academicJournals

Vol. 12(25), pp. 3968-3977, 19 June, 2013
DOI: 10.5897/AJB2013.12405
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biochemical responses during the pathogenesis of *Sclerotium rolfsii* on cowpea

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Accepted 31 May, 2013

The hypersensitive reaction (HR), one of the most efficient and visible parts of the defense mechanisms in nature against invading pathogens, is associated with a coordinated and integrated set of metabolic alterations which are instrumental in impeding further pathogen ingress or alleviating stress. It includes a variety of novel proteins and secondary metabolites. This study aimed to examine the induction of different stress related enzymes like phenyl alanineammonia lyase (PAL), chitinase, β-1,3 glucanase, oxidative enzymes like peroxidases (POD), poly phenol oxidases (PPO) and phenolics after inoculation of *Sclerotium rolfsii* in collar region of 30 days old cowpea plant. Scanning electron microscopy strengthened the presence of mycelial network in xylem vessel of infected collar region of cowpea at three days after inoculation. Cowpea plants inoculated with *S. rolfsii* isolate showed significantly increased POD, PPO, PAL, chitinase and β-1, 3-glucanase activities at different days after inoculation. In the present study, there was a greater accumulation of total phenol in cowpea plants observed up to five days after inoculation. The highest activity of POD, SOD was found in three days after inoculation and PPO activity was greater in five days after inoculation and thereafter, the activities of such enzymes steadily decreased. It was due to susceptible interaction of *S. rolfsii* ingression in cowpea. The correlation study between disease progression and changes in activity of different defense related enzymes showed that POD and chitinase were significantly associated with susceptible host pathogenic interaction in cowpea against *S. rolfsii*.

Key words: Cowpea, *Sclerotium rolfsii*, defense-related enzymes, phenolics, pathogenesis related proteins, scanning electron microscopy.

INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp.) is a food legume of significant economic importance worldwide. It is grown in North and South America, Africa, Europe, and Asia, primarily in the semi-arid and humid tropical regions lying between 35 °N and 30 °S of the equator. It is estimated that cowpea is now cultivated on at least 12.5 million hectares with an annual production of over 3 million tonnes of grains worldwide (Singh et al., 1997). In India, cowpea is grown on about 0.5 million ha with an average productivity of 600 to 750 kg grains/ha. Like most crop plants, cowpea production is limited by numerous biotic and abiotic factors.

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Abbreviations: HR, Hypersensitive reaction; **PAL,** phenyl alanineammonia lyase; **PPO,** poly phenol oxidases; **SEM,** scanning electron microscopy.

Collar and stem rot of cowpea has been reported in many countries (Sing et al., 1997; Aveling and Adandonon, 2000). *Sclerotium rolfsii* was considered to be the main causal agent of cowpea collar and stem rot due to its wide distribution, high incidence and predominance on plants with collar and stem rot symptoms. Stress alleviation or disease control remains one of the most challenging issues to be addressed, which is especially true for cowpea considering the largely undefined area of cowpea self-defense mechanisms. The hypersensitive reaction (HR), one of the most efficient and visible parts of the defense mechanisms in nature against invading pathogens, is associated with a coordinated and integrated set of metabolic alterations which are instrumental in impeding further pathogen ingress or alleviating stress. It includes a variety of novel proteins and secondary metabolites. Active research on various aspects of defense/stress response in various crops has resulted in the identification of a variety of response proteins and antifungal secondary metabolites induced in response to pathogen attack. In this context we aimed a pioneer study in cowpea regarding changes of several defense related enzymes and phenolics upon *S. rolfsii* attack. We have also strengthened the infection progress through scanning electron microscopy and correlated the enzyme activity with symptom progression in the collar region of cowpea. These studies will provide novel insight into these responses and mark a major advance in our understanding on the cowpea self-defense mechanisms against *S. rolfsii*, which ultimately will throw a focus on development of sustainable disease management strategy on long term basis.

MATERIALS AND METHODS

Inoculation of cowpea cv. *Kashi-kanchan*

A pure culture of *S. rolfsii* was obtained by isolation from the infected collar region of cowpea. The pathogen was grown on potato dextrose broth (PDB-Hi Media) medium in a conical flask, and four-day-old fungal mat (0.2 g) was inoculated at the collar portion of 30-day-old cowpea variety Kashi-kanchan grown in pots in the greenhouse by the fungal mycelium inoculation method. The inoculated portion was covered with moist absorbent cotton and held tightly by a rubber band. Un-inoculated collar portions of similar age were used as control. The inoculated and un-inoculated plants were maintained in temperature, light and humidity-controlled growth chamber (Adaptis A1000) at 28°C along with 95% RH under controlled growth chamber condition with 12/12 L/D cycle. The lession length (cm) was recorded at different days after inoculation (DAI).

Scanning electron microscopy

The samples for scanning electron microscopy (SEM) were collected from both healthy and infected collar region at different hours after inoculation in cowpea plant. The collar portions were transversely sectioned in12 to15 µm thick on a sliding microtome. The sections were fixed for 2 h in 25% (v/v) glutaraldehyde solution and then dehydrated in different strength of ethyl alcohol solutions (20%-absolute) for 10 min in each. The sections were taken on SEM stubs and gold ionization was done through IB-2 Ion coater prior to scanning electron microscopy (Hitache-S530). Photographs were captured at different magnifications shown in the Figure 1.

Preparation of enzyme extract:

0.5 g of diseased and healthy plant tissue was crushed separately with 1 ml of sodium-phosphate buffer (pH-7) for peroxidase (POD), superoxide dismutase (SOD), polyphenol oxidase (PPO); sodium-acetate buffer (pH-5) for β-1,3-glucanase; Borate buffer (pH-8.7) for phenylammonia lyase (PAL); McIlvaine buffer (pH-4) for chitinase and 80% ethyl alcohol or phenolics in a pre-chilled mortar and pestle at 4°C. The homogenate was centrifuged (Biofuge stratos) at 12,500 rpm for 20 min at 4°C. Supernatant was used as enzyme source.

Spectrophotometric assay of enzyme:

Peroxidase (POD) (E.C. 1.11.1.7) assay

Orthodinisidin and hydrogen peroxide were used as substrate for enzymatic reaction and Spectrophotometric reading was taken at 430 nm following the method of Malik and Singh (1980). Enzyme activity was expressed as changes in absorbance min^{-1}g of fresh wt^{-1}. Each experiment was done with three replications.

Superoxidedismutase (SOD) (E.C. 1.15.1.1) assay

Potassium-phosphate buffer (pH-7.8), 100 mM methionine, 10 mM riboflavin, 5 mM EDTA, and 750 µM nitroblue tetrazolium were used as substrate for enzymatic reaction of SOD according to the modified method described by Madamanchi et al. (1994). One unit of SOD activity was defined as the enzyme which caused 50% inhibition of initial read of reaction in absence of enzyme. Enzyme activity was defined as SOD activity unit min^{-1}g. of fresh wt^{-1}. Each experiment was done with three replications.

Polyphenol oxidase (PPO) (E.C. 1.14.18.1) assay

Spectrophotometric assay was done at 265 nm according to the method of Esterbaner et al. (1977) and Hammerschmidt et al. (1982). Enzyme activity was expressed as changes in absorbance min^{-1}g. of fresh wt^{-1}. Each experiment was done with three replications.

Phenyl ammonia lyase (PAL) (E.C. 4.3.1.24) assay

Spectrophotometric assay was done at 290 nm according to the method of Dickerson et al. (1984). Enzyme activity was defined as µg trans-cinnamic acid produced min^{-1}g of fresh $wt.^{-1}$. Each experiment was done with three replications.

Chitinase (E.C.3.2.1.14) assay

Colloidal chitin was used as substrate for enzymatic assay and prepared according to the method of Sun et al. (2006). Chitinase activity was estimated by the method of Chen and Lee (1995). The amount of released N-acetyl glucosamine (NAG) was determined from a calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of NAG min^{-1}g. fresh wt^{-1}. Each experiment was done with three replications.

Table 1. Progression of lesion length and expression of disease after different days of inoculation.

Days after inoculation (DAI)	Average lesion length (cm)	Disease symptom(s)
1	0.00	-
3	1.95	Progress of brown discolouration (1.95 cm) and tissue degradation initiated.
5	3.20	Progression of infected areas (3.20 cm) along with tissue degradation, drooping of leaves.
7	4.60	Progression of infected areas (4.60 cm), tissue degradation and shrinkage of collar region, drooping of leaves and initiation of yellow discolouration of leaves.
10	5.30	Drooping, yellowing and wilting of the plant.

β- 1, 3 Glucanase (EC 3.2.1.39) assay

Spectrophotometric assay of β-1, 3-glucanase was determined by the procedure described by Noronha et al. (2000). During the enzymatic assay, production of reducing sugar was determined following the method of Miller (1959). One unit of β -1,3-glucanase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar min^{-1}g. of fresh wt^{-1}. Each experiment was done with three replications.

Assay of phenol

Assay of phenol was done according to Malik and Singh (1980) and expressed as mg. g. fresh $tissue^{-1}$. Each experiment was done with three replications.

Native polyacrylamide gel electrophoresis (Native PAGE) and staining of different iso-enzymes

Peroxidase (POD) (E.C. 1.11.1.7)

Electrophoresis of peroxidase was done in 10% polyacrylamide gel. Staining of peroxidase was done according to method of by Welter and Dyck (1983). Gel was stained with 1% H_2O_2 and orthodinisidin (dissolved in acetic acid).

Superoxidedismutase (SOD) (E.C. 1.15.1.1:)

Electrophoresis of SOD was done in 11% polyacrylamide gel. Staining was done by 1.23 mM NBT, and 28 mM TEMED in potassium-phosphate buffer (pH 7) (Madamanchi et al., 1994).

Statistical analysis

The data collected during these investigations were analyzed in the Duncan multiple range test (DMRT) (p = 0.05) of the Univariate ANOVA model in SPSS statistical tool 10.0. A correlation study was done with lesion length and activities of different enzymes at different DAI using one tailed simple correlation menu of SPSS Statistical Tool 10.0.

RESULTS

The data presented in Table 1 indicate that the visible symptom appeared from three days onward and initial symptom appeared at 36 h after inoculation. After three days of inoculation, the progression of the symptoms was associated with brownish discolouration, tissue degradation and shrinkage of the collar region. The drooping of the leaves was observed from five days onwards and the drooping of the leaves along with yellowing and wilting was observed from seven days onward after inoculation at 28°C along with 95% RH under controlled growth chamber condition with 12/12 L/D cycle.

Scanning electron microscopy strengthened the presence of mycelial network in xylem vessel of infected collar region of cowpea at three days after inoculation. Though initial infection hampers the cortical tissues, the main machinery of solute transport was occupied by the pathogens at three days after infection (Figure 1a to d).

POD activity

The present study reveals that the induction in POD activity was significantly up-regulated from one day onwards after inoculation and reached at its peak at 3 DAI (54.50 EU) when 2.44 fold increase in enzymatic activity was noticed (Figure 2a). A sudden decrease in POD activity was noticed from 5 DAI and maximum reduction in its activity was observed at 10 DAI. POD activity in infected cowpea collar region was closed to that of the control plant of 30 days old even at 10 DAI.

SOD activity

An early and significantly increased SOD activity was evidenced after *S. rolfsii* inoculation in cowpea plant. Though the induction of SOD activity was noticed form 1 DAI but the highest increase in SOD activity was revealed in 3 DAI. A 2.67 and 2.36 folds increase in SOD activity was observed at 3 and 5 DAI, respectively. However, a progressive and rapid decrease in SOD

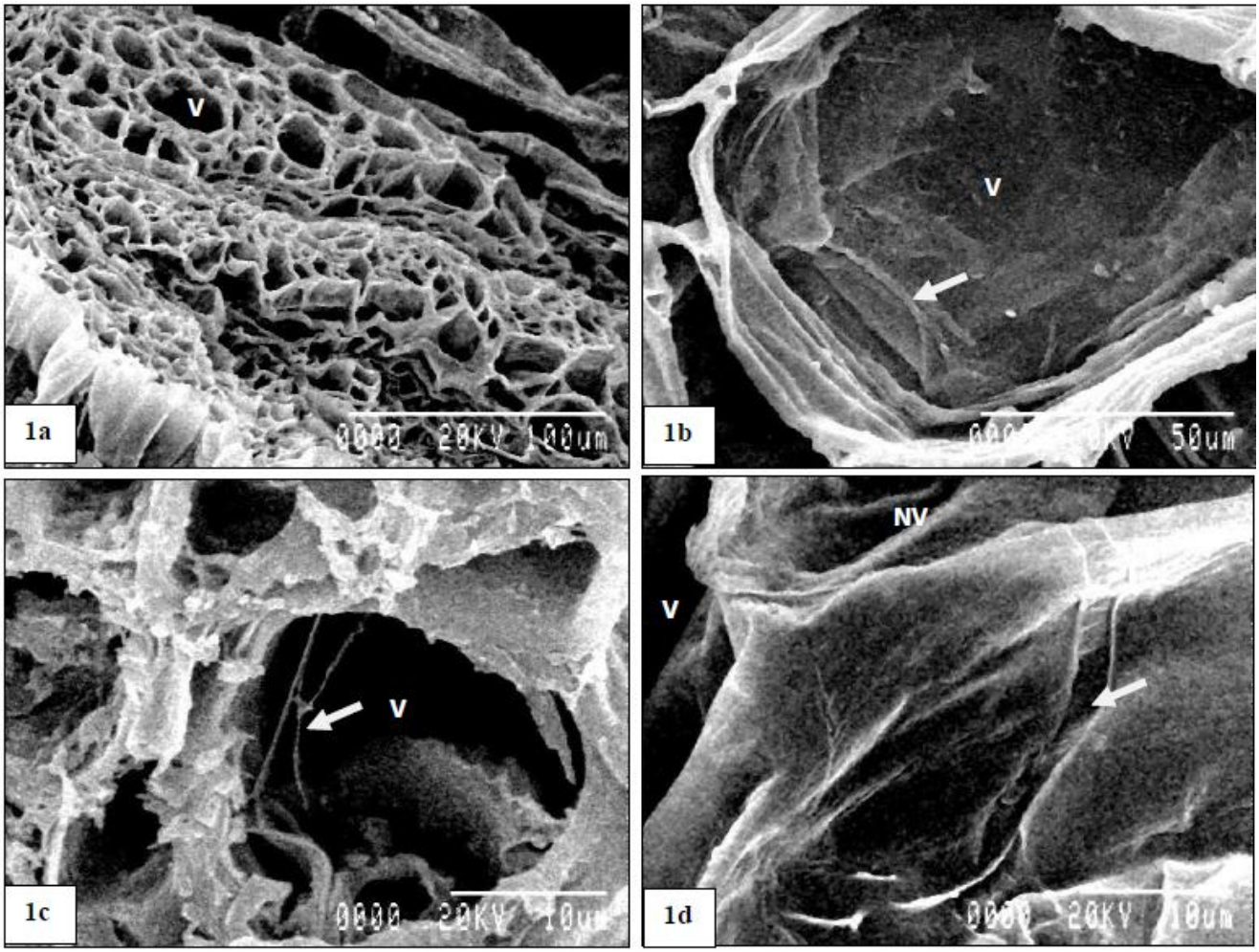


Figure 1. SEM image of location of *Sclerotium rolfsii* in infected stems, and features of cellular responses (Cross sectional view). **(a)** Healthy stem anatomy showing the compactness of xylem vessels (V) 500X. **(b)** Xylem vessels (V) occupied by *S. rolfsii* mycelia (White arrow) at 3 DAI. 1000X. **(c)** Xylem vessels (V) occupied by *S. rolfsii* mycelia (White arrow) at 5 DAI. 3000X. **(d)** *S. rolfsii* mycelia (White arrow) is passing from one xylem vessel to neighboring vessel (NV) at 7 DAI. 3000X. Note: Vessel cell wall degradation is associated with infection progress from 3 to 7 DAI (b-d). DAI, Days after inoculation.

activity was noteworthy in 7 and 10 DAI of *S. rolfsii* (Figure 2b).

PPO activity

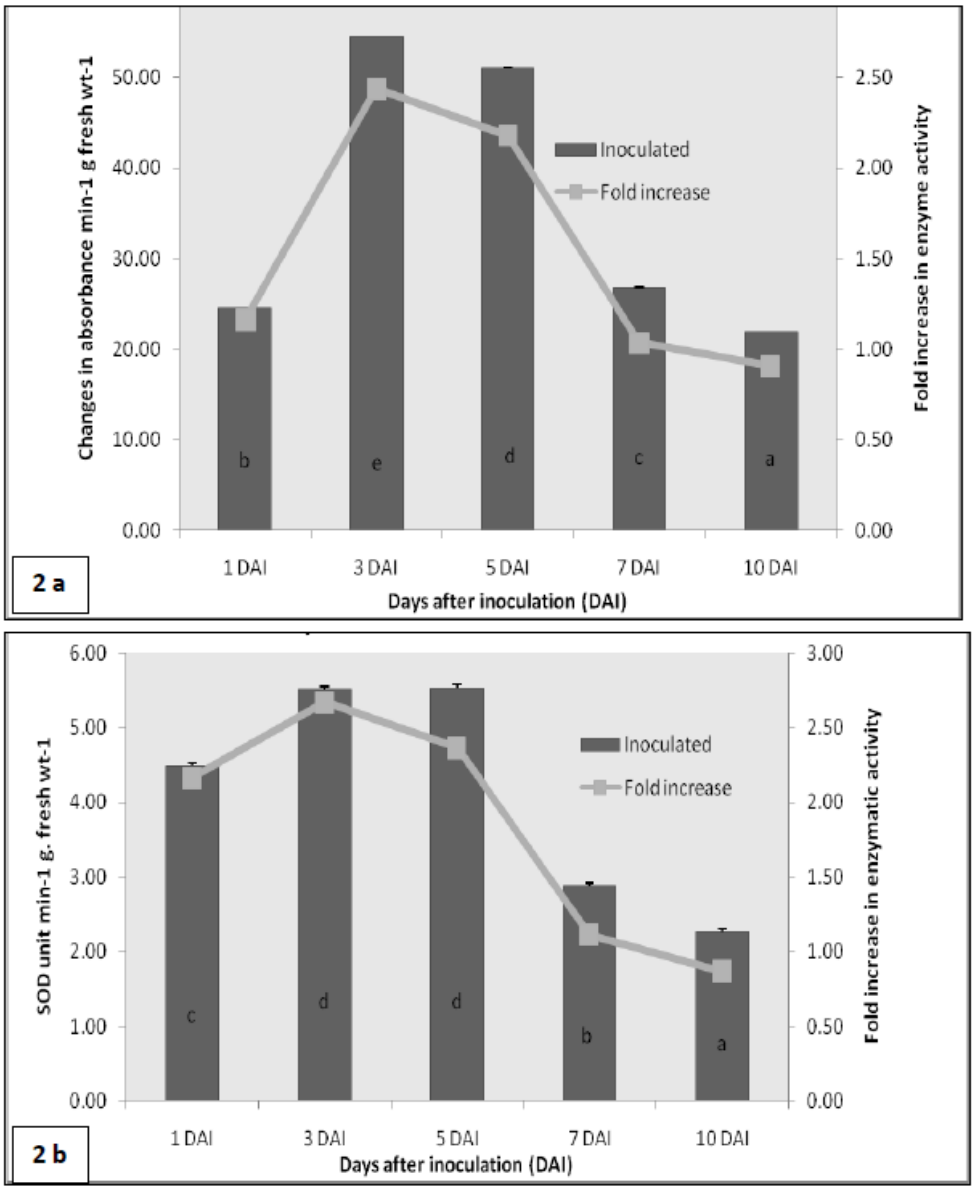
Cowpea *S. rolfsii* interaction system revealed that the PPO activity increased from 1 DAI and reached its peak at 5 DAI. Though the PPO activity was highest in 5 DAI, the enhancement of activity in 3 DAI was highest over the control plant of same age (fold increase). A sudden and rapid decrease in activity of this enzyme started after 5 DAI and continued up to 10 DAI. Enhancement of PPO activity by 3.90 and 3.20 folds was noticed in *S. rolfsii* inoculated cowpea at 3 and 5 DAI, respectively as compared to water treated un-inoculated control plant of respective ages (Figure 2c).

PAL activity

S. rolfsii inoculated plants showed induction in PAL activity from 1 DAI and the activity after inoculation reached its peak at 5 DAI compared to the control (Figure 2d). PAL activity was higher by 2.69 fold in *S. rolfsii* inoculated susceptible tissue compared to water treated control plant at five days after inoculation (Figure 2d). Decline in PAL activity was observed after five days of inoculation and continued till 10 DAI.

Chitinase activity

Steady increase in chitinase activity was observed from one day after inoculation and chitinase activity reached its peak at 3 DAI. The activity was enhanced by 4.37 fold



Figures 2. Changes in defense related enzymes and phenolics at different days after inoculation (DAI) of *S. rolfsii* in cowpea collar region. A common letter (on the dark shed column) means they are not significantly different (p= 0.05) by DMRT. Vertical bar shows the Critical difference (CD) value calculated on three replications (p = 0.05). The Fold increase of enzymatic activity calculated by dividing the activity in inoculated plant with the same of control plant in each DAI. **(a)** POD activity. **(b)** SOD activity. **(c)** PPO activity. **(d)** PAL activity. **(e)** Chitinase activity. **(f)** β-1, 3-glucanase activity. **(g)** Accumulation phenolics.

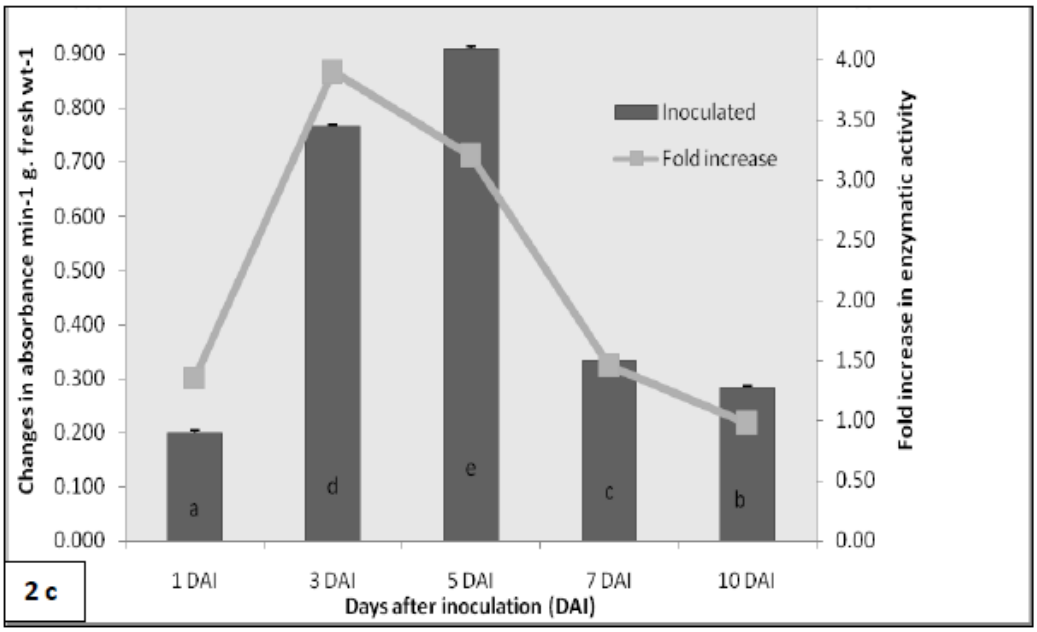
at 3 DAI when compared with the water treated control (Figure 2e). Though the activity steadily declined after 3 DAI, a high level of its activity was maintained upto the end of the experimental period.

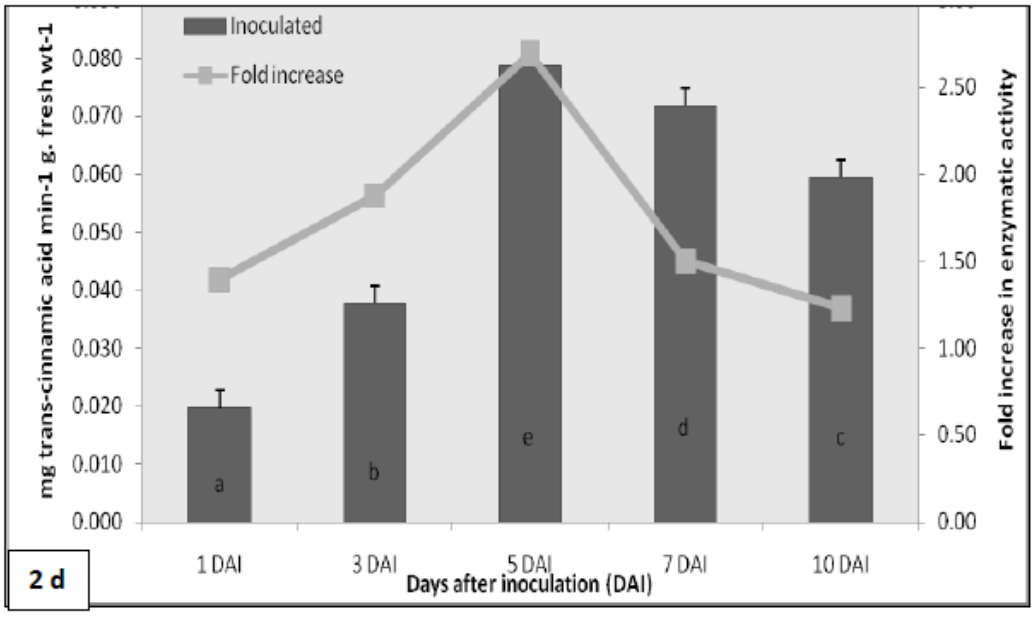
β 1-3 Glucanase activity

β 1-3 Glucanase activity reached at its peak at five days after inoculation and thereafter there was significant fast decline in its activity in the susceptible host-pathogenic interaction. There was an increase of 5.07 folds in β 1-3 glucanase activity at 5 DAI in the infected cowpea plant compared with the water treated control (Figure 2f).

Phenol accumulation

A steady increase in phenolics accumulation was observed up to 5 DAI and maximum accumulation took





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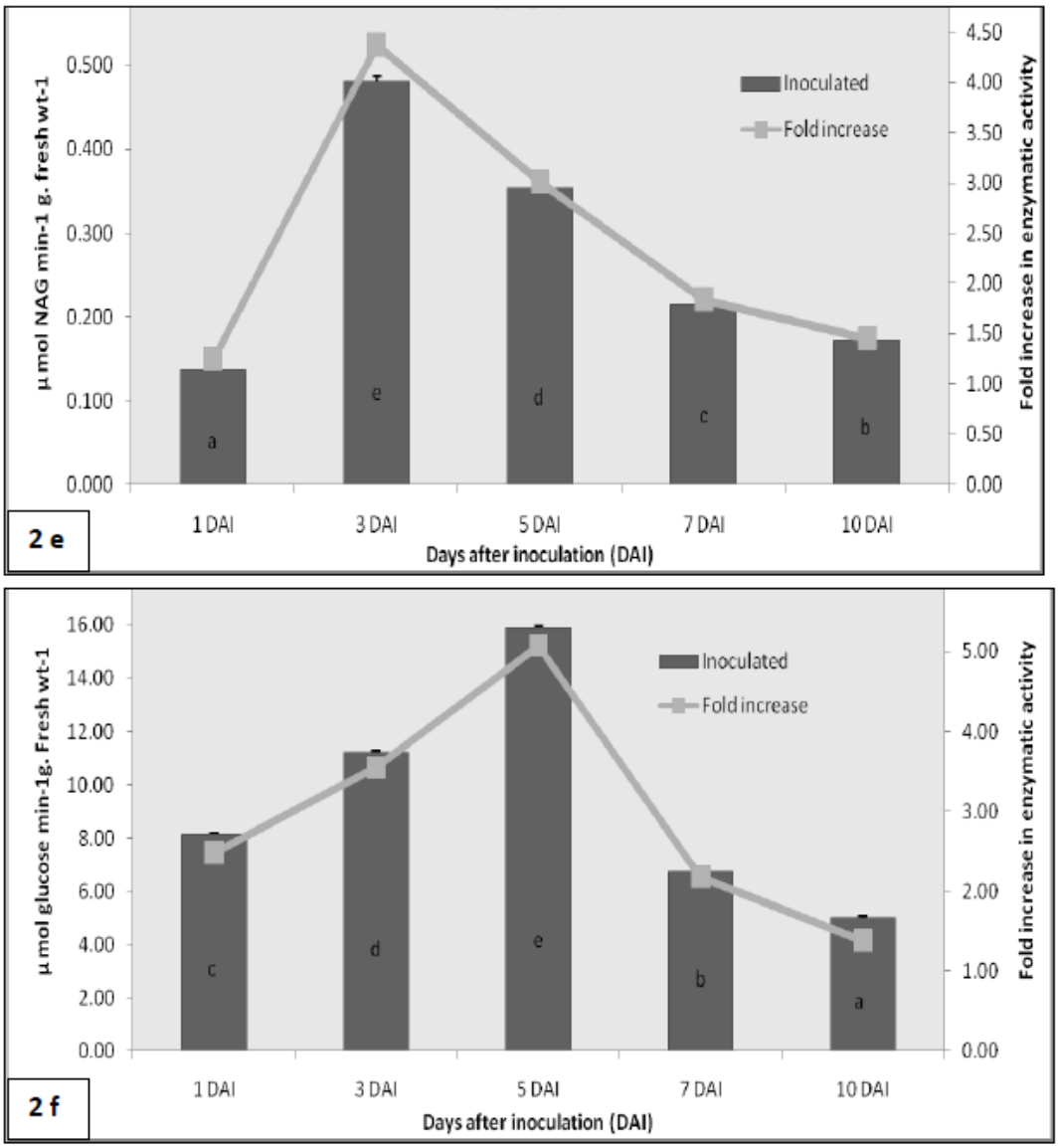
place in five days after *S. rolfsii* inoculation (2.434 mg phenol g. fresh wt^{-1}). Though the decline in phenol accumulation was noteworthy after 5 DAI, a significant higher amount of phenol was maintained even at 10 DAI(1.09 fold increased phenol content) (Figure 2g).

Electrophoresis of isozymes

POD isozyme analysis indicated that, five isoforms designated as POD1 to POD5 could be identified in infected cowpea collar region up to 10 days of inoculation. While induction of isomers POD- 5 (~20 kD., mf- 0.69) which was observed in 3 and 5 DAI, they disappeared at 7 DAI onwards in the infected collar region suggesting that this isomer may be associated with only the susceptible cowpea host- *S. rolfsii* interaction (Figure 3a).

SOD isozyme analysis recorded the appearance of three iso-forms designated as SOD1 to SOD-3 up to 10 days of inoculation. The isomers SOD- 2 (~66 kD, mf- 0 .46) was observed at three and five days after inoculation, there-



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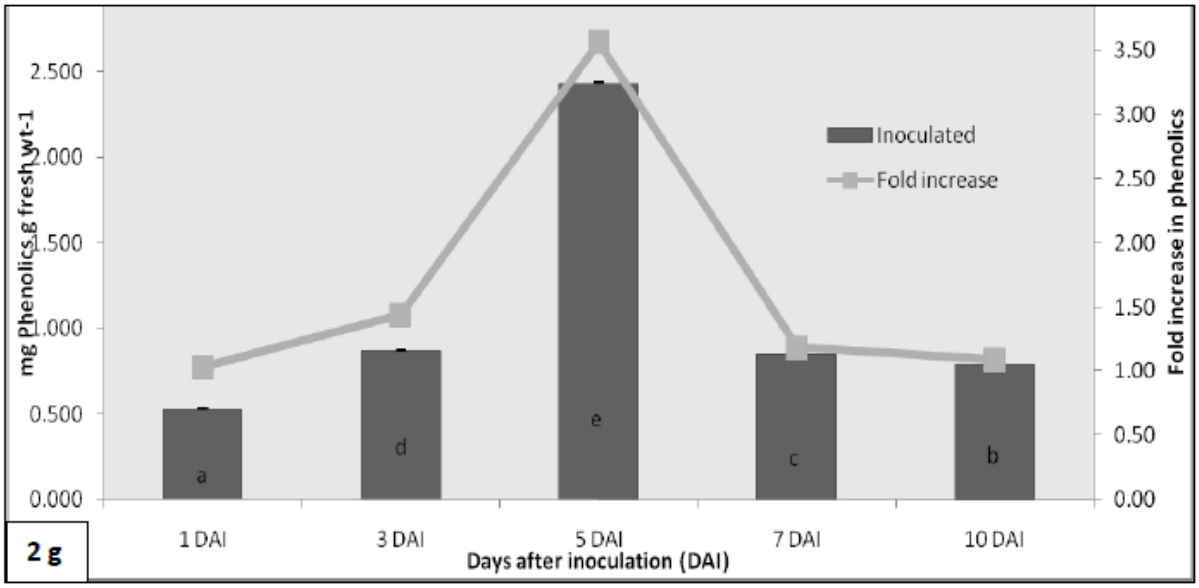
after this isomer disappeared (Figure 3b).

Correlation study

The correlation study between disease progression and changes in activity of different defense related enzymes showed that the chitinase and peroxidase activity significantly influenced the susceptible host-pathogenic interaction in cowpea-*S. rolfsii* system at 1 and 5% level, respectively (Table 2).

DISCUSSION

Plants have developed a highly sophisticated antioxidative defense system to cope with many biotic and abiotic stresses (Heidari, 2009). Utilization of plant's own defense mechanism against stress is an attractive strategy that enables the plants to thrive well in hostile environment. Plant products play an important role as antifungal, antibacterial and antiviral agents. The products of activated defense genes which are synthesized *de novo* during periods of pathogenic stress has been well documented in several host-pathogen interactions but such information is really lacking in cowpea-*S. rolfsii* system. The rapid elicitation of plant's defense responses mandate that successful pathogenic fungi must have evolved strategies to suppress and/or avoid the responses of potential hosts (Jackson and Taylor, 1996). The necrotropic pathogens normally launch the plant into cell-death program, thereby short



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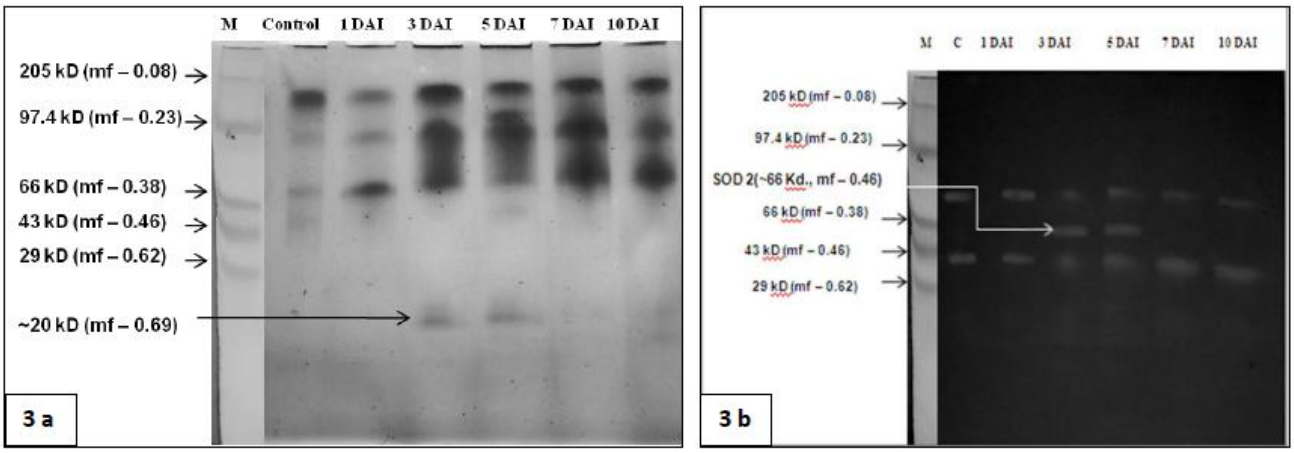

Figure 3. Native PAGE of isozymes. M= Size marker (Broad range-Genei); C= control/healthy collar tissue region of cowpea; 1-10 DAI= infected collar regions of 1-10 days after inoculation respectively. (a) POD (black arrow: POD 5). (b) SOD (white arrow: SOD 2).

Table 2. Significance level of changes in different enzymes activity and phenol content with disease progression in cowpea - *S. rolfsii* host pathogenic interaction.

Parameter	LL[#] (cm)	PO activity	PPO activity	SOD activity	PAL activity	Glucanase activity	Chitinase activity	Phenolic amount
LL[#] (cm)	**1**	p=0.038*	p=0.150	p=0.065	p=0.532	p=0.271	p=0.003**	p=0.718

[#]Lesion length,*Significant at 5 % level of probability; **Significant at 1 % level of probability; p = probability value (in parentheses).

circuiting a strong defense response and providing a ready source of pathogen nourishment (Swords et al., 1999). Several reactive oxygen species are produced during infection by pathogen. These active oxygen species species have been shown to be associated with the hypersensitive response in plants (Grant et al., 2000). A strong but positive correlation has been found between oxidative state of the host plant and resistance against different stress (War et al., 2011). This oxidative state of the host plant is mediated through the production of ROS and their subsequent elimination by antioxidative enzymes. It was reported that POD, PPO, PAL and phenol

are involved in the cowpea-*R. solani* and Tea-*S. rolfsii* interaction system respectively (Bhagat and Chakraborty, 2010).

Peroxidase has been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxyl cinnamyl alcohols into free radical intermediates, which subsequently are coupled to lignin polymer. Furthermore, peroxidase is involved in the production or modulation of active oxygen species which may play various roles directly or indirectly in reducing pathogen viability and spread (Passardi et al., 2005). Earlier studies suggest that peroxidases are important PR proteins and the plant expresses POD activity during host-pathogen interaction (Saikia et al., 2004). In our study, we observed that POD activity reached at its peak at three days after inoculation and maximum reduction of its activity was observed 10 days after inoculation. POD activity and isozymes studies indicate that the suppression of POD after 5 DAI leads to the weakening of defense mechanisms in *S. rolfsii*-inoculated cowpea plants. This helps in the further spread of the pathogen and eventually severe collar rot symptoms are expressed. Our results suggest that suppression of peroxidase was found to be one of the important factor responsible for the successful pathogenesis in cowpea *S. rolfsii* system.

One new isomer POD-5 (~20 kD, mf = 0.69) was induced in the inoculated cowpea at 3 and 5 DAI and latter disappeared at 7 DAI onward. Thus ~20 kD (mf = 0.69) POD isomer may be associated with the susceptible host-pathogenic interaction in cowpea-*S. rolfsii* system.

The SOD activity increased steadily up to five days after *S. rolfsii* infection and thereafter progressively decreased SOD activity was observed upto 10 DAI. The enhanced activities of SOD upto 5 DAI may have helped in scavenging of the reactive oxygen derivatives which in turn may have led to susceptibility of cowpea to *S. rolfsii* infection. Increases in the activities of superoxide dismutase in plants have been correlated with increased susceptibility to pathogens (Durner and Klessig, 1995). It was reported that the levels of SOD in bean leaves was enhanced during the establishment of the symptoms in the susceptible cultivars following infection with *Uromyces phaseoli* (Buonaurio et al., 1987). Induction of one new isomer SOD- 2 (mf- 0.46, ~66 kD) was observed at three and five days after inoculation. Hence, SOD 2 isomer is very important in the case of pathogen and susceptible host interaction.

The mitochondrial electron transport system (ETS) is the major site of ROS production in non-photosynthesizing plant cells. Depending on the mitochondrial respiratory states, a small portion of the consumable oxygen is partially reduced to generate ROS (Smith et al., 2004). The monoelectronic reduction of oxygen by ETS leads to the production of superoxide radicals ($O_2^{\cdot-}$) that can be dismutated by SOD, producing hydrogen peroxide (H_2O_2), and further decomposed by catalase and/or ascorbate-glutathione peroxidase cycles (Møller, 2001). An imbalance between the ROS production and antioxidant defenses can lead to an oxidative stress condition (Camacho-Pereira et al., 2009). In this present work, it is noteworthy that, after the fungal infection, the activity of SOD and POD were highest up to 5 DAI. The gradual increase of superoxide radicals and hydrogen peroxide is clear evidence of a higher respiration rate. However, on the 5th day of treatment, the increase in SOD activity was higher than that of POD. Due to this imbalance, an oxidative stress condition appeared where the concentration of hydrogen peroxide increased. The undesirable accumulation of ROS causes oxidative damage of mitochondrial proteins and leads to the collapse of mitochondrial membrane potential (Qin et al., 2011) which leads to plant cell apoptosis and symptom expression.

PPO catalyzes the last step in the biosynthesis of lignin and other oxidative phenols. PPO activity reached its peak at five days after inoculation and afterwards a steady decrease was recorded up to 10 DAI. This finding has close similarity with rice-*Rhizoctonia* interacting system (Mondal et al., 2012). Hence these results indicate that the oxidative enzyme converts phenolic compounds of plants to polyphenols and quinones which are toxic substances to the extracellular enzymes produced by the pathogens. Moreover, POD and PPO play a central role triggering the hypersensitive reaction (HR) in crosslinking and lignifications of the cell wall and in transducing signals to adjacent non-challenged cells. Strengthening of cell wall barrier synthesis involves the influence of POD and PPO enzymes which may restrict further entry by the pathogen (Bruce and West 1989).

PAL is an enzyme of the general phenyl propanoid metabolism and controls a key branch point in the biosynthetic pathways of flavonoid phytoalexins which are antimicrobial compound. In this present study, the highest PAL activity was found at 5 DAI and though, thereafter a slow decrease in PAL activity was noteworthy but it maintained higher activity up to 10 DAI also. Reduction of phenyl propanoid levels by co-suppression of PAL increases disease susceptibility (Maher et al., 1994). Significant decrease in PAL activity level after 5 DAI suggests the role of this enzyme in susceptible host pathogenic interaction.

The pathogenesis related protein (PRP) -2 family consists of β-1,3-glucanases, which are able to hydrolyze β-1,3-glucans, a biopolymer found in fungal cell walls. The PRP-3, 4, 8 and 11 families consist of chitinases belonging to various chitinase classes (I to VII). The substrate of chitinases, chitin, is also a major structural component of fungal cell walls. Chitinases hydrolyze the β-1,4-linkage between N-acetylglucosamine (NAG) residues of chitin. In the present study, enhanced activity of chitinase and β 1-3 glucanase was observed in cowpea plant infected with *S. rolfsii* up to 3 and 5 DAI. Similarly, a

correlation in between the systemic induction of chitinase and β -1,3-glucanase and resistance in bean plants by binucleate *Rhizoctonia* species was demonstrated (Xue et al., 1998).

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. The hyphae of the pathogen surrounded by phenolics substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. The present study shows higher accumulation of phenolics in cowpea collar region up to 5 DAI and thereafter a rapid degradation. In many instances, phenols serve as plant defense mechanisms against predation by insects, herbivores and microorganisms (Beckman, 2000). Phenolics that occur constitutively and function as preformed inhibitors are generally referred to as phytoanticipins, and those that are produced in response to infection by the pathogen are called phytoalexins and constitute an active defense response.

A coordinated defense response system is activated in plants and defense related proteins or pathogenesis related proteins (PR proteins) are produced in the host during the host-pathogen interaction. Biochemical analyses of various disease-responsive components have thrown light on the pivotal role of different enzymes and phenolics in cowpea -*S. rolfsii* interaction. The suppressed activity of defense arsenals suggests their role in disease development. Moreover, the correlation study suggest the multifaceted effect of POD, SOD and chitinase in symptoms expression in cowpea by *S. rolfsii* up to 10 DAI. Further, individual components of the defense pathway should be enlightened for a broad spectrum resistance.

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