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Role of Riboflavin in Induced Resistance against *Fusarium* Wilt and Charcoal Rot Diseases of Chickpea

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Riboflavin caused induction of systemic resistance in chickpea against *Fusarium* wilt and charcoal rot diseases. The dose effect of 0.01 to 20 mM riboflavin showed that 1.0 mM concentration was sufficient for maximum induction of resistance; higher concentration did not increase the effect. At this concentration, riboflavin neither caused cell death of the host plant nor directly affected the pathogen's growth. In time course observation, it was observed that riboflavin treated chickpea plants were inducing resistance 2 days after treatment and reached its maximum level from 5 to 7 days and then decreased. Riboflavin had no effect on salicylic acid (SA) levels in chickpea, however, riboflavin induced plants found accumulation of phenols and a greater activities of phenylalanine ammonia lyase (PAL) and pathogenesis related (PR) protein, peroxidase was observed in induced plant than the control. Riboflavin pre-treated plants challenged with the pathogens exhibited maximum activity of the peroxidases 4 days after treatment. Molecular weight of the purified peroxidase was 42 kDa. From these studies we demonstrated that riboflavin induced resistance is PR-protein mediated but is independent of salicylic acid.

Keywords **:** riboflavin, induced resistance, Fusarium wilt, charcoal rot, salicylic acid

Plants can defend themselves against disease caused by different pathogens through a wide variety of mechanisms that may be local or systemic, inducible or constitutive (Ryals et al., 1996; van Loon et al., 1998; Saikia et al., 2005). This phenomenon is termed as induced resistance (Hammerschmidt, 1995). There are several mechanisms like the hypersensitive response (HR), the production of phytoalexins and pathogenesis related (PR) proteins, the deposition of lignin, etc. which contribute to induced resistance in plants. Induction of plant defense reactions is very attractive because induced disease resistance enhances protection against different types of pathogens and utilizes the plants own defense mechanisms, which is a natural, safe, effective, persistent and durable alternative to pesticides in controlling plant diseases (Van Wees et al., 1997). Applications of *Pseudomonas* spp. are capable of triggering plant mediated resistance response in above ground plant parts (Van Peer et al., 1991). This type of induced resistance is termed rhizobacteria-mediated induced systemic resistance (ISR; van Loon et al., 1998). Phenotypically ISR resembles pathogen-induced resistance, in which non-infected parts of previously pathogen infected plants acquire more resistance to further infection. This later form of induced resistance is termed systemic acquired resistance (SAR; Ross, 1961). ISR is distinguished from SAR by different signalling pathways and resistance expression (Pieterse et al., 1996). Furthermore, ISR is independent of the accumulation of salicylic acid (SA). However, ethylene or jasmonic acid play an important role for triggering ISR (van Loon et al., 1998). Maurhofer et al. (1994) had shown that ISR induced by *P. fluorescens* strain CHA0 was associated with PR-protein accumulation. Later reports informed that ISR may be controlled by a SA-independent pathway (Van Wees et al., 1997; Pieterse et al., 1998). It was also reported that siderosphores (Leeman et al., 1996), lipopolysaccharides from the outer membrane of bacterial cells (Van Peer and Schippers, 1992), Jasmonic acid (JA), and Ethylene (ET) (Pieterse et al., 1998) might be involved in the induction of ISR. Saikia et al. (2003) reported that strains of *P. fluorescens* producing salicylic acid (SA) causes ISR to chickpea against *Fusarium* wilt disease.

In recent years, the importance of vitamins as nutrients and as control agent for different diseases has been demonstrated (Dong and Beer 2000; Andrews et al., 2001; Beyer et al., 2002; Ahn et al., 2005). Ahn et al. (2005) demonstrated that thiamine (vitamin B_1) induces SAR and vitamin B_1 functions as an activator of plant disease resistance. They described that thiamine treated rice, *Arabidopsis thaliana* and vegetable crops showed resistance to fungal, bacterial and viral infections and that thiamine treatment induced the transient expression of PR-genes in

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rice and other plants through the SA and Ca^{2+} related signaling pathways.

Vitamin B_2 (riboflavin) produced by plants and microbes acts as a coenzyme in many physiological reaction in plants, microbes and animals (Gastaldi et al., 1999; Wolinsky and Driskell, 1997). It also participates in antioxidation as well as in peroxidation (Packer et al., 1996; Zubay, 1998). In these processes the production of reactive oxygen intermediates (ROIs) results in an oxidative burst and consequently leads to hypersensitive response (Alvarez et al., 1998; Delledonne et al., 1998 and Jabs et al., 1996). Some studies observed that foliar application of riboflavin effectively controlled several diseases of tobacco (Dong et al., 1995; Dong and Beer, 2000). There were also reports that following the treatment of riboflavin, *A. thaliana* developed systemic resistance to *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato,* and tobacco plants developed systemic resistance to tobacco mosaic virus (TMV) and *Alternaria alternata* (Dong and Beer, 2000). Delaney (1997) and Ryals et al. (1996) presented evidence that riboflavin acts as a novel signaling pathway leading to systemic resistance, activating PR-genes in *A. thaliana* and tobacco, and induces resistance to pathogens.

Previously, we reported that *P. fluorescens* produces SA in chickpea roots and causes systemic resistance against *Fusarium* wilt of chickpea (Saikia et al., 2003). We also reported that salicylic acid could induce systemic resistance in chickpea and control *Fusarium* wilt. It was shown that exogenously applied salicylic acid stimulated systemic resistance against *Fusarium* wilt and reduced the disease severity significantly. Our preliminary studies showed that riboflavin causes induced resistance in chickpea against *Fusarium* wilt and charcoal rot diseases (Saikia et al., 2004). In continuation of our previous work, the present study was carried out for detailed investigations to see whether riboflavin can induce resistance systemically, the effect of riboflavin on plants and pathogens *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina* and activation of PR-proteins in chickpea.

Materials and Methods

Pathogens. The pathogens *F. oxysporum* f. sp. *ciceri* Rs1 (*Foc*Rs1) and *Macrophomina phaseolina* Rs420 (*Mph*-Rs420) were obtained from the culture collection repository of the National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, U.P. 275101, India. Cultivar of chickpea cv JG-62 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 then rinsed in sterile distilled water (SDW). Seeds were then sown in earthen pots (radius 18 cm, 3 seedling pot^{-1}) and maintained in a green house at 10 hrs light and 14 hrs dark.

Evaluation of pathogen infection and disease resistance. Fifteen days old seedlings were injected with 20 µl of riboflavin by sterile syringe at the base of stem with different concentrations of riboflavin (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 mM). Three days after riboflavin treatment, the rhizosphere was inoculated with conidial suspension of *Foc*Rs1 or *Mph*Rs420 (10^3 conidia ml^{-1}, 500 μl^{-1} pot). In another set of experiments, 15 days old seedlings were sprayed with these concentrations of riboflavin and after 5 days, seedling were spray inoculated with the pathogen, *Mph*Rs420. The whole experiment was designed as complete randomized block design (CRBD). Disease severity (%) was examined for the next 27 days by the formula as given by Saikia et al. (2003).

Time course effect of riboflavin. Dose effect of riboflavin showed that concentration of 1.0 mM was effective, so this concentration was used for the study. Fifteen days old seedlings were injected with 20 µl of riboflavin by sterile syringe at the base of stem; after 0 to 15 days of riboflavin treatment, soil of the seedlings were inoculated with the conidial suspension of *Foc*Rs1 or *Mph*Rs420 (10^3 CFU ml^{-1}, 500 ul^{-1} pot). The disease severity (%) was recorded as mentioned above.

Effect of riboflavin on pathogen growth. A Petri plate assay was carried out to observe the direct effect of riboflavin on the growth of *Foc*Rs1 and *Mph*Rs420. Actively growing *Foc*Rs1 and *Mph*Rs420 (5 mm mycelial disc) were placed at the center of each riboflavin-amended plates containing sterilized potato dextrose agar medium (PDA, pH 5.5). The riboflavin concentration in the medium was adjusted to 0, 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 15 and 20 mM ml^{-1}. The diameter of the colony on riboflavin amended plates was measured 7 days after inoculation from the inoculum disc edge to the growing colony edge.

Effects of riboflavin on plants. To assess the phytotoxicity of riboflavin on chickpea, chickpea seedling (20 days old) were sprayed with riboflavin at 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 15 and 20.0 mM respectively. One week after treatment, stems of chickpea were detached and sections were cut and stained with trypan blue in lactophenol and examined under microscope.

Determination of systemic resistance. A split root technique was used to observe whether riboflavin induces resistance systemically. For this purpose, Y-shaped PVC tubes were used in a gnotobiotic system in the green house.

The roots of 15 days old plants were split with a razor and grown into PVC tube such that the root system was spread into the two parts of the PVC tube. 5 days later, one side of the root system was treated with 20 µl of riboflavin (0.5 or 1.0 mM) or with SDW (control). After 3 days, the other side of the root system was challenged with either 100 µl of a conidial suspension of (ca. 10^3 conidia ml^{-1}) *Foc*Rs1 and *Mph*Rs420 respectively. Tubes were covered with plastic bags to prevent pathogen contamination. Seedlings were watered daily with sterile water. Disease severity (%) was checked every day for 27 days.

In an another set of experiments, 3 lower leaves of 20 days old chickpea seedlings which were grown in earthen pots were treated with riboflavin (20 µl $leaf^{-1}$, 1.0 mM concentration) or SDW by spraying and after 5 days untreated upper leaves were inoculated with conidial suspension (50 µl $leaf^{-1}$; 10^3 CFU ml^{-1}) of *Mph*Rs420. Whole tests were repeated 5 times.

Effect of riboflavin on salicylic acid (SA) level. To evaluate the effect of riboflavin on SA content in chickpea roots, the split root technique was used as mentioned earlier with 1.0 mM riboflavin. Root samples were taken at an interval of one day from the riboflavin treated site and a distant site till 7 days. Sterile distilled water treated roots served as control. One gram of riboflavin treated and non-treated roots were separately homogenized in liquid nitrogen. The homogenate was rinsed with 2.5 ml of 90% methanol, sonicated and centrifuged at 2800 g for 20 min. The pellet was extracted again with 2 ml of methanol. The supernatants were dried using a stream of nitrogen. The residue was resuspended in 2 ml of 5% trichloroacetic acid (TCA) and centrifuged at 2800 g for 15 min. The supernatant was partitioned twice with an extraction medium containing ethyl acetate: cyclopentane: isopropanol in the ratio (100: 99:1). The top phase was combined and dried under a nitrogen stream (Raskin et al., 1989). Residues were resuspended in 1 ml of 23% methanol in 20 mM sodium acetate buffer and the solution was passed through a 0.2 µm nylon membrane via vacuum at 250 mm Hg.

SA was analyzed by HPLC at 280 nm with a Bondapak C18 column (3.9 mm × 30 cm), with a mobile phase flow rate at 0.5 ml min^{-1}. SA was separated isocratically with 23% methanol (v/v) in 20 mM sodium acetate buffer (pH 5). Ten µl of each sample was injected into the column. Retention time of the isolated SA was compared with standard SA (Sigma). The SA was estimated as ng g^{-1} fresh weight.

Effect on phenol content, PAL and peroxidase activity. To observe the accumulation of phenolic compounds, PAL and peroxidase content, 15 days old seedlings were treated with riboflavin (1.0 mM, 100 µl $plant^{-1}$) and SDW in place of riboflavin and after 2 days one set of the riboflavin and SDW treated plants were challenge inoculated with 2 ml of *Foc*Rs1 or *Mph*Rs420. The following treatments were made (i) control- treated with SDW only; (ii) pathogens control- treated with *Foc*Rs1/*Mph*Rs420 and (iii) plant treated with riboflavin and after 2 days challenge inoculated with the pathogens. The phenol content, PAL and peroxidases activities were estimated after 0, 1, 2, 3, 4, 5, 6 and 7 days from each treatment.

To assess phenolic content, 1 g fresh plant sample was homogenized in 10 ml 80% methanol and agitated for 15 min. at 70°C. One ml of the extract was added to 5 ml of distilled water and 250 µl of 1 N Folin-Ciocalteau reagent and the solution was kept at 25°C. The absorbance was measured with a spectrophotometer (Thermospectronic, USA) at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in µg g^{-1} fresh tissue.

PAL activity was measured with riboflavin treated plants as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson et al., 1984). One gram of plant material was homogenized with 5ml of sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 g of polyvinyl pyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 g for 30 min. The supernatant was used to measure enzyme activity. 0.4 ml of enzyme extract 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 the reference cuvette, 0.4 ml of enzyme extract was taken along with 1.0 ml borate buffer. The amount of trans-cinnamic acid synthesis was calculated by using its extinction coefficient of 9630 $M^{-1}cm^{-1}$ as described by Dickerson et al. (1984). Enzyme activity was expressed on a fresh weight basis as amount of transcinnamic acid (nmol $min^{-1}g^{-1}$) synthesized.

To observe the accumulation of the PR-protein, peroxidase in riboflavin treated chickpea plants, 1g of 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 6000 g at 4°C for 20 min and this supernatant served as an enzyme source.

Peroxidase activity was determined according to the procedure given by (Hammerschmidt et al., 1982). To a spectrophotometer sample cuvette, 1.5 ml of pyrogallol (0.05 M) and 100 µl of enzyme extract were added. In the reference cuvette, enzyme extract inactivated by boiling (100 µl) was taken along with 1.5 ml of pyrogallol (0.05 M). The reading was adjusted to zero at 420 nm. To initiate the reaction, 100 µl of hydrogen peroxide (1%) (v/v) was

added to the sample cuvette and the absorbance values read at 420 nm. The enzyme activity was expressed as change in absorbance $min^{-1}g^{-1}$ fresh sample.

SDS-PAGE for crude peroxidase. SDS-PAGE of the crude peroxidase was carried out according to Laemmli (1970) with 15% acrylamide. The denaturing gel was run for 8 h at 10°C, 30 mA in an Electrophoresis Unit (Genei, Bangalore, India). Protein bands were visualized by staining with coomassie brilliant blue R 250.

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 6.0) as elution buffer (Ramanathan et al., 2001). Five ml fractions were collected and analyzed for peroxidase activity. Peroxidase activity was estimated through a spectrophotometic method and those fractions showing peroxidase activity were pooled and applied to a DEAE- Sephadex column (1.5 cm × 30 cm) (Sigma, USA) equilibrated with 0.05 M Tris-HCl, pH 8.0. Peroxidase was eluted from the column with a linear salt gradient of 0 to 0.2 M NaCl in a total volume of 150 ml. Fractions containing peroxidase activity were pooled, dialyzed four times against distilled water and lyophilized. The molecular weight of purified peroxidase was determined by SDS-PAGE with protein standards of known molecular weight.

Results

Riboflavin induces disease resistance. A mark reduction in infection by *F. oxysporum* f. sp. *ciceri* and *M. phaseolina* was observed in riboflavin treated chickpea plants. While, SDW treated plants exhibited heavy infection by both of the pathogens (Fig. 1). The number of infected plants treated with riboflavin was significantly lower than those plants treated with SDW. The dose effect of riboflavin showed that the concentration of 1.0 mM was maximum effective and sufficient for induction of resistance and control of these diseases. Higher concentrations did not show increase in resistance (Fig. 1). However, less than 1.0 mM concentration was less effective which indicated that below 1.0 mM concentration, it is directly proportional to the reduction of induction of resistance.

The time course resistance in riboflavin treated chickpea became apparent after two days and reached maximum level at 5 to 7 days after treatment (Fig. 2). However, the duration of resistance to the two pathogens differed. In case of Fusarium wilt resistance declined quickly after the 9th day and in case of *M. phaseolina*, it was reduced after the 10th day of treatment with riboflavin.

Riboflavin has not directly affected the growth of the

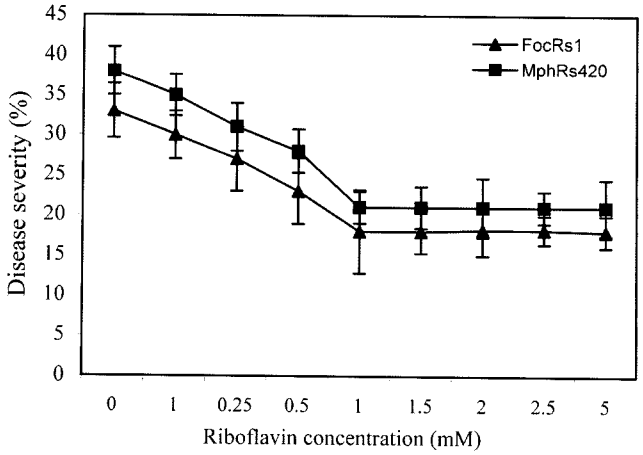

Fig. 1. Influence of riboflavin in pathogen infection.

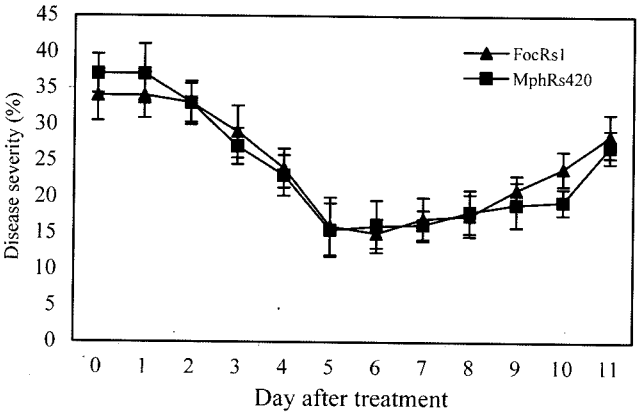

Fig. 2. Time course effect of riboflavin on pathogen infection.

pathogens, which was observed *in vitro* by growth of these pathogens on potato dextrose agar (PDA) plates, supplemented with different concentrations of riboflavin (0 to 20 mM) (data not shown). Both pathogens grew well at almost all concentrations, as similar colony diameters of the fungal culture on PDA plates were observed. The possible effect of phytotoxicity on chickpea was determined by treating the plants with 0.1 to 20 mM riboflavin. Treated plants did not exhibit any abnormality when observed under the microscope or examined visually (Fig. 3). Both, riboflavin treated or untreated tissue showed similar appearance and no morphological or physiological effect was seen.

Kinetics of riboflavin mediated induced resistance. Resistance induction in chickpea against *F. oxysporum* f. sp. *ciceri* and *M. phaseolina* developed systemically. In split root experiments, it was observed that when one side of the root system was treated with riboflavin and the other untreated side of the root system was challenge inoculated with these pathogens, the disease severity was significantly reduced in comparison to the control plant (Table 1). In case

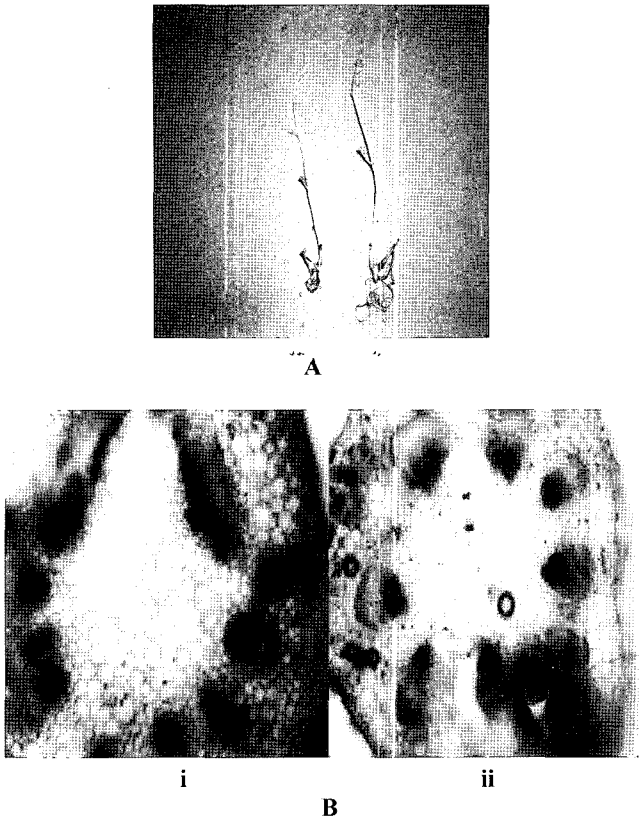

Fig. 3. Macroscopic and microscopic appearance of chickpea plant-A. (left) plant treated with sterile distilled water. (right) treated with riboflavin (20 mM). B. (i) tissue of control plant. (ii) tissues of riboflavin (20 mM) treated plant.

Table 1. *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina pheseolina* infection in chickpea plants in split root system

Treatment	Disease severity (%)
SDW + *Foc*Rs1	47.0 ± 2.2
SDW + *Mph*Rs420	51.0 ± 3.0
Riboflavin (0.5 mM) + *Foc*Rs1	32.0 ± 2.7
Riboflavin (1.0 mM) + *Foc*Rs1	27.0 ± 3.6
Riboflavin (0.5 mM) + *Mph*Rs420	35.0 ± 2.3
Riboflavin (1.0 mM) + *Mph*Rs420	29.6 ± 3.0

Critical difference (CD) = 15.31; C.D. computed at $P = 0.05$

of charcoal rot disease of chickpea, we found that foliar application of riboflavin developed resistance systemically throughout the plants as based on the reduction in the disease severity on the untreated upper leaves or plant parts following treatment of lower leaves with riboflavin (data not shown).

Effect of riboflavin on salicylic acid (SA) levels. From our observations, it was found that riboflavin has no effect on SA levels in chickpea. SA levels were nearly the same in

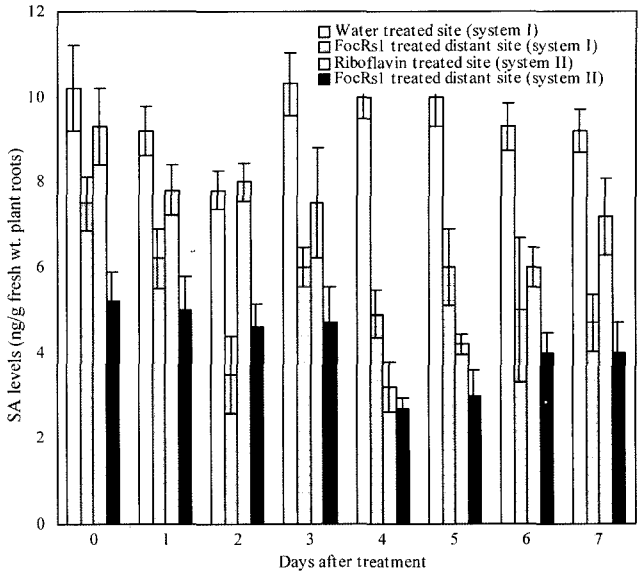

Fig. 4. Effect of riboflavin on salicylic acid levels in chickpea root.

riboflavin treated plants and control plants which were treated with sterile distilled water (Fig. 4). Moreover, levels of SA were not significantly different between riboflavin treated sites and non-treated distant sites and no time-course effect of SA accumulation were observed.

Effect on phenol content, PAL and peroxidase activity. Accumulation of phenolic compounds, PAL and the pathogenesis related (PR) protein, peroxidase was also studied in induced plants. Riboflavin did not show significant role to accumulation of phenolic compounds, PAL and peroxidase when treated alone. However, riboflavin pre-treated chickpea plants challenge inoculated with the pathogens showed rapid increase in the activity of defense related components (Fig. 5a, b and c). There was no significant effect in accumulation of phenolic content as well as PAL activity in riboflavin treated plants, however, when these plants were challenged with these pathogens, *Foc*Rs1 and *Mph*Rs420, Phenolic compounds and PAL accumulation was significantly increased. Maximum level of phenolic compounds and PAL were recorded at 6th and 4th day respectively which decreased thereafter (Fig. 5a, b). However, in plant inoculated with these pathogens alone, maximum phenolic content was exhibited at 5th day and then decreased progressively.

It was observed that riboflavin had no role to increase the peroxidase activity in chickpea when applied alone, however, riboflavin pre-treated plant challenge inoculated with these pathogens showed a significant increase the activity than control plant as well as riboflavin treated plant (Fig. 5c) and during time–course activity, maximum

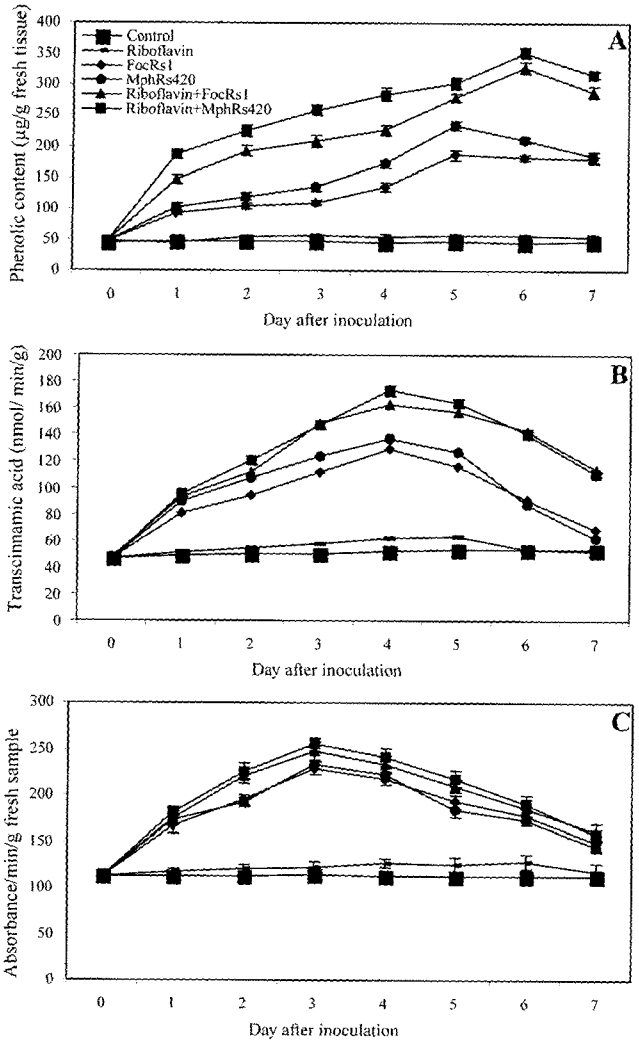

Fig. 5. Accumulation of phenolic content (A), PAL (B), peroxidase (C) by riboflavin against challenge inoculation with *F. oxysporum* f. sp. *ciceri* and *M. phaseolina*.

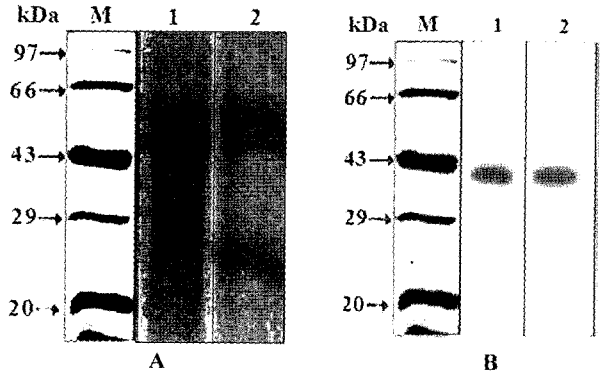

Fig. 6. SDS-PAGE showing protein profile in induced chickpea. (A) Crude protein, M. size marker, lane 1. plant treated with riboflavin + *Foc*Rs1, lane 2. Plant treated with riboflavin + *Mph*Rs420. (B) Purified protein, M. size marker, lane 1. plant treated with riboflavin + *Foc*Rs1, lane 2. plant treated with riboflavin + *Mph*Rs420.

accumulation of this PR-protein was found at 4th day and its activity was high upto the 6th day. However, in pathogen inoculated plants highest activity was recorded at 3rd day, and then decreased progressively.

SDS-PAGE for crude and purified peroxidase. Crude PR-protein, peroxidase profiles showed that no protein profile change was exhibited in riboflavin treated chickpea plants (data not shown) whereas in the challenge inoculated plants with *Fusarium* and *Macrophomina* separately the plant showed similar banding pattern e.g. 25, 42, 55 and 58 kDa (Fig. 6a, b) and purified peroxidase showed a single band of 42 kDa molecular weight.

Discussion

The results from this study demonstrate that riboflavin induce resistance against *Fusarium* wilt and charcoal rot diseases of chickpea. Several mechanisms that mediate the disease protection induced by different chemicals have been demonstrated, including blocking of disease cycle, the direct inhibition of pathogen growth (Thompson et al., 2000; Vicentini et al., 2002) and the induction of resistance to plant against pathogen infection (Kachroo et al., 2003; Nakashita et al., 2003; Ahn et al., 2005).

Riboflavin is an antioxidant (Packer et al., 1996) and other antioxidants also induce resistance in plants against pathogen (Norris, 1991). The possibility of riboflavin directly inhibiting the growth of these pathogen's can be denied because even at high concentration (20 mM) the pathogens could grow (vigorously). These results imply that riboflavin induces resistance in chickpea plants to infection by *F. oxysporum* f. sp. *ciceri* and *M. phaseolina*. We have determined the development of resistance in chickpea following application of riboflavin in a split root experimental system and after foliar application. Disease severity was significantly reduced in the induced plant. It was suggested that riboflavin mediated induced resistance is systemic. Similar reports had also been demonstrated by Dong and Beer (2000).

The influence of riboflavin on defense related components like phenolic compounds, PAL and the PR-protein, peroxidase accumulation in chickpea plant was observed. In this study, it was showed that induced plants resulted in a significant increase in phenolic content, PAL and peroxidase activities and these components begin to accumulate 1 day after treatment and reached maximum levels at 4, 6 and 4 days for PAL, phenol and peroxidase respectively and activity was persistent upto 7 days. Riboflavin was not found to enhance these defenses related components when applied alone. However, when riboflavin pretreated plants were challenge inoculated with the pathogens, accumu-

lation of phenol, PAL and peroxidase was significantly increased. In these studies, we have found comparatively least disease severity at 5 to 7 days, this indicated that riboflavin mediated induced resistance in chickpea plants was related with the increase in phenolic compounds, PAL and peroxidase.

Peroxidase is considered an important PR-protein (Van Loon et al., 1994). Plants express peroxidase activity during host pathogen interaction (Young et al., 1995; Saikia et al., 2004). In this study, we observed increased peroxidase activity in riboflavin pre- treated chickpea plants challenged inoculated with the pathogens, *Foc*Rs1 and *Mph*Rs420. An increase in the amount of peroxidase accumulation was observed, however, SA levels did not increase, indicating that riboflavin induced resistance is PR-protein mediated but is independent of SA. The peroxidase was purified and found to be a 42 kDa protein from induced chickpea plants, which was in accordance with, similar results as described by Bhatti et al. (2006). Unlike many systemic acquired resistance (SAR) elicitors which induce hypersensitive cell death as a necessary step in the development of SAR (Ryals et al., 1996), riboflavin has not caused either macroscopic or microscopic cell death in chickpea plants at concentrations that effectively induce resistance. Therefore, riboflavin resistance mechanisms in chickpea plants are not HR mediated.

Many earlier studies indicated that riboflavin has potential for practical use to control different diseases (Dong et al., 1995; Packer et al., 1996; Wang and Tzeng, 1998). Our studies also indicated that riboflavin could be practically used to control two major diseases of chickpea. The present study suggests that riboflavin is not phytotoxic at concentrations much higher than that required for induction of resistance. In this experiment, treatment of chickpea plants with riboflavin significantly reduced disease severity in both *Fusarium* wilt and charcoal rot diseases.

The kinetics of riboflavin-induced resistance shows the requirement of accumulation of defense related components (Hammerschmidt and Kuc, 1995; Gregory, 1998). Consequently, accumulation of PR-protein took 4 to 6 days to attain highest level and so, ensued the greatest phenotypic resistance. This is in agreement with the previous work of Mori and Sakurai (1996) and Gastaldi et al. (1999) who reported that external application of riboflavin promotes certain physiological processes in plants. For example, Mori and Sakurai (1996) observed that addition of riboflavin to suspension-cultured cells of strawberry increased synthesis of a plant pigment, anthocyanin that has pharmacological effects. Our findings suggest that this is the first report of riboflavin mediated induced resistance of *Fusarium* wilt and charcoal rot diseases of chickpea.

Overall, our findings suggest that (i) riboflavin systemically induced resistance in chickpea plants and showed the accumulation of phenolic compounds, PAL and PR-protein, peroxidase of molecular weight 42 kDa (ii) riboflavin has not caused either macroscopic or microscopic cell death in chickpea plants, and (iii) riboflavin mediated induced resistance is independent of SA and is HR-independent.

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