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Biochemical responses of soil-borne necrotroph *Macrophomina phaseolina* during the pathogenesis on chickpea

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Abstract
Charcoal rot caused by *Macrophomina phaseolina* is a potential threat to 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 (JG315) and another susceptible genotype (K850) and two isolates of *M. phaseolina* i.e., highly aggressive (Dark MP4J) and least aggressive (CP3). Both isolates were inoculated on both genotypes and observation of enzymatic activity was done. Catalase activity was highest in case of resistant genotype inoculated with highly aggressive isolate at 24 hai but it decrease at 72 hai. There was depletion in catalase and superoxide dismutase activity but enhancement in activity of peroxidase and ascorbate peroxidase whether it was inoculated with highly or least aggressive isolate. 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 superoxide dismutase during host-parasite interaction might be due to induction of antioxidant enzyme in plant which leads to oxidative stress and multiplication of pathogen.

Keywords: Peroxidase, ascorbate peroxidase, superoxide dismutase, catalase, *Macrophomina phaseolina*, chickpea

Introduction
Macrophomina phaseolina, a global devastating soil borne necrotrophic fungal pathogen having a wide host range of about 500 cultivated and wild plant species worldwide (Khan, 2007) [9]. Wide range of diseases caused by *M. phaseolina* includes color rot, damping off, charcoal rot, stem rot, root rot, and seedling blight in economically important crops (Babu *et al*., 2007) [2]. *M. phaseolina* infects plants on almost all growth stages of plant which instigated by seed, soil and plant residues (Reuveni *et al*. 1983) [15]. It is difficult to control *M. phaseolina* due to its persistence as sclerotia in the soil and plant debris. However, low levels of productivity in chickpea are due to drought and are aggravated by charcoal rot disease. Here, chickpea is cultivated with very low input and therefore the production remains very low as response. Cultivation of chickpea is done as an alternate crop on those fields that were affected with flood and sowing of wheat is not profitable. Due to recurrence of diseases, the farmers are shifting from chickpea to lentil cultivation.
M. phaseolina induces the production of reactive oxygen species (ROS) including superoxide radical, hydrogen peroxide and hydroxyl radical which results in destruction of cellular organelles and damage of cell membrane that cause cell death due to oxidative stress and damage to nucleic acids. To repair the *M. phaseolina* induced inhibitory effects of ROS, plants possess the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Kanazawa *et al*. 2000) [7]. Among, antioxidative enzymes, superoxide dismutase constitutes primary step of cellular defense and dismutates -O_2 to H_2O_2 and O_2. To protect themselves against these toxic ROS, plants have evolved efficient systems which include ROS-scavenging antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX).
M. phaseolina affects the plant by secreting an array of cell wall degrading enzymes which depolymerize the cell wall components such as cellulose, xylan, pectin, polygalacturonic acid, and other proteins (Javaid and Saddique, 2012) [5]. Lipases are also produced by *M. phaseolina* and cause hydrolysis of the fats, mono and diglycerides into free fatty acids and glycerol (Kakde and Chavan, 2011) [6]. It also produces certain toxins such as phaseolinone and botryodiplodin which facilitate the infection (Ramezani, 2008; Bressano *et al*., 2010) [14, 4]. The production of hydrolytic enzymes has been reported to play a crucial role in the development of disease (Kaur *et al*., 2012) [8].

Any pathogen's attack stimulates the plant defense mechanism through hypersensitive response (HR). As a result of HR, reactive oxygen species (ROS) such as hydroxyl radicals, superoxide radicals, and hydrogen peroxide (H_2O_2) are produced. The unbalanced production of ROS could damage the plants severely. Hence, the production of ROS stimulates the deployment of antioxidant enzymes which scavenge ROS to maintain a balance. Thus, activity of antioxidant enzymes could serve as good marker for estimating oxidative stress in certain plant caused by pathogen (Anthony *et al*., 2017) [1].

Material and Methods

The diseased plants exhibiting characteristic symptoms of charcoal rot 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) [11]. The selected highly aggressive (Dark MP4J) and least aggressive (CP3) 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 (JG315) and susceptible genotype (K850) 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.

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 0.54 units/min/g/FW and at 72 hai was 0.64 units/min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 0.64 units/min/g/FW and at 72 hai was 1.69 units/min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 1.04 units/min/g/FW and at 72 hai was 1.35 units/min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 1.28 units/min/g/FW and at 72 hai was 1.50 units/min/g/FW was recorded. It means peroxidase activity always increases period of time whether it is inoculated on resistant genotype or susceptible genotype.

Peroxidase has been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxyl cinnamyl alcohols into free radical inter-mediates, 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) [13]. Earlier studies suggest that peroxidases are important PR proteins and the plant expresses POD activity during host-pathogen interaction (Saikia *et al*., 2004) [16]. In our study, we observed that POD activity reached at its peak at three days after inoculation

Catalase (CAT)

Resistant genotype with highly aggressive isolate show at 24 hai was 131 units/min/g/FW and at 72 hai was 125 min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 11 units/min/g/FW and at 72 hai was 10 min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 46 units/min/g/FW and at 72 hai was 44 min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 39 units/min/g/FW and at 72 hai was 35 min/g/FW was recorded. This shows when resistant genotype inoculated with highly aggressive isolate CAT activity was highest but when resistant genotype inoculated with least aggressive isolate CAT activity was lowest. When susceptible genotype was inoculated with highly as well as least aggressive isolate there was slight difference in decrease of CAT activity, but in all case 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 *M. phaseolina* system. The CAT found in peroxisomes, cytosol and mitochondria as tetrameric heme protein (Krych *et al*., 2014) [10]. The major function of CAT within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes mainly that of the electron transport pathway (Montalbini, 1991) [12].

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 charcoal rot 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 19.28 units/min/g/FW and at 72 hai was 28.92 unit/min/g/FW, resistant genotype with least aggressive isolate at 24 hai was 22.50 units/min/g/FW and at 72 hai was 38.57 unit/min/g/FW, susceptible genotype with highly aggressive isolate at 24 hai was 16.50 units/min/g/FW and at 72 hai was 18.85 unit/min/g/FW, susceptible genotype with least aggressive isolate at 24 hai was 31.60 units/min/g/FW and at 72 hai was 32.57 unit/min/g/FW was recorded. It shows resistant genotype inoculated with least aggressive isolate has highest amount of APX activity after 3 days of inoculation. This also shows APX activity increases with time interval. APX activity indicates that the unconstraint of APX after 72 hai leads to the weakening of defense mechanisms in *M. phaseolina*-inoculated chickpea genotype. This helps in the further spread of the pathogen and eventually severe charcoal rot symptoms are expressed. Similar result was found in 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. Baker and Orlandi (1995) [3] also has 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.

Superoxide Dismutase (SOD)

Resistant genotype with highly aggressive isolate show at 24 hai was 37.27 units/min/g/FW and at 72 hai was 36.61 unit/ml, resistant genotype with least aggressive isolate at 24 hai was 39.06 units/min/g/FW and at 72 hai was 38.57 unit/ml, susceptible genotype with highly aggressive isolate at 24 hai was 41.42 units/min/g/FW and at 72 hai was 40.79 unit/ml, susceptible genotype with least aggressive isolate at 24 hai was 37.71 units/min/g/FW and at 72 hai was 37.27 unit/ml was recorded. It shows there is significant decrease in SOD activity with increase of time whether resistant or susceptible genotype is inoculated with highly or least aggressive isolates, but the amount of reduction is slightly low in case of susceptible genotype inoculated with least aggressive isolate. The lower 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 was considered as first line of defense against reactive oxygen species (ROS) and was the major O2− scavenger.

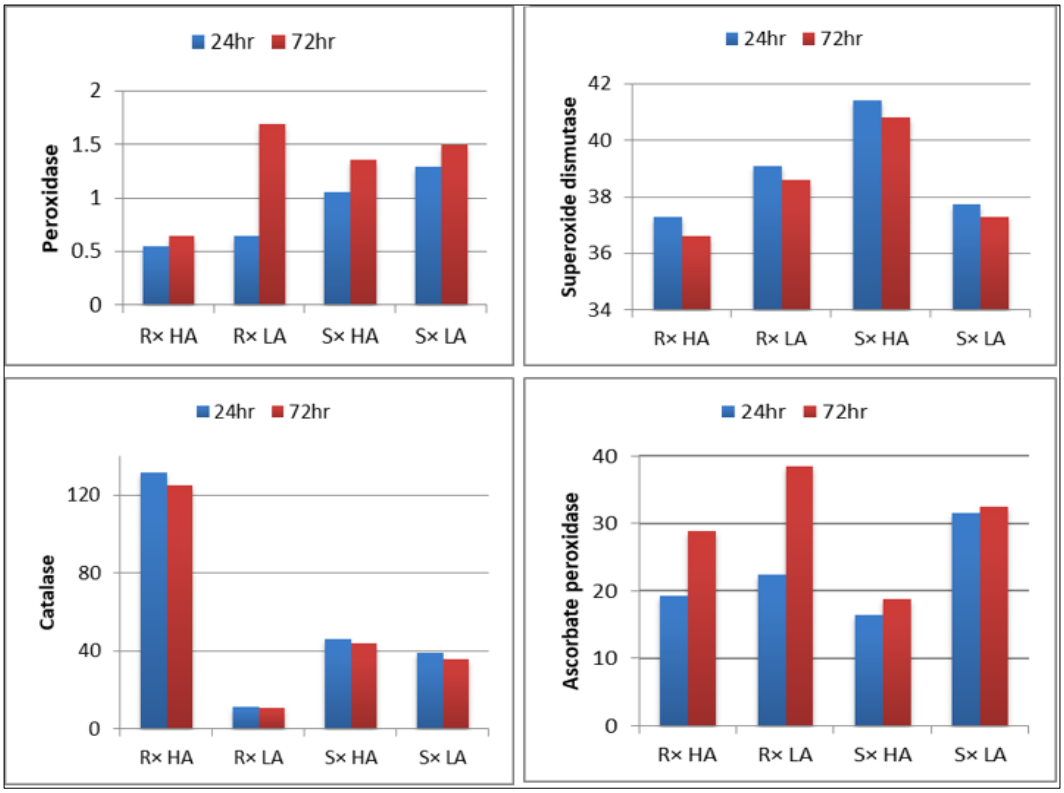

Fig 1: Peroxidase, Superoxide dismutase, Catalase and ascorbate peroxidase activity in *M. phaseolina* inoculated chickpea genotype

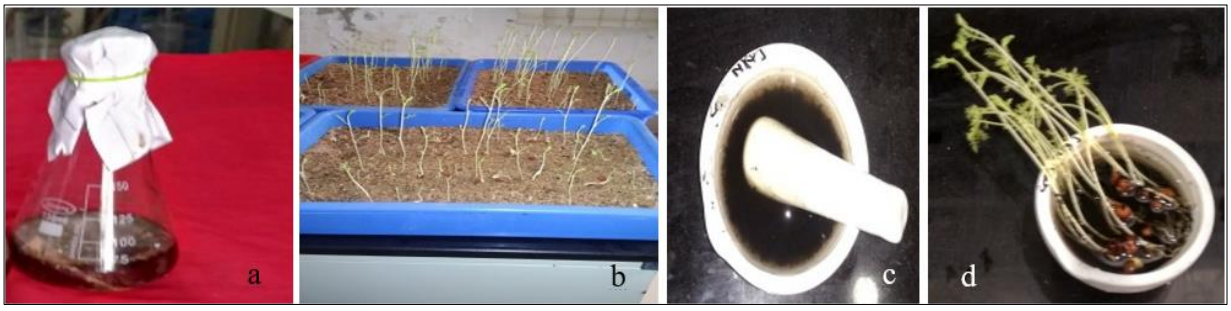

Fig 2: (a) Mycelial mat of isolate, (b) Chickpea genotype, (c) Crushed mycelial mat and (d) Dipping of chickpea genotype in mycelial mat

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

Conclusions

There was an increase in the activity of peroxidase and ascorbate peroxidase in all the genotypes whether it was inoculated with highly aggressive isolate or least aggressive isolate, but there was reduction in catalase as well as superoxide dismutase activity in both genotype when inoculated with highly and least aggressive isolate. 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 and Ascorbate 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. This shows that 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 Superoxide dismutase in all the chickpea genotypes upon inoculation with highly and least aggressive isolate. The depletion of CAT and SOD 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. Our study indicates that the antioxidant enzyme POD is actively involved in imparting resistance to charcoal rot of chickpea. It was observed that upon inoculation CAT expression was higher in resistant var. JG315 inoculated with highly aggressive isolate Dark MP_4J, as compared to inoculated with least aggressive isolate CP_3. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to charcoal rot of chickpea. Increased POD and APX enzyme activity during host-pathogen interaction is well correlated with imparting resistance to charcoal rot of Chickpea.

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