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Biochemical responses of soil-borne necrotroph *Sclerotium rolfsii* during the pathogenesis on chickpea

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Abstract
Southern blight caused by *Sclerotium rolfsii* is a fast spreading and destructive disease of chickpea yield. This disease cause severe damage to plant on almost all growth stage. In our study we have selected two genotype of chickpea i.e one is resistant genotype (GNG1958) and another susceptible genotype (L550) and two isolates of *S. rolfsii* i.e., highly aggressive (C4) and least aggressive (L9). Both isolates were inoculated on both genotypes and observation of enzymatic activity was done. Peroxidase activity was highest in case of susceptible genotype inoculated with least aggressive isolate. There was depletion in catalase and ascorbate peroxidase activity and enhancement in activity of peroxidase whether it was inoculated with highly or least aggressive isolate, but there was variation in superoxide dismutase activity. The enhanced biochemical activities during plant pathogen interaction triggers the defense related enzymes such as wall-bound phenolics, flavonoids and induction of hypersensitive reaction (HR) etc., which resulted in cell strengthening and enhances resistance to pathogen. The depletion of catalase and ascorbate peroxidase during host-parasite interaction might be due to induction of antioxidant enzyme in plant which leads to oxidative stress and multiplication of pathogen. In case of superoxide dismutase activity when resistant genotype inoculated with least aggressive isolate there was increase in activity but when susceptible genotype inoculated with least aggressive isolate there was decline in superoxide dismutase activity. Which shows superoxide dismutase activity could improve superoxide scavenging system of cells and favor accumulation of superoxide which mainly contributes in damaging the concentration and damage to cell membrane.

Keywords: Peroxidase, ascorbate peroxidase, superoxide dismutase, catalase, *Sclerotium rolfsii*, chickpea

Introduction
Southern blight caused by the soilborne necrotrophic pathogen, *Sclerotium rolfsii* is one of the most important devastating soil-borne diseases of chickpea (*Cicer arietinum*) infecting usually the collar region of the plant. *S. rolfsii* survives on dead plant material in the soil by forming sclerotia which later germinate and attack young plants, causing necrosis by attacking the cell walls. The pathogen infects all portions of the plant in contact with the soil, and sclerotia that are produced can remain viable for many years and provide the primary inoculum for epidemics. It also produces oxalic acid, which in synergistic action with enzymes causes injury to plant tissue (Aycock, 1961, 1966) [4, 5]. *S. rolfsii* induces the production of reactive oxygen species (ROS) including superoxide radical, hydrogen peroxide and hydroxyl radical which may results in membrane damage and the destruction of cellular organelles and biomolecules i.e., cause cell death due to oxidative stress such as membrane lipid peroxidation which is reflected by increased malondialdehyde (MDA) concentration, enzyme inhibition and damage to nucleic acids. To repair the *S. rolfsii* induced inhibitory effects of ROS, plants possess the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and peroxidase (POD) (Kanazawa *et al*., 2000) [14]. In oxidative stress, ROS scavengers and antioxidant enzymes are highly activated to neutralize the negative effect. The fungal inoculation also enhanced the activity of ROS scavenging enzymes CAT, POD, SOD and APX which advocated the presence of high oxidative stress during fungal infection. Similar findings on other hosts under different pathogens stress have been reported (Anthony *et al*., 2017) [2].

Among, antioxidative enzyme, superoxide dismutase constitutes the primary step of cellular defense and dismutates O_2 - to hydrogen peroxide (H_2O_2) and O_2. Further, the accumulation of H_2O_2 is restricted through the action of catalase, where ascorbate peroxidase is converted to H_2O. The current study was undertaken to see the biochemical response of two genotypes of chickpea against the southern blight pathogen *S. rolfsii* and to examine the correlation in susceptible and resistant genotype and *S. rolfsii* isolates i.e., highly and least aggressive isolates induced damage to genotypes.

In this context we aimed to study in chickpea regarding changes of several defense related enzymes such as peroxidase, superoxide dismutase, catalase, and ascorbate peroxidase upon *S. rolfsii* attack. These studies will provide novel insight into these responses and mark a major advance in our understanding on the chickpea self-defense mechanisms against *S. rolfsii*, which ultimately will throw a focus on development of sustainable disease management strategy on long term basis.

Material and Methods

The diseased plants exhibiting characteristic symptoms of Southern blight was collected from different fields and brought to the laboratory at Bihar Agricultural College, Sabour for isolation. Disease samples collected from different locations of Bihar were stored in a refrigerator (4 °C) for 1-2 days. Pathogenicity of samples was tested and selection of highly aggressive isolate and least aggressive isolate were done (Kumari and Ghatak, 2018) [17]. The selected highly aggressive (C4) and least aggressive (L9) isolates were maintained on PDA slants throughout the investigation. Mycelium from slants were inoculated on PDA plates and allowed to grow for 3-4 days. The active mycelium from the edge of the colony were cut into 4-5 bits (5 mm diameter), and transferred to potato dextrose broth (PDB). The inoculated broth was incubated according to the mentioned incubation temperature. After 5-7 days of incubation, mycelial mat were developed on PDB. Seeds of chickpea resistant genotype (GNG1958) and susceptible genotype (L550) were sown in sterilized soil. The seedling of 4 weeks were dipped sterile distilled water for few seconds and then dipped in the suspension of mycelial mat for 5 minutes and then wrapped in the wet blotter paper. Each isolate was inoculated on resistant as well as susceptible genotypes of chickpea and the biochemical activity was estimated 24 hai and 72 hai. Level of various antioxidant enzymes activity in plant extracts were measured spectrophotometrically.

Peroxidase activity assay was conducted as described by Singh and Jha (2016). Briefly, the phosphate buffer (0.1 M, pH 7.0), pyrogallol (0.1 mM), and H_2O_2 (5 mM) were mixed with 100 mL of crude extract. The mixture was incubated at 25°C for 5 min. A 1.0 ml of 2.5 N H2SO4 was used to stop the reaction. The absorbance was read at 436 nm. Catalase activity was determined by following the method of Sarkar *et al*. (2014). Briefly, the crude extract was mixed with potassium phosphate buffer (50mM, pH 7.5) and H_2O_2 (0.1 mM). The absorbance was measured at 240 nm. CAT activity was calculated on the basis of H_2O_2 utilization (extinction coefficient = 43.6 $M^{-1}cm^{-1}$) (Aebi, 1984). Activity of APX enzyme was assayed as described by Sarkar *et al*. (2014). The reaction mixture was prepared by mixing potassium phosphate buffer (50 mM, pH 7.0), H_2O_2 (0.1 mM), and ascorbate (0.5 mM). The crude extract was added to the mixture to initiate the reaction and H_2O_2 -dependent oxidation of ascorbate was measured at 290 nm. Superoxide dismutase (SOD) activity was assayed using the modified method of Maral *et al*. (1977). Enzymatic reaction was estimated by measuring decrease in H_2O_2 absorption at 560 nm. The proline content in the samples was analyzed by the method suggested by Bates *et al*. (1973). Enzymatic reaction was estimated by measuring decrease in H_2O_2 absorption at 520 nm.

Results and Discussion

Peroxidase (POD)

Significantly maximum POD activity observed in resistant genotype of pathogen inoculated with least aggressive isolate at 72 hai. Resistant genotype showed low amount of peroxidase activity when it is inoculated with highly aggressive isolate. When comparison was done between 24 hai and 72 hai of genotype, there was significant difference in peroxidase activity. Results recorded for resistant genotype with highly aggressive isolate at 24 hai was 0.47 units/min/g/FW and at 72 hai was 1.35 units/min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 0.38 units/min/g/FW and at 72 hai was 1.26 units/min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 0.54 units/min/g/FW and at 72 hai was 1.09 units/min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 1.85 units/min/g/FW and at 72 hai was 2.40 units/min/g/FW was recorded. It shows POD activity always increases with increase of period of time, whether it is inoculated on resistant genotype or susceptible genotype. POD is involved in the production and modulation of active oxygen species which may play various roles directly or indirectly in reducing pathogen viability and spread (Passardi *et al*., 2005) [21]. Earlier studies suggest that peroxidases are important PR proteins and the plant expresses POD activity during host-pathogen interaction (Saikia *et al*., 2004) [24]. In our study, we observed that POD activity reached at its peak at 72 hai. POD is a metallo enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, amino acids and inorganic compounds (Balasimaha, 1982) [7]. Various naturally occurring and synthetic substances, growth regulator and environmental factors influence the activity of these peroxidases. Akhtar and Garraway (1990) [1] observed increased POD activity in susceptible cultivars compared to the resistant one when inoculated with *Botrytis maydis*. On the other hand there are also reports of increased POD activity due to induction of resistance (Chen *et al*. 2000) [8]. The existence of multiple molecular forms of POD activity in tea has also been reported (Sharma and Chakraborty 2004) [25].

Catalase (CAT)

Resistant genotype with highly aggressive isolate show at 24 hai was 166 units/min/g/FW and at 72 hai was 161 min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 88 units/min/g/FW and at 72 hai was 87 min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 144 units/min/g/FW and at 72 hai was 140 min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 177 units/min/g/FW and at 72 hai was 163 min/g/FW was recorded. This shows when susceptible genotype inoculated with least aggressive isolate CAT activity was highest but when resistant genotype inoculated with least aggressive isolate CAT activity was lowest. In all cases CAT activity reduced with time interval. Our results suggest that suppression of CAT was found to be one of the important

factors responsible for the successful pathogenesis in chickpea *S. rolfsii* system and differentiation inhibiting action of H_2O_2 is also cell proliferating. The low levels of CAT activity confirm the high levels of H_2O_2. In conclusion, the cell proliferating effect of H_2O_2 in the fungi is exerted at low H_2O_2 cytoplasmic concentrations, which has been associated with oxidative stress (Halliwell and Gutteridge 1999; Georgiou *et al*. 2006) [11, 9]. The major function of CAT is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes within cells (Montalbini, 1991). Organisms possess enzymatic antioxidant defense against H_2O_2 via CAT and glutathione peroxidase (already studied in these fungi: Patsoukis and Georgiou 2007b; Patsoukis and Georgiou 2008b) [22, 23].

Ascorbate peroxidase (APX)

Induction of ascorbate peroxidase is usually occurs in the cell surrounding infection area and there is close correlation between enzyme activity and induced resistance. The observation recorded here suggested that increases in lipid peroxidation and antioxidative enzymes activity in roots can be associated with resistance to southern blight in chickpea. Maximum activity of APX was found in case of resistant and susceptible genotype inoculated with least aggressive isolate. Resistant genotype with highly aggressive isolate show at 24 hai was 40 units/min/g/FW and at 72 hai was 38 unit/min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 12 units/min/g/FW and at 72 hai was 19 unit/min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 32 units/min/g/FW and at 72 hai was 37 unit/min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 21 units/min/g/FW and at 72 hai was 21 unit/min/g/FW was recorded. In this observation we found that there is decline in APX activity with interval of time when highly aggressive isolate inoculated on both genotypes, resistant as well susceptible. When susceptible genotype inoculated with least aggressive isolate there was no change in activity of APX, but in case of resistant genotype inoculated with least aggressive isolate there was increase in APX activity at 72 hai. APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants (Gill, 2010) [10]. Among peroxidases, ascorbate peroxidases are well known for their role in H_2O_2 detoxification in plant (Apel and Hirt, 2004) [3]. Infection of sweet orange leaves by *Xanthomonas axopodonis* subsp citri showed increase in activity of ascorbate peroxidase activity (Kumar, 2011) [16]. The change in antioxidant enzyme activity in tomato leaves infected by *Fusarium* was reported (Mandal, 2008) [19]. APX activity indicates that the unconstraint of APX which leads to the weakening of defense mechanisms in *S. rolfsii* inoculated chickpea genotype. This helps in the further spread of the pathogen and eventually severe southern blight symptoms are expressed.

Superoxide Dismutase (SOD)

Resistant genotype with highly aggressive isolate show at 24 hai was 41.4 units/min/g/FW and at 72 hai was 41.5 unit/ml, resistant genotype with least aggressive isolate at 24 hai was 37.7 units/min/g/FW and at 72 hai was 39.5 unit/ml, susceptible genotype with highly aggressive isolate at 24 hai was 37.5 units/min/g/FW and at 72 hai was 37.8 unit/ml, susceptible genotype with least aggressive isolate at 24 hai was 42.6 units/min/g/FW and at 72 hai was 40.3 unit/ml was recorded. The result shows there is variation in SOD activity with increase of time whether resistant or susceptible genotype is inoculated with highly or least aggressive isolates, when highly aggressive isolate inoculated on both genotype i.e., resistant as well as susceptible genotype there was no change in SOD activity. When resistant genotype inoculated with least aggressive isolate there was enhancement in SOD activity but when susceptible genotype inoculated with least aggressive isolate there was decline in SOD activity. The SOD activity could improve superoxide scavenging system of cells and favor accumulation of superoxide which mainly contributes in damaging the concentration and damage to cell membrane. Superoxide dismutase is considered as first line of defense against reactive oxygen species (ROS). The potential accumulation of H_2O_2 by SOD activity may have several important effects on the host-pathogen interaction (Nilima *et al*., 2015). Hence, an increase in SOD activity was observed in resistant genotypes after inoculation least aggressive isolate. Upon inoculation in resistant genotypes H_2O_2 accumulates due to SOD activity. This may inhibit the growth and viability of the pathogen. This in turn may impart resistance to chickpea genotype against southern blight disease in resistant genotype. SOD plays an important role in antioxidant defense system. It is known to catalyze the production of H_2O_2 by scavenging superoxide radicals (O_2). The production of ROS is one of the earliest cellular responses following successful pathogen recognition. ROS was considered as the first defense line against pathogen and may act as direct antimicrobial agent against phytopathogen attack.

Conclusions

There was an increase in the activity of peroxidase in all the genotypes whether it was inoculated with highly aggressive isolate or least aggressive isolate, there was declination in catalase as well as ascorbate peroxidase activity in both genotype when inoculated with highly and least aggressive isolate, but there was variation in activity of superoxide dismutase when inoculated on both genotypes. Highest enzymatic activity was recorded in resistant genotypes inoculated with highly aggressive isolate at 24 hai as compared to susceptible genotype. The enhanced activity of Peroxidase is linked with production of defense related products such as lignin, suberin, wall-bound phenolics, induction of hypersensitive reaction (HR) etc., which resulted in cell strengthening and then enhanced resistance to pathogen penetration. Peroxidase is considered as one of the important PR proteins and plants express enzyme activity during host–pathogen interaction. Peroxidases are involved in the defence of plants against pathogens either by their direct participation in the cell wall reinforcement or by their role as antioxidants in oxidative stress generated during plant–pathogen interaction. Increase in peroxidase activity has been correlated with resistance in many plants, including rice and wheat. In our study, significant increase in peroxidase activity in chickpea genotype inoculated with *S. rolfsii* isolates suggests that it may be one of the expressions of defence reactions in plants mechanism. This shows when plant cells were subjected into infection, it switches from normal primary metabolism to secondary metabolism defense pathway and activation of novel defense enzymes and genes takes place which inhibit fungal development, or indirectly by their implication in the metabolic ways associated with resistance to diseases. There was decrease in the activity of Catalase and Ascorbate peroxidase in all the chickpea genotypes upon inoculation with highly and least aggressive isolate. The depletion of CAT and APX during host-parasite interaction

might be due to induction of antioxidant enzyme in plant might be correlate to lack of colonization of isolate in these genotype, which could relate to oxidative stress and pathogen spread. SOD contributes to later accumulation of hydrogen peroxide and the hydrogen peroxide produced is metabolized by APX and also by POD. SOD seems to play a key role in the apparition and extension of the necrotic reaction. The induction of SOD activity was frequently reported in the plants in response to pathogen invasion and constitutes a reaction often associated to the plant resistance (Jetiyanon, 2007) [12]. Our study indicates that the antioxidant enzyme POD is actively involved in imparting resistance to southern blight of chickpea. It was observed that upon inoculation CAT expression was higher in resistant genotype GNG1958 inoculated with highly aggressive isolate C4, as compared to inoculated with least aggressive isolate L9. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to southern blight of chickpea. Increased POD enzyme activity during host-pathogen interaction is well correlated with imparting resistance to southern blight of Chickpea. This finding could have a practical biotechnological potential for the development of novel non-toxic antifungal pathogen agents designed on the basis of biochemical response of genotypes of chickpea inoculated with pathogen. The induction of ROS-scavenging enzymes, such as peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are the most common mechanism for detoxifying ROS synthesized during stress responses. Based on the above results it may be concluded that high activity of antioxidative enzymes of the chickpea appear to be important biochemical constituents in imparting resistance to *S. rolfsii* southern blight disease.

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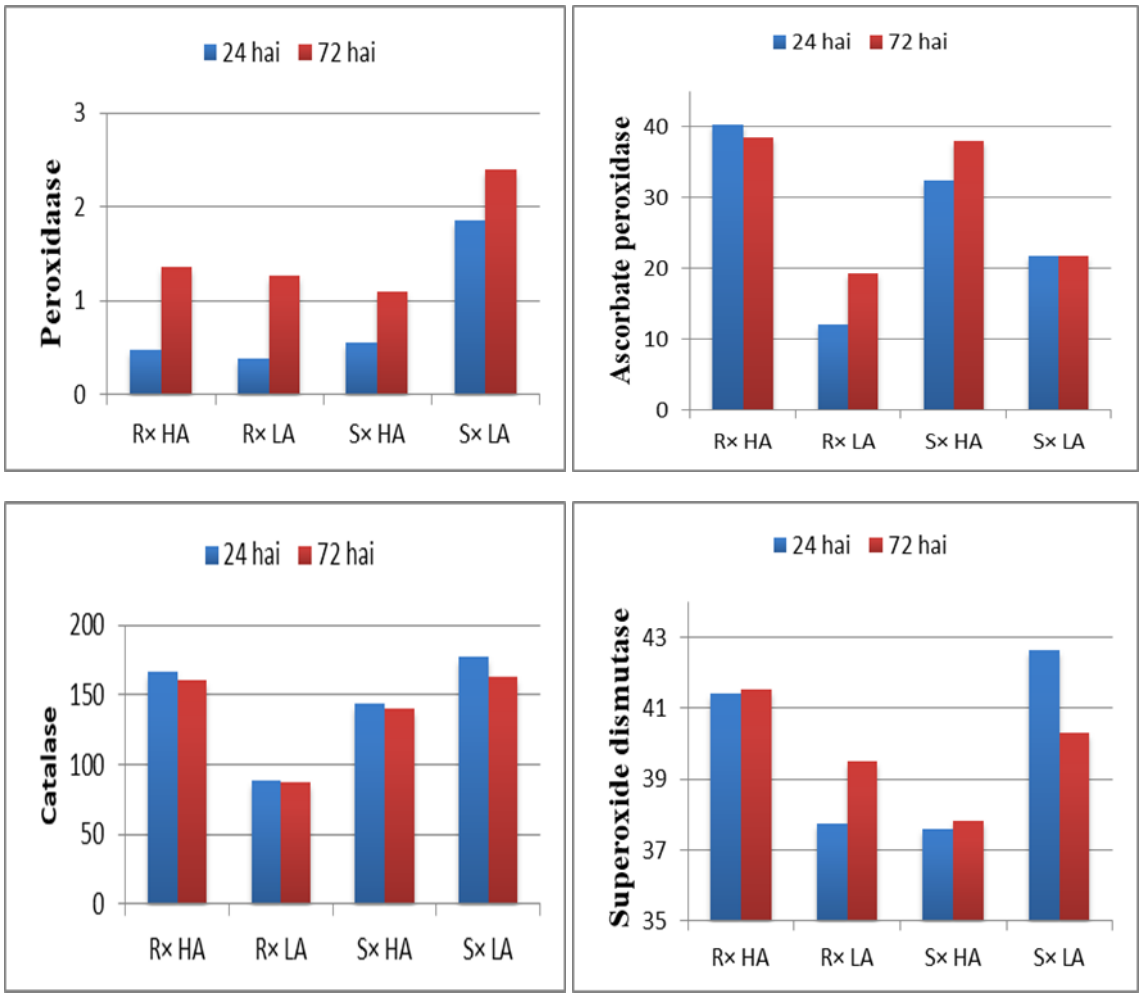

Fig 1: Peroxidase, Superoxide dismutase, Catalase and ascorbate peroxidase activity in *S. rolfsii* inoculated chickpea genotype (HA = Highly aggressive, LA = Least aggressive isolate)

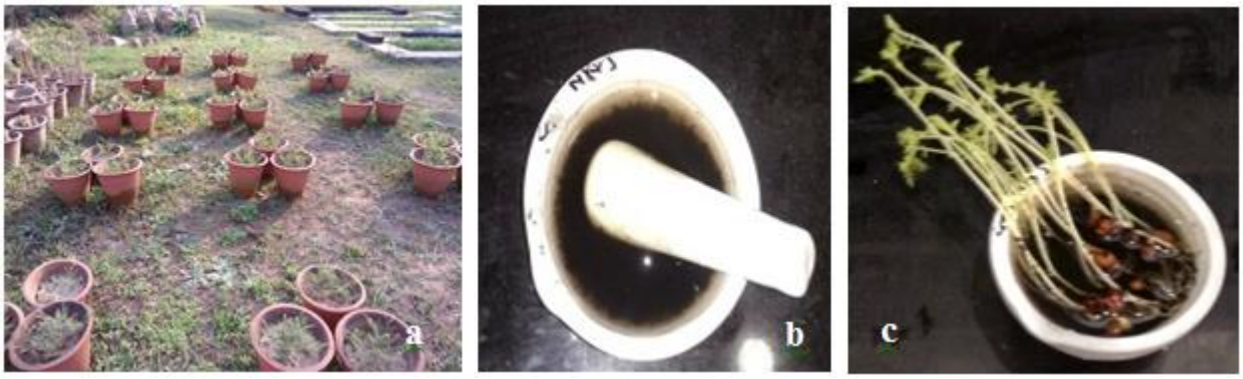

Fig 2: (a) Chickpea genotype, (b) Crushed mycelial mat and (c) Dipping of chickpea genotype in mycelial mat

Fig 3: (a) Blotter paper technique for infection of genotype, (b) Diseased genotype

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