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Biochemical Characterization of Defense Response of *Vigna aconitifolia* Against the Fungal Pathogen *Macrophomina phaseolina*

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Abstract

Charcol root rot disease of *Vigna aconitifolia* (mothbean) caused by pathogen *Macrophomina phaseolina* is the most destructive disease. The investigation was carried out to know the biochemical basis of defense mechanism of three (two susceptible RMO-40, CZM-3 and one resistant FMM-96 varieties) of *Vigna aconitifolia* inoculated with *Macrophomina phaseolina*. The activity of Polyphenol, Flavonoid, PAL, Total protein content, Proline content and oxidative enzymes involved in the removal of reactive oxygen species (ROS) such as, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) and histochemical detection of hydrogen peroxide (H_2O_2) using DAB were recorded at different time intervals after pathogen inoculation. Two age groups of plants that are 15 days and one month were taken for experiments. The increase in production of these biochemical parameters was higher in the inoculated plants compared to control plants and their response in the resistant cultivar was faster and higher than in the susceptible cultivars. Resistant variety FMM-96 exhibited maximum increase in these parameters. The finding revealed that FMM-96 is comparatively more resistance against infection of *Macrophomina phaseolina* then other observed cultivar.

Keywords: *Vigna aconitifolia*, 3,3'-Diamino benzidine (DAB), phenylalanine ammonia lyase (PAL), polyphenol, flavnoid, Total protein content, Proline content, Reactive Oxygen Species (ROS), peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD), *Macrophomina phaseolina*.

1. Introduction

Plant pathogens are a real threat to worldwide agriculture. Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. More than 70% of all major crop diseases are caused by fungi (Agrios, 2005). Plants provide long-lasting systemic immunity to secondary infection by a range of biotrophic and necrotrophic pathogens (Grant and Lamb, 2006). The interaction between plants and pathogens induces a variety of defense mechanisms which includes cell wall strengthening (Bradley *et al*., 1992), *de novo* production of antimicrobial compounds such as pathogenesis response proteins and secondary metabolites (Gupta *et al*., 2010a) and rapid localized cell death etc (Alvarez, 2000).

Plant defense responses have been broadly grouped as localized acquired response (LAR) and systemic acquired response (SAR). The LAR is characterized by localized cell death and necrosis at the site of infection. In addition to cell death, LAR is characterized by an increase in the production of cell wall phenolics, release of active oxygen species (AOS) such as superoxide anion radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), production of phytoalexins and synthesis of salicylic acid (Hahlbrock and Scheel, 1987). In addition to causing accumulation of antimicrobial compounds, such as phenolic compounds and phytoalexins (Ortega *et al*., 2005), the LAR also leads to an increase in the activity of antioxidant enzymes such as peroxidases (Kortekamp and Zyprian, 2003) and polyphenol oxidases enzymes (Agrios, 1997) involved in defense responses (Thipyapong *et al*., 2004). LAR is also known as

hypersensitive response (HR). HR leads to SAR against a wide range of pathogens (Metraux, 2001). SAR is a mechanism of induced resistance in plants that provides them with extended temporal and spatial protection against a wide range of microbes.

Moth bean (*Vigna aconitifolia*) is an important kharif pulse as well as a fodder crop in arid and semiarid regions, particularly in north western states. It belongs to the family *leguminaceae* and subfamily *papillionaceae*. Moth bean is drought resistant, hard and is able to survive under dry conditions. The plant conserves moisture, protects soil erosion and fixes atmospheric nitrogen through symbiosis with nodule bacteria. The main contribution of moth bean as a food is based on its protein content, which ranges from 20 to 30%. This plant is infected by fungi like *Fusarium sp., Macrophomina phaseolina, Phythium sp.* etc. *Macrophomina phaseolina* is a common root parasite found in warmer lands. It is omnivorous and is a widely infecting pathogen. Mycelium spreads through soil causing seedling blight, stem and root rot of herbaceous plant and also decay of woody plant. *Macrophomina phaseolina* is responsible for causing charcoal root rot disease in mothbean cultivars.

The aim of this study is to evaluate the defense mechanisms of *Vigna aconitifolia* in response to plant fungal pathogens, by analyzing the occurrence of the hypersensitive response, changes in the levels of total phenolic compounds and the activity of the Phenylalanine ammonia-lyase (PAL) enzyme. Susceptible and resistant mothbean cultivars inoculated with *Macrophomina phaseolina*, were used for understanding biochemical mechanism of disease resistance.

2. Material and Methods

Plant material and growth conditions

Three different cultivars of Mothbean viz., var. FMM-96 (resistant) and RMO-40, CZM-3 (susceptible) were procured from SKN College of Agriculture, Jobner (Rajasthan, India) and from Central Zone Research Institute (CAZRI), Jodhpur (Rajasthan, India). Seeds were surface sterilized in 0.1% $HgCl_2$ and grown in pots containing steam-sterilized garden soil in plant growth chamber with a photoperiod of 14 hr light and 10 hr dark with (28±2˚C) temperature and 60% relative humidity under control conditions. Two age groups of plants that are 15 days and one month were taken for experiments. The Polyphenol , Flavnoid, PAL, Total protein content, Proline content and oxidative enzymes activity was determined at 0, 2, 4, 24, 48, 72, 96, 120, 144 and 168 h after inoculation of moth plants with the pathogen in 15 days and one month old plants.

Preparation of spore suspension and mode of inoculation

The fungal strain of *Macrophomina phaseolina* (MTCC NO. 2165) was procured from IMTECH, Chandigarh. The lyophilized fungal strain was activated on potato dextrose broth (PDB) under proper aseptic conditions in the laminar flow. The flasks were incubated in incubator shaker (28±2˚C) for 120 h at 120 rpm. Activated fungal strain was then streaked on potato dextrose agar (PDA) slants. Fungal spore suspension was prepared in sterilized water at a concentration of10^5 spores ml^{-1} under aseptic conditions and kept in the incubator shaker (28±2˚C) at 120 rpm for 1 h to obtain a uniformly distributed spore suspension. For the plant infection the leaf and stem surfaces of the plants were injured mildly with an abrasive to facilitate entry of spores on spraying with fungal spores using a sprayer. The plants sprayed with autoclaved distilled water without fungal spores were served as control.

Extraction and Assay of Polyphenols

The determination of polyphenols was done using the method of (Mc Donald *et al*., 2001). One g tissue i.e. leaf and stem portions of the plants were homogenized in 10 ml of 50% methanol. The supernatant was filtered and centrifuged at 5000 rpm in a cooling centrifuge (Remi cooling compufuge, CPR 24) for 25 min at 4˚C. The pellet was discarded and the supernatant was used for further assay. 0.5 ml of the plant extract was mixed with 5 ml of Folin's reagent and 4 ml sodium carbonate. The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. (ECIL, UV-VIS spectrophotometer). The standard curve was prepared using gallic acid in methanol: water (50:50, v/v).

Extraction and Assay of Flavonoid

The determination of flavonoids was done using the method of (Chang *et al*., 2002). The flavonoid content was determined by aluminum chloride colorimetric method. One g tissue i.e. leaf and stem portions of the plants were homogenized in the conc. of (1:25 v/v) with 95% ethanol. The supernatant was filtered and centrifuged at 5000 rpm in a cooling centrifuge (Remi cooling compufuge, CPR 24) for 25 min at 4˚C. The pellet was discarded and the supernatant was used for further assay. 0.5 ml of extract was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation

at room temperature for 30 min, the absorbance of the reaction mixture was read at 415 nm with a Shimadzu UV-2450 spectrophotometer (Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Quercetin was used to make the calibration curve.

Extraction and Assay of Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) activity was determined using the method of (Camm and Towers, 1973). 1 gm plant tissue was homogenized in 15 ml 0.05 M borate buffer, pH 8.8. The homogenate was filtered and centrifuged at 10,000 rpm (Remi cooling compufuge, CPR 24) for 15 min at 4˚C. The pellet was discarded and supernatant was used for the assay. 0.1 ml enzyme extract was mixed with 0.3 ml 50 mM L-phenylalanine. The total volume was adjusted to 3 ml with 0.05 M borate buffer, pH 8.8. The reaction mixture was incubated at 30˚C for 15 min. The absorbance was recorded at 290 nm in UV-VIS spectrophotometer. The amount of the product formed was calculated using the standard value obtained from the standard curve of cinnamic acid. The PAL activity was calculated in µkatals per gram fresh weight using the standard value obtained from the standard curve of cinnamic acid. The specific activity was calculated for all the control and pathogen inoculated samples of the three varieties viz. RMO-40, CZM-3 (susceptible) and FMM-96 (resistant) by determining the protein content.

Extraction and Assay of peroxidase

One gram of fresh plant tissue of moth bean plants was homogenized in 3 ml of 0.1 M phosphate buffer (pH 7.0) in a prechilled mortar and pestle. The homogenate was centrifuged at 18,000 g for 15 min at 4˚C. The supernatant as used for enzyme assay. The reaction mixture of peroxidase consisted of 1.8 ml phosphate buffer, 100µl guaiacol, 50 µl hydrogen peroxide and 100 µl sample extract. The reaction mixture was mixed well in the cuvette and placed in the spectrophotometer set at 436 nm. Initially the absorbance was allowed to increase by 0.05 from the first OD then the time required in minutes (Δt) to increase the absorbance by 0.1 was recorded against water as blank. Most accurate value was obtained when Δt was between 1 and 3 min. Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39 per micromole, the enzyme activity per liter of extract was calculated as given below:

Enzyme activity units µkatals/liter/gfw = $3.18\times0.1\times1000/6.39\times1\times\Delta t\times0.1 = 500/\Delta t$

Enzyme activity was expressed in terms of rate of increased absorbance per unit time per mg protein.

Histochemical detection of hydrogen peroxide using 3, 3'-Diamino benzidine (DAB)

The presence of hydrogen peroxide (H_2O_2) was done histochemically by the method of Christensen *et al*. (1997). Leaves of 15 days and one month old control and inoculated plants of the var. viz., FMM-96, RMO-40 and CZM-3 were placed in 20 ml solution of DAB of 1 mg/ml concentration in petri-plates for 8 h. These were then subsequently shifted in boiling 96% ethanol for 10 min. Then samples were transferred and stored in 96% ethanol. Reddish–brown colored patches were formed at places where hydrogen peroxide was present. The treated leaves were then mounted on a slide in 60% glycerol and the whole mount were observed under light microscope (Motic DMB-1 digital microscope; Motic Images 2000 1.1 software) to clearly visualize the reddish-brown patches at the site of hydrogen peroxide synthesis.

Extraction and Assay of catalase

Catalase activity was determined using the method of Luck (1974) with some modification. Catalase has double function as it first decomposes hydrogen peroxide into water and oxygen and then oxidizes H donor with the consumption of one mole of peroxide. One gram of fresh plant tissue of moth was homogenized in 10 ml of M/150 phosphate buffer (pH 7.0) in a prechilled mortar and pestle. The homogenate was centrifuged at 18,000 g for 15 min at 4˚C. The supernatant was taken for enzyme assay. For determination of catalase activity 3 ml H_2O_2-phosphate buffer was taken in cuvette and to this 400 µl of sample extract was added. The absorbance was read in the UV-VIS spectrophotometer at 240 nm. The absorbance was allowed to decrease from 0.45 to 0.4 and the time required in seconds (Δt) was noted against M/150 phosphate buffer as blank. Δt should be less than 60 seconds. One gram plant tissue was homogenized in 10 ml M/150 phosphate buffer and 0.4 ml was taken for assay. The time required for absorbance at 240 nm to decrease from 0.45 to 0.4 was taken.

Extraction and Assay of superoxide dismutase activity

The activity of super oxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1986). One gram plant tissue was

homogenized at 4˚C in 3 mL of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15 000 g for 15 min at 4°C. Enzyme extract was added to 3 ml of reaction mixture containing, 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA and 0.2 mM riboflavin. Riboflavin was added at the end and the tubes were shaken and placed 30 cm below a light source consisting of two 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 15 min. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. The absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity was defined as the exact volume of enzyme causing half maximal inhibition (50%) of the NBT reduction under the assay condition.

Extraction and Assay of proline

Proline was determined by the method of Bates *et al*. (1973). For the determination of proline 0.5 g of plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate centrifuged at 5000 g for 15 min at 4˚ C. One ml of filtrate was reacted with one ml acid-ninhydrin and one ml of glacial acetic acid in a test tube for 1 h at 100˚ C, and the reaction was terminated by transferring to ice bath. The reaction mixture was extracted with two ml toluene, mixed vigorously with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene as blank. The proline concentration was determined from a standard curve of proline and calculated on fresh weight basis as follows:

[(µg proline/ml × ml toluene) / 115.5 µg/µmole]/ [(g sample)/5] = µmoles proline/gfw.

Extraction and Assay of total protein

The total protein was extracted using the method of Van Loon and Van Kammen (1970) in control and pathogen inoculated plants of all the three varieties viz. RMO-40, CZM-3 and FMM-96. One gram of fresh leaves of plants were homogenized in 4 ml of Tris-HCl buffer, pH 8.0. The homogenate was centrifuged at 12,000 g in a cooling centrifuge for 15 min at 4˚ C. The supernatant was used for further assay. The protein content was determined using the method of Lowry *et al*. (1951). A standard curve was plotted using bovine serum albumin (BSA) as standard. Different aliquots of BSA solution (200µg/ml) viz. 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml were taken in separate test tube and made to 1 ml with distilled water. 1 ml DDW alone was used as blank. 5 ml of freshly prepared reagent C was added, mixed well and allowed to stand for 10 min. Then 0.5 ml of 1 N Folin-Ciocalteau reagent was added to each tube, mixed well and incubated at room temperature in the dark for 30 min. The developed blue colour was read at 660 nm against water as blank. 0.1 ml of samples of different time intervals were taken and the volume was made upto 1 ml using DDW. The absorbance of the samples was recorded at 660 nm after addition of reagent C and 1 N Folin-Ciocalteau reagent. The amount of protein in different samples was calculated from the standard graph of BSA.

Characterization of total proteins by SDS-PAGE

For SDS-PAGE, method of Laemmili (1970) was followed. The glass plates, spacers and comb were cleaned with ethanol. The spacers were assembled properly between the plates using petroleum jelly and clamps to hold the spacers in place. The 10% resolving gel mixture was prepared and poured between the plates and allowed to polymerized, the stacking gel mixture was prepared and poured on top of the separating gel. The comb was placed on top of the stacking gel and allowed to polymerize. After the stacking gel polymerized, the spacer at the bottom of the plates and comb were removed. The gel was installed carefully after removing the clips and placed in the electrophoresis apparatus (midi vertical gel electrophoresis system, Genei). The apparatus was filled with the electrode buffer and connected to the electrophoresis power supply (Genei, minipack-250, PS 100). The samples were mixed with 1X loading dye in 1:1 ratio and loaded into wells using a micropipette. About 40 µg of protein was loaded per well. Medium range molecular protein marker was used for comparing the bands obtained by separation. The gel was electrophoresed at 100 volts till the tracking dye migrated to the bottom of the gel. After electrophoresis, the plates were disassembled. The gel was carefully removed from between the plates and stained with Coomassie brilliant blue stain, overnight. Destaining was done by 4-5 changes of destain with slight shaking on a table top shaker.

Statistical analysis

All the quantitative estimations of Polyphenol, Flavonoid, PAL, Total protein content, Proline content and oxidative enzymes such as, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were based on single experiment, with three replicates. Values are expressed as mean±standard error.

3. Results and Discussion

The maximum polyphenol content was obtained at 96 h after pathogen inoculation in 15 days and one month old plants of varieties viz. CZM-3, RMO-40 and FMM-96 moth bean fig. 1. However, the polyphenol content was higher in resistant var. FMM-96 when compared to susceptible var. RMO-40 and CZM-3. Significant increase in flavonoid activity in 15 days old plants was detected in both resistant var. FMM-96 and susceptible var. RMO-40 and CZM-3 cultivars of *Vigna aconitifolia*, 96 h after pathogen induction and in one month old plants, significant increase in flavonoid activity was detected in both resistant FMM-96 and susceptible RMO-40 and CZM-3 cultivars of *Vigna aconitifolia*, 72h after pathogen induction fig. 2. However, the flavonoid content was higher in resistant var. FMM- 96 when compared to susceptible var. RMO-40 and CZM-3.

The present investigation is in confirmatory with the investigation of (Poiatti *et al.,* 2009), who reported increased activity of phenolic compounds in leaves inoculated with *E. carotovora*. A similar increase occurred in the flavonoid levels, when leaves were inoculated with *X. axonopodis* (48 and 96 hpi)). This is an agreement with results reported by (Ruelas *et al.,* 2006) Changes in phenolic acid content in tomato fruits in response to pathogen attack was determined. The induction of phenolic compounds as part of the defense system against pathogen using elicitors has also been demonstrated by (Pearce *et al.,* 1998).

The PAL activity increased at 2 h after pathogen inoculation in both 15 days and one month old plants of moth bean than the corresponding control fig. 3. However, the enzyme activity was higher in resistant var. FMM- 96 when compared to susceptible var. RMO-40 and CZM-3. The activation of PAL enzyme was more rapid and to a greater extent in plants of (FMM-96) than in RMO-40 and CZM-3. The maximum enzyme activity was recorded at 2 h after the induction and thereafter a gradual decline in enzyme activity was noticed. The levels of enzyme activity were higher in resistant cultivar than in the susceptible cultivar. PAL catalyzed first reaction of phenylproponoid pathway, phenylalanine to t-cinannamic acid, which results accumulation of phenolics and other antimicrobial compounds (Slatnar *et al.,* 2010). PAL is involved in phytoalexin and phenolic compound biosynthesis. Increased PAL activity has been demonstrated in many plant-pathogen systems.

The peroxidase activity is highest in 15 days old plants at 48 h with 3.87, 3.79 and 5.35 nkat.mg^{-1} protein after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which are 3.68, 3.26 and 4.31 fold higher in inoculated plants as compared to the control plants. The decrease in peroxidase activity is 3.65 fold in var. RMO-40, 3.47 in CZM-3 and 1.85 in FMM-96 at 168 h after pathogen inoculation when compared to the maximum activity. In one month old plants, the highest peroxidase activity was obtained at 48 h with 1.20, 1.77 and 2.74 nkat.mg^{-1}protein after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which is 3.15, 3.10 and 3.60 fold higher in inoculated plants as compared to the control plants. Initially a gradual increase in peroxidise activity was observed upto 48 h, thereafter a decrease was observed upto 168 h. The decrease in peroxidase activity is 1.10 fold in var. RMO-40, 1.23 in CZM-3 and 2.51 in FMM-96 at 168 h after pathogen inoculation when compared to the maximum activity fig. 4. Similar results have been reported by Manonmani *et al*. (2009) in acid lime plants infected by canker pathogen. Increased activity of cell wall bound peroxidases has been elicited in different plants such as rice (Reimers *et al*., 1992), tomato (Mohan *et al*., 1993) and tobacco (Ahl Goy *et al*., 1992) due to pathogen infection. In bean, rhizosphere colonization of various bacteria induced the peroxidase activity (Zdor and Anderson, 1992). Chen *et al*. (2000) reported the higher PO activity in cucumber roots treated with *Pseudomonas corrugata* challenged with *Pythium aphanidermatum*. These results correlate with our finding that var. FMM-96 is more resistant than var. RMO-40, CZM-3 and is considered to play an active role in contributing to disease resistance in plant after pathogen inoculation.

Kortekamp and Zyprian (2003) observed either a higher basal level or a rapid enhancement of peroxidase activity in grapevines resistant to the fungus *Plasmopara viticola*. In cucumbers, lignification mediated by POX activity was one of the most important defense responses (Nicholson and Hammerschmidt, 1992). Similarly, De Ascensao and Dubery (2000) reported cell wall reinforcement in banana roots in response to elicitors from *Fusarium Oxysporum* f. sp. *cubense*. POX catalyzes the cell-wall crosslinking, promoting the strengthening of cell walls, and therefore acting against the pathogen attack.

This correlates the present findings where *Macrophomina phaseolina* inoculated moth bean plants having higher peroxidase activity at 48 h in all the three varieties. This might be due to the fact that the host plant when challenged with the pathogen secretes more enzymes for defense but at the steady state of infection of plant itself deters the acitivy of the pathogen resulting in the decline of anzyme activity. Varietal differences could also be observed

as the peroxidase activity is higher in resistant var. FMM-96 when compared to susceptible var. RMO-40 and CZM-3. Peroxidase activity is significantly higher in 15 days old plants compared to one month old plants.

Histochemical detection of hydrogen peroxide using 3, 3'-Diamino Benzidine (DAB)

The presence of H_2O_2 was also detected histochemically in control and pathogen inoculated leaves of moth bean plants using 3,3-diaminobenzidine. DAB polymerizes instantly and locally as soon as it comes in contact with H_2O_2 in the presence of peroxidase and by allowing the leaf to take up this substrate, *in vivo* and *in-situ* detection of H_2O_2 can be done at subcellular levels (Thordal-Christensen *et al*., 1997).

The subcellular localization of H_2O_2 can be observed in control and pathogen inoculated leaves as reddish brown patches fig. 4.1 and 4.2. Appearance of brownish colouration suggests the accumulation of H_2O_2 as DAB polymerization depends on the presence of H_2O_2 and peroxidase activity. A distinct colouration in the inoculated leaves as compared to the control leaves evidences that H_2O_2 accumulation is more in the inoculated plants compared to the control plants. DAB is taken by the living plant tissue where it polymerizes instantly and locally at sites of peroxidase activity into a reddish-brown polymer.

Orozco-Cardenas and Ryan (1999) assayed tomato plants for the production of H_2O_2 in response to wounding by DAB colouration method. H_2O_2 was detectable as early as 1 hr after wounding with the colour deepening for about 4-6 hours and then declining. The whole mounts of DAB treated control and pathogen inoculated leaves also confirmed the presence of H_2O_2 which can be observed as reddish brown patches.

Similar detection of superoxide and H_2O_2 was demonstrated by Yang *et al*. (2005) in transgenic rice plants upon infection with avirulent and virulent strains of the blast fungus. The highly localized accumulation of H_2O_2 is consistent with its direct role as an antimicrobial agent and as the cause of localized membrane damage at sites of bacterial (*Pseudomonas syringae* pv. *phaseolicola*) attachment in lettuce plants (Bestwick *et al*., 1997). Park *et al*. (2005) also demonstrated accumulation of H_2O_2 by DAB staining in Chinese cabbage leaves excised 24 hrs after infiltration with sterile water or *Pseudomonas syringae* pv. *tomato* (*Pst*). Synthesis of intercellular peroxidase might be involved in many events during the early stages of infection including hypersensitive response (HR) (Wojtaszek, 1997).

In moth bean-*M. phaseolina* plant-pathogen system also it can be inferred that the reddish brown patches obtained in control and pathogen inoculated plants are due to the localized accumulation of H_2O_2 which is actually the site of membrane damage facilitated for pathogen entry in leaves of moth bean plants. Thus, the determination of peroxide activity and detection of H_2O_2 by DAB method confirms the synthesis of H_2O_2 in our plant-pathogen system.

The catalase activity is highest in 15 days old plants at 24 h with 4.23, 3.76 and 7.78 nkat.mg^{-1}protein after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which is 1.98, 1.51 and 2.70 fold higher in inoculated plants as compared to the control plants. The catalase activity was 1.23, 1.17 and 1.70 fold higher in var. RMO-40, CZM-3 and FMM-96, respectively in pathogen inoculated plants as compared to the catalase activity at 0 h. In one month old plants the catalase activity was highest at 24 h with 4.07, 3.50 and 5.78 nkat.mg^{-1} protein after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which are 2.49, 2.17 and 3.15 fold higher in inoculated plants as compared to the control plants. The decrease in catalase activity at 168 h after pathogen inoculation is 1.20, 1.25 and 1.24 fold higher in var. in RMO-40, CZM-3 and FMM-96, respectively when compared to the maximum catalase activity at 24 h fig. 5.

Our findings are similar with the earlier studies of Blilou *et al*. (2000), where they have observed, in tobacco colonized by *Glomus mosseae*, the transient induction of CAT and ascorbate peroxidase (APX) during appressoria formation likely indicates a defense response during the early stages of symbiosis development Similarly, Baker and Orlandi (1995) reported that there was an increase in antioxidant enzymes such as APX, CAT SOD etc in tomato plants inoculated with *Meloidogyne javanica* and induction of the antioxidant enzymes and oxidative stress are quite general defense responses.

Our study indicates that the antioxidant enzyme CAT is actively involved in imparting resistance to charcoal root rot of moth bean. It was observed that upon inoculation CAT expression was higher in resistant var. FMM-96, as compared to RMO-40 and CZM-3 susceptible varieties. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to charcoal root rot of moth bean. Increased CAT enzyme activity during host-pathogen interaction is well correlated with imparting resistance to charcoal root rot of moth bean. Samia and Khalla, (2007) reported that infection by *Fusarium oxysporum* significantly increased SOD, APX and CAT activities in leaves of tomato plants at different stages of growth as

compared with non-infected control plants. Enhanced catalase activity was observed in resistant variety of *Capsicum annuum* against the pathogen *Phytophthora capsici* L. in comparison to the susceptible one as reported by Koc and Ustun (2012). These results are similar with our finding that the catalase activity was higher in var. FMM-96 as compared to RMO-40 and CZM-3, susceptible varieties. Overall, on comparing the two age groups of moth bean plants the catalase activity was higher in 15 days old plants as compared to one month old plants.

In 15 days old plants of moth bean, the maximum SOD activity at 4 h was 2.51, 1.92 and 3.49 fold higher in var. RMO-40, CZM-3 and FMM-96, respectively, as compared to control. Further, the SOD activity at 4 h was obtained 3.22, 3.26 and 7.33 µkat.mg^{-1}protein which is 1.50, 1.58 and 2.45 fold higher as compared to SOD activity obtained at 168 h for inoculated moth bean plants of var. RMO-40, CZM-3 and FMM-96, respectively. The One month old moth bean plants indicate a higher SOD activity in pathogen inoculated plants compared to control plants. The maximum SOD activity at 4 h was 4.26, 4.07 and 4.88 fold higher in var. RMO-40, CZM-3 and FMM-96, respectively, as compared to control. However, with further increase in inoculation time beyond 4 h, no further increase in the SOD activity is observed, rather a gradual decrease up to 168 hrs is observed fig. 6.

Our results are similar to the results reported by Babitha *et al.* (2002) who observed the differential induction of superoxide dismutase in downy mildew-resistant and susceptible genotypes of pearl millet (*Pennisetum glaucum*) on inoculation with *Sclerospora graminicola*. SOD activity increased by 2·3-fold in resistant seedlings upon inoculation. Similarly, Kong *et al*. (2000) has also reported induced SOD activity in roots of *Pinus massoniana* after infection with *Pislithus tinctorius*.

From this study it appears that SOD is involved in imparting resistance to moth bean against *M. phaseolina*. The precocity of necrosis apparition and its restriction in the wheat leaves of the resistant cultivars has been observed in the incompatible and compatible interaction of wheat-*Puccinia striiformis* f. sp. *tritici* host-parasite interactions (Wang *et al*., 2007). Overall, on comparing the two age groups of moth bean plants the SOD activity was higher in 15 days old plants as compared to one month old plants. In the present study of *Vigna aconitifolia-Macrophomina phaseolina* plant-pathogen system the SOD activity of control plants was significantly less than the inoculated plants. The SOD activity is higher in var. FMM-96, a resistant variety as compared to var. RMO-40 and CZM-3, susceptible varieties.

This is an agreement with results reported by Yarullina *et al*. 2005; Troshina *et al*. 2007 who reported that in soft wheat, the reactive oxygen species were produced by oxalate oxidases, which increase during the host-pathogen interactions and the restriction of *Septoria tritici* in the incompatible interactions were associated with the production of hydrogen peroxide by SOD (Shetty *et al*., 2007).

The proline content is highest in 15 days old plants at 48 h with 7.33, 9.43 and 11.9 mg.g^{-1}fw after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which is 1.76, 2.45 and 2.62 fold higher in inoculated plants as compared to the control plants. The proline content for one month old plants was highest at 48 h with 4.88, 5.55 and 9.41 mg.g^{-1}fw after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which are 1.77, 1.92 and 3.14 fold higher in inoculated plants as compared to the control plants fig. 7. From the data it is clear that the proline content is higher in the pathogen inoculated samples compared to the control plants. Varietal differences are also observed from the data, the proline content is higher in resistant var. than the susceptible var. against pathogen *M. phaseolina*. The proline content is significantly higher in 15 days plants as compared to one month old plants.

The present investigation is in confirmation with the investigation of Grote *et al.* (2006) who reported proline accumulation in *Lycopersicon esculentum* plants inoculated by *Phytophthora nicotianae*. A similar increase occurred in proline level of the Widusa cultivar after the inoculation with *Colletotrichum lindemuthianum* race 23 probably due to oxidative stress, in which the pathogen invasion provokes the formation of free radicals that are controlled by the osmoprotection mechanism. This amino acid has capacity to minimize the damaging effects provoked during biotic and abiotic stresses imposed to plant, besides it is utilized to intermediate the gene activation (Fabro *et al*., 2004; Lobato *et al*., 2008b).

The total protein content was determined for 15 days and one month old moth bean plants showed a similar trend of higher total protein content in pathogen inoculated plants compared to the control. In 15 days old plant the total protein content is highest at 48 h with 5.92, 8.28 and 14.5 mg.g^{-1}fw after pathogen inoculation for var. RMO-40, CZM-3 and FMM-96, respectively, which is 1.40, 1.92 and 2.79 fold higher in inoculated plants as compared to the control plants. The total protein content is highest in one month old plants at 48 h with 5.36, 5.74 and 12.3 mg.g^{-1}fw after pathogen inoculation for var. RMO-40, CZM-3 and

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FMM-96, respectively, which is 1.88, 2.15 and 3.30 fold higher in inoculated plants as compared to the control plants fig. 8.

Similar results of higher accumulation of two PR proteins (β-1,3-glucanase and chitinase) in infected chickpea plants against the pathogens *Fusarium udum*, *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina* were reported by Saikia *et al*. (2005). Our results are supported by Liang *et al*. (2005) who reported increase in β-1,3-glucanase activity in peanut (*Arachis hypogaea*) seed is induced by inoculation with *Aspergillus flavus*.

Ashry and Mohamed (2011) reported the total protein content in flax leaves increased significantly in resistant variety with the progress of infection. Changes in protein occur when the pathogen penetrates the host cells resulting in disturbances in protein and related metabolisms. The increase in protein content may be because of increase in PR protein after infection with pathogen (Bailey and Mansfield, 1992; Ebel, 1986). Similar results have been reported in soybean root nodules against the pathogen *Bradyrhizobium japonicum*, indicating the synthesis of PR proteins under pathogen inoculated conditions (Mohammadi and karr, 2002). The plant pathogen system of *Vigna aconitifolia*-*Macrophomina phaseolina* also correlates with the above studies where the total protein content increases after pathogen inoculation.

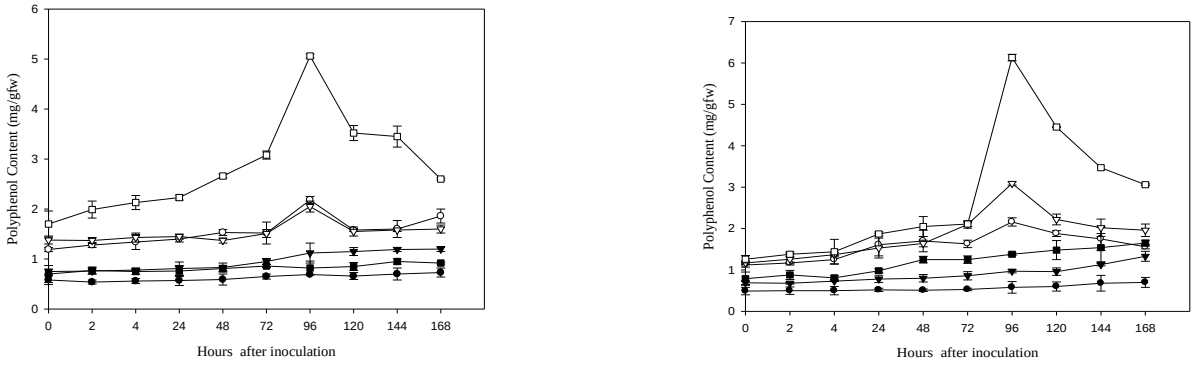
Varietal differences are also observed as the total protein content is higher in var. FMM-96, a resistant variety as compared to RMO-40 and CZM-3, susceptible varieties of moth bean. Further total protein content is significantly higher in 15 days old plants as compared to one month old plants.

Qualitative analysis of total proteins using SDS-PAGE

The total protein content of control and pathogen inoculated 15 days and one month old moth bean plants were characterized by SDS-PAGE in 10% resolving gels. A medium range protein molecular weight marker was used to determine the molecular weight of various protein bands obtained by SDS-PAGE.

The protein bands separated by SDS-PAGE were analysed using the KODAK electrophoresis documentation and analysis system (EDAS) 290. The molecular weight, area and probable identity of bands of control and pathogen inoculated plants of moth bean were determined by comparing the unknown protein bands with the standard molecular weight protein markers. The protein profile revealed the presence of a number of bands in the range of 14.3-97.4 kDa in control and inoculated samples of 15 days and one month old plants var. RMO-40, CZM-3 and FMM-96 extracted after different time intervals of pathogen inoculation i.e. 0, 48, 72, 96, 120 and 144 h fig. 8.1.

Based on above results it may be concluded that high activity of polyphenol, flavonoids and PAL, antioxidant enzyme activity and protein content of the *Vigna aconitifolia* appeared to be important biochemical constituents in imparting resistance to charcoal root rot disease.



(A) (B)

Fig. 1. Polyphenol content in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.

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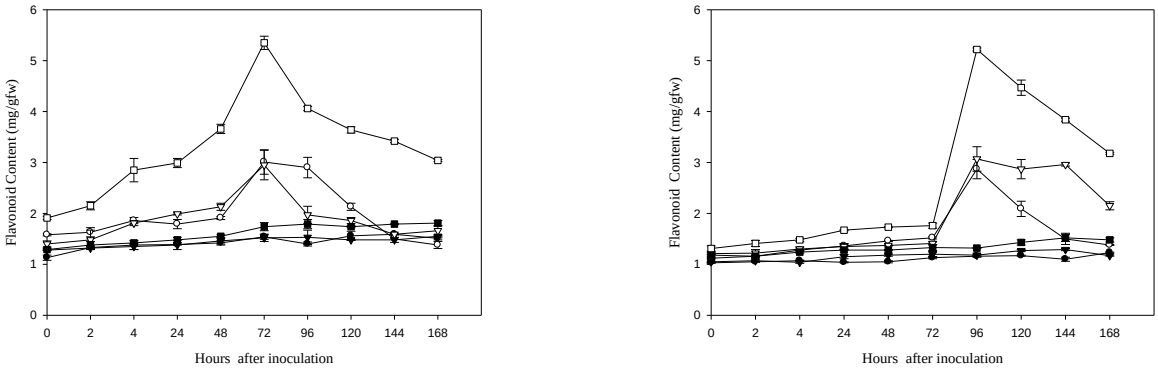
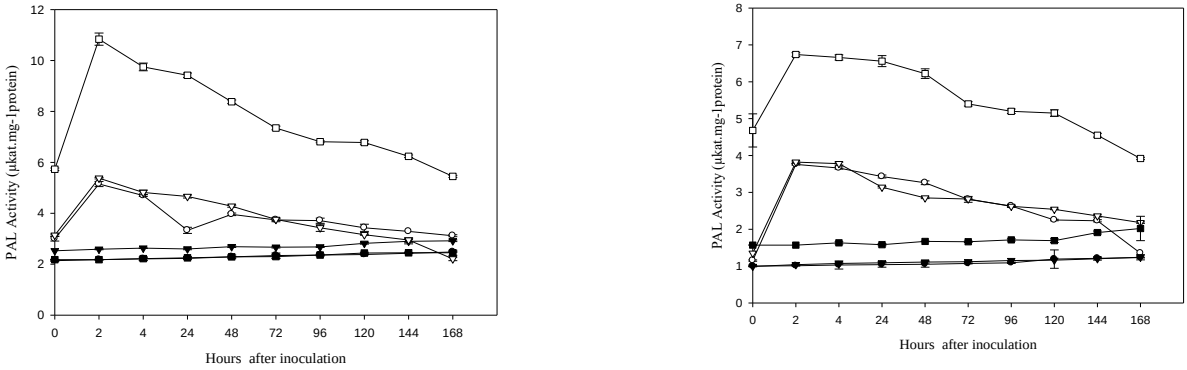


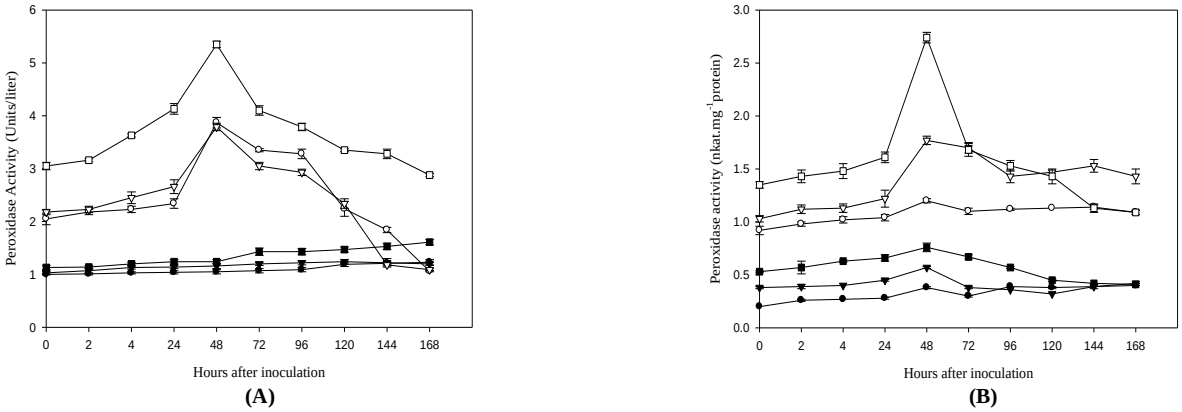
(A) (B)

Fig. 2. Flavonoid content in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.



(A) (B)

Fig. 3. PAL activity in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.



(A) **(B)**

Fig. 4. Peroxidase activity in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.

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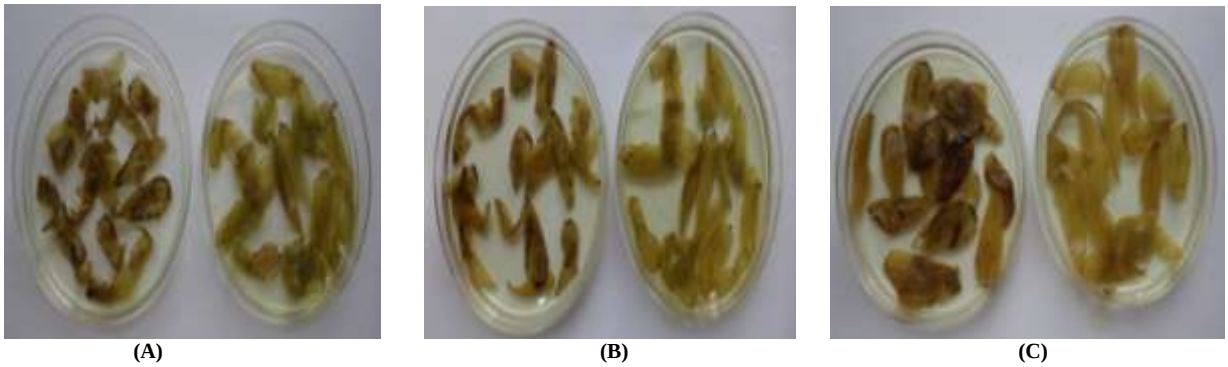

Fig. 4.1. Whole mount of pathogen inoculated leaves of moth bean (*Vigna aconitifolia*) plants showing hydrogen peroxide accumulation after DAB staining as visualized under light microscope. (A) Leaves of var. RMO-40, 48 hrs after pathogen inoculation, (B) Leaves of var. CZM-3, 48 hrs after pathogen inoculation, (C) Leaves of var. FMM-96, 48 hrs after pathogen inoculation.

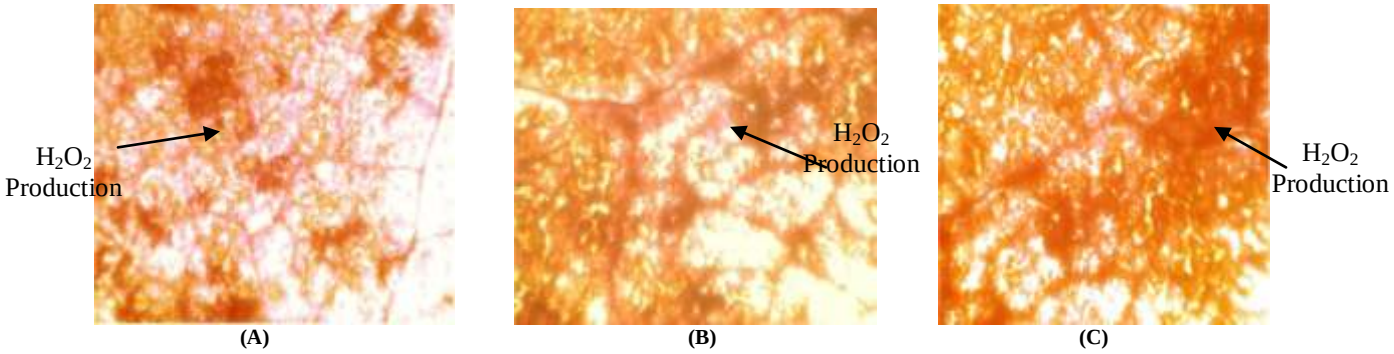

Fig. 4.2. Histochemical detection of hydrogen peroxide using DAB in control and pathogen inoculated leaves of moth bean (*Vigna aconitifolia*) plants. (A) Leaves of var. RMO-40, 48 hrs after pathogen inoculation, (B) Leaves of var. CZM-3, 48 hrs after pathogen inoculation, (C) Leaves of var. FMM-96, 48 hrs after pathogen inoculation.

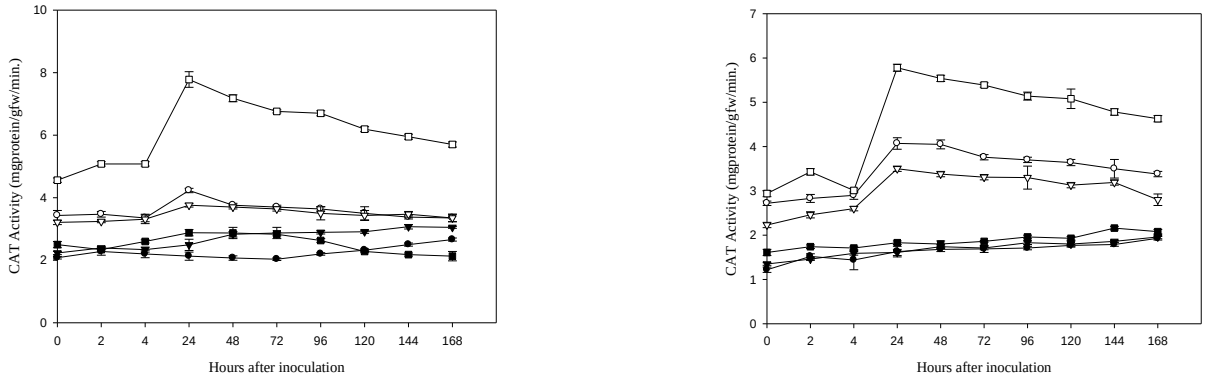

Fig. 5. Catalase activity in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.

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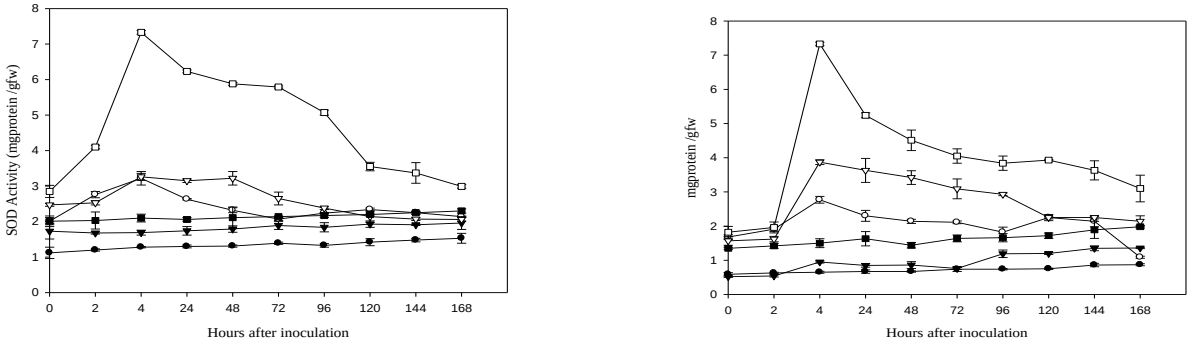

Fig. 6. Superoxide dismutase activity in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.

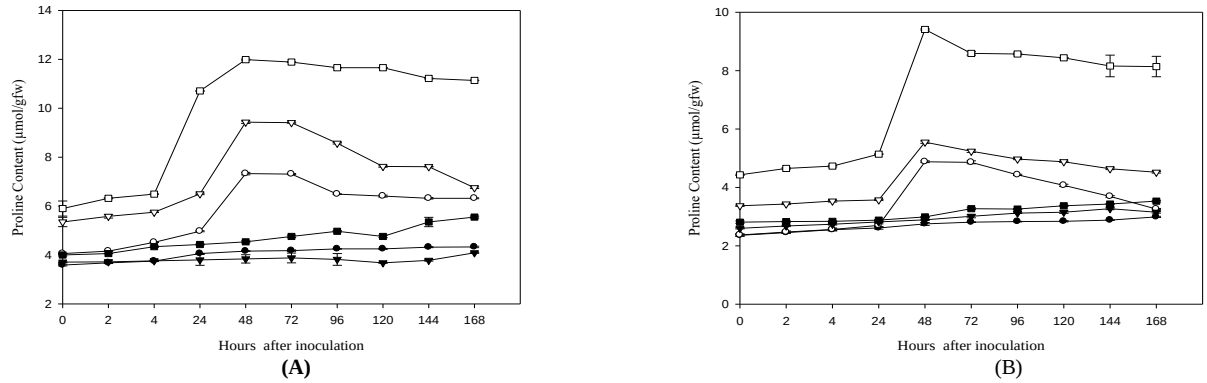

Fig. 7. Proline content activity in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○- RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ - FMM-96 control.

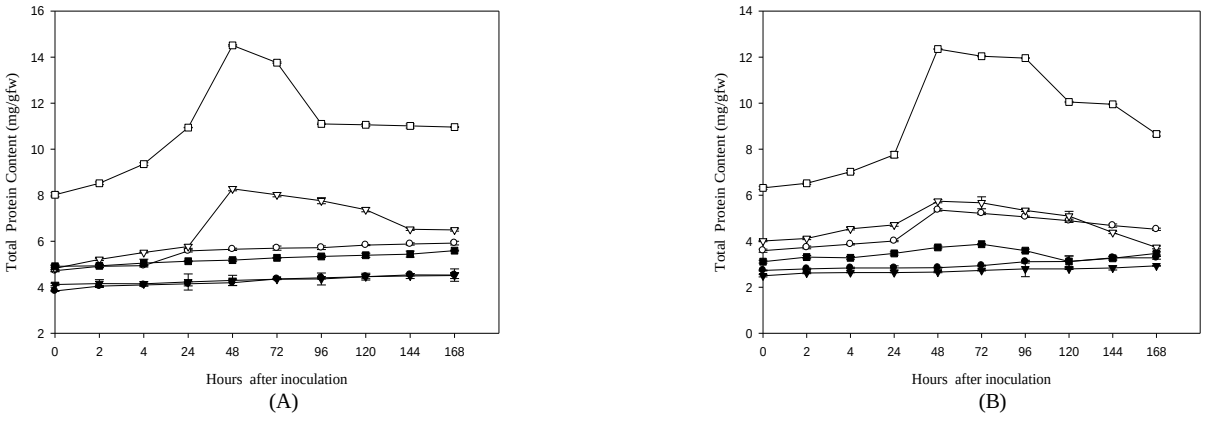

Fig. 8. Total protein content in (A) 15 days old and (B) one month old moth bean plants. -●- RMO-40 control, -○-RMO-40 inoculated, -▼- CZM-3 control, -∇- CZM-3 inoculated, -■- FMM-96 control, - □ -FMM-96 control.

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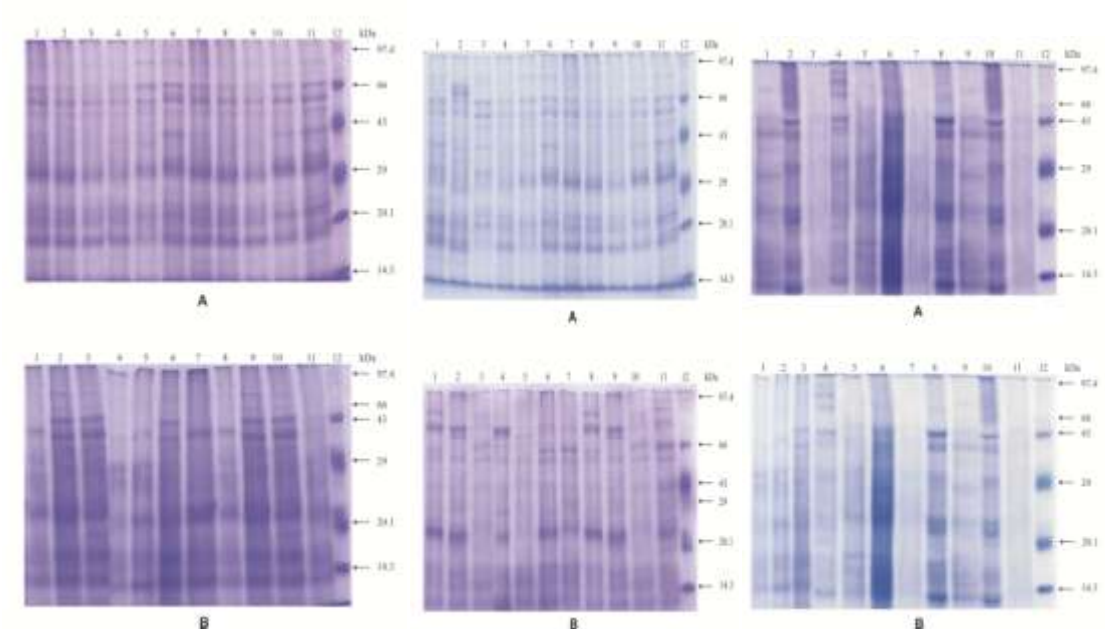

RMO-40 **CZM-3** **FMM-96**

Fig. 8.1. SDS-PAGE profile of total proteins in 10% gels stained with Coomassie brilliant blue. 40 □g protein of control and pathogen inoculated samples were loaded in 15 days (A) and one month (B) old *Vigna aconitifolia* of var. RMO-40, CZM-3 and FMM-96. Lane 1: 0 h control, Lane 2: 48 h control, Lane 3: 48 h inoculated, Lane 4: 72 h control, Lane 5: 72 h inoculated, Lane 6: 96 h control, Lane 7: 96 h inoculated, Lane 8: 120 h control, Lane 9: 120 h inoculated, Lane 10: 144 h control, Lane 11: 144 h inoculated, Lane 12: Medium range molecular weight marker.

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