of PAL and PR-proteins (chitinases, glucanases and peroxidases) was studied in induced plants (Figs. 1 & 2). In all the treatments, accumulation of phenolic com-

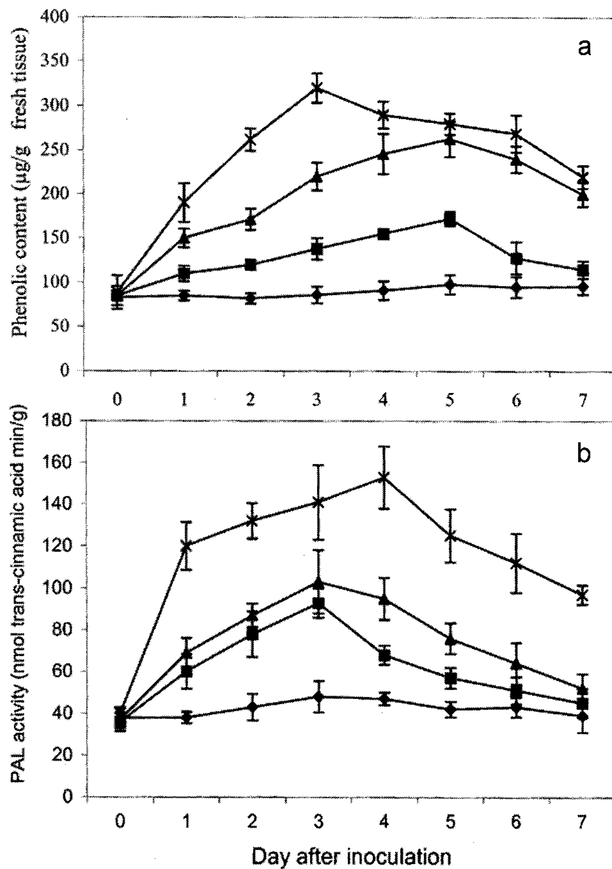

Fig. 1. a. Accumulation of phenolic content; b. changes in PAL activity in chickpea by *P. fluorescens* against challenged inoculation with *F. o.* f. sp. *ciceri*. Symbols represent: ◆ Control; ■ *Foc*Rs1; ▲ *Pf*1-94; × *Pf*1-94 + *Foc*Rs1.

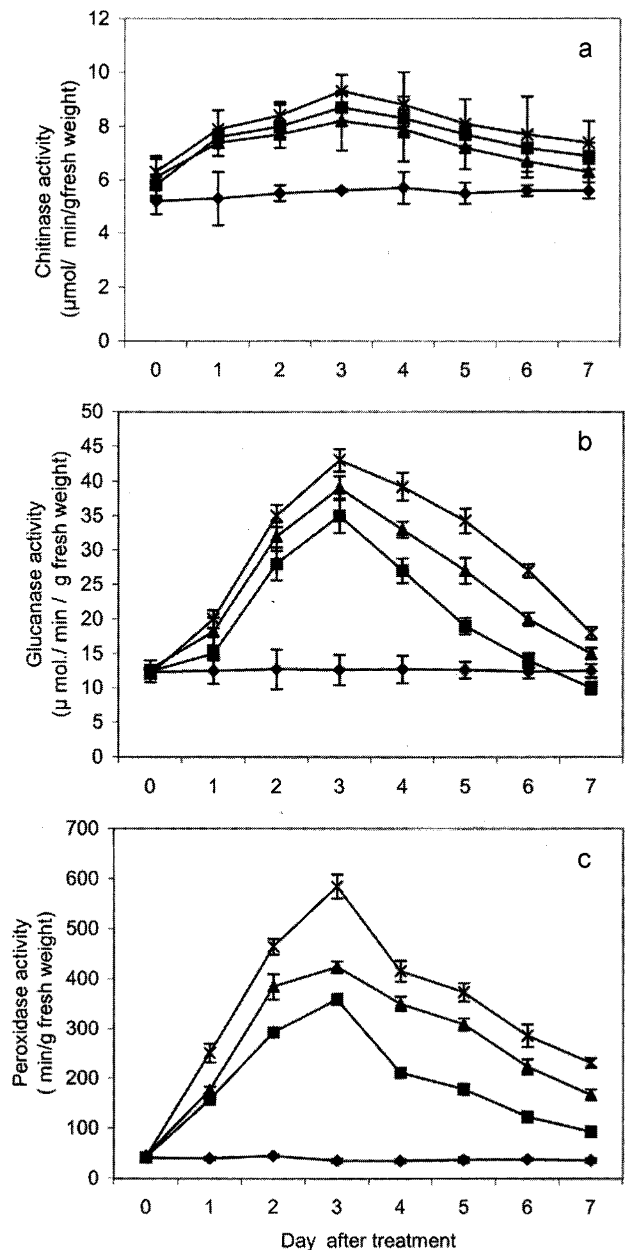

Fig. 2. Change of PR-proteins activities in chickpea by *P. fluorescens* against challenged inoculation with *F. o.* f. sp. *ciceri*; a. chitinase; b. glucanase; c. peroxidase. u Control; ◆ *Foc*Rs1; ▲ *Pf*1-94; × *Pf*1-94 + *Foc*Rs1.

pounds and activities of PAL and PR-proteins in *Pf*1-94 treated plants increased significantly ($P = 0.05$) than control. In general, maximum accumulations of these enzymes were observed in *Pf*1-94 pre-treated plant challenged by *Foc*Rs1. Increase in phenolic content and PAL activity was started from one day after inoculation and maximum level was exhibited at day 4, thereafter decreased progressively (Fig. 1). However, in *Pf*1-94 or *Foc*Rs1 inoculated plants, maximum accumulation was recorded at days 6 and 3, respectively. In control plant no such activity was recorded.

Maximum activities of PR-proteins were observed at day 3 in all induced plants (Fig. 2). For example, activities of chitinases was 1.6 fold higher at day 3 in bacterized plants challenged with the pathogen. In all the treatments, the activity of β-1,3-glucanase was also increased significantly (LSD, $P = 0.05$). A 2.7-3.4 fold increase was recorded at day 3, and thereafter a slow decrease was observed. Peroxidase activity increased in induced plants from the day 1 of post-inoculation until day 3 (more than 14-fold), and decreased rapidly after day 4. No such activity was recorded in control plants (Fig. 2c).

Five pathogenesis related (PR) anionic peroxidases were detected in induced chickpea under laboratory conditions. These peroxidases could not detect in control plants. The highest expression of these peroxidases was also exhibited in *Pf*1-94 pretreated plants challenged by inoculation with the pathogen, *Foc*Rs1 (Fig. 2c). Purified peroxidase showed similar pattern of migration in native and denaturing acrylamide and molecular weight of these peroxidases were 20, 29, 43, 66 and 97 kDa (Data not shown).

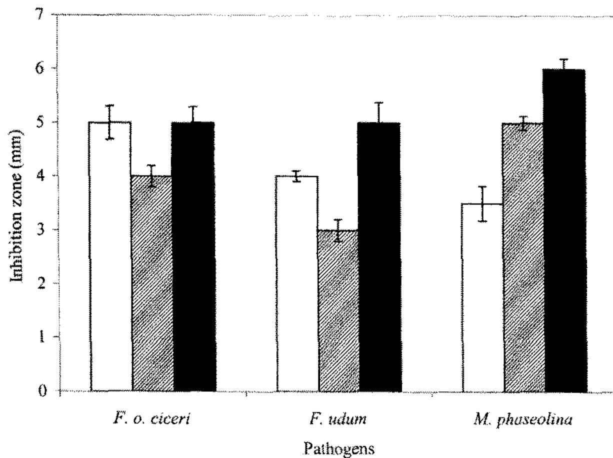

Fig. 3. Antifungal activity of purified peroxidase extracted from induced chickpea. Symbols represent: □ *Foc*Rs1; ▨ *Pf*1-94; ■ *Pf*1-94 + *Foc*Rs1.

The purified peroxidase also exhibited antifungal activity by inhibiting the radial growth of different fungal species e.g. *F. oxysporum* f. sp. *ciceri*, *F. udum* and *M. phaseolina*. The peroxidase extracted from *Pf*1-94 pretreated plants challenged with the pathogen showed the highest antifungal activity against these pathogens and maximum inhibiting zone (6 mm) was observed with *M. phaseolina* (Fig. 3).

Discussion

The results from this study demonstrated that *P. fluorescens* 1-94 induced systemic resistance against Fusarium wilt of chickpea. Induced plants resulted in a significant increase of phenolic content and activities of PAL and PR-proteins (chitinases, glucanases and peroxidases). *Pseudomonas* pretreated-plants challenged by inoculation with the pathogen resulted in a maximum increase of these components, whereas other treatments (e.g. treated with *Foc*Rs1 and *Pf*1-94) were less effective in inducing the resistance by increasing the level of phenolic content, PAL and PR-proteins.

Induced systemic resistance has been demonstrated by *P. fluorescens* on several plants (Maurhofer *et al.*, 1994; Raupach, *et al.*, 1996; van Loon *et al.*, 1998; Saikia *et al.*, 2003). Different mechanisms of *P. fluorescens* may be involved in reducing plant diseases such as-accumulation of phenolic compounds, increasing activity of PAL and PR proteins and lysis of the host structure by secretion of extra-cellular lytic enzymes (Beffa *et al.*, 1990; Xue *et al.*, 1998; Srivastava *et al.*, 2001). ISR by *P. fluorescens* involves increasing physical and mechanical strength of the host cell wall and causing biochemical and physiological changes leading to synthesis of PR-proteins (van Loon and van Strien, 1999; Kim *et al.*, 2001; Ramamoorthy and Samiyappan, 2001). Our findings suggested that *P. fluorescens* could cause ISR against *F. oxysporum*. f. sp. *ciceri* via synthesis and accumulation of phenolic content, PAL and PR-proteins. The result of time-course study showed that at least 1 day after inoculation was required for induction of resistance. These are consistent with work of Meena *et al.* (2000) and Ramamoorthy and Samiyappan (2001).

Peroxidases was considered as an important PR-proteins (van Loon *et al.*, 1994). Plant expressed peroxidase activity during host pathogen interaction and its activity was increased (Young *et al.*, 1995). In this study, we detected five anionic peroxidase isozymes in induced chickpea and these peroxidases showed antifungal activity against different pathogenic fungi like *F. oxysporum* f. sp. *ciceri*, *F. udum* and *M. phaseolina*. Several peroxidase isozymes were detected in rice (Thordal-Christensen *et al.*, 1992; Kim *et al.*, 2001), tobacco (Ye *et al.*, 1990), green gram (Ramanathan *et al.*, 2001) due to the infection with fungal pathogen. Mohan and Kolattukudy (1990) reported that two tomato anionic peroxidase genes were expressed in response to elicitor treatment and fungal attack. Similar observations were also reported by some earlier workers (Ye *at al.*, 1990; Graham and Graham, 1991). Ramanathan *et al.* (2001) detected two pathogenesis related peroxidases in greengram leaves and cultured cells induced by *M. phaseolina* and its elicitor. The molecular masses of these peroxidases were 27 and 38 kDa respectively.

Overall, our findings indicated, that (i) induced chickpea plants showed activities of defense related enzymes and proteins, (ii) *P. fluorescens* could cause ISR against *F. oxysporum* f. sp. *ciceri* via synthesis and accumulation of phenolic compounds, PAL and PR-proteins, (iii) induced chickpea plants produced five anionic peroxidases, and (v) molecular weight of these purified peroxidases were ranged within 20, 29, 43, 66 and 97 kDa, and purified peroxidases also exhibited antifungal property against pathogenic fungi.

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