Oxidative stress and role of antioxidant machinery in two cultivars of sorghum (*Sorghum bicolor* L.) to combat the damage induced by *Macrophomina phaseolina*

Nilima Kumari, Isha Sharma, Afroz Alam, Vinay Sharma

**Abstract**

The presence of *M. phaseolina* affects the sorghum cultivars and constrains the crop productivity. A pot experiment was performed using two cultivars (PJ-1430 and SU-1080) of *S. bicolor* to evaluate the difference in their response to *M. phaseolina* infection at different time intervals. The malondialdehyde quantity enhanced in the roots of sorghum plants treated with *M. phaseolina* and the damage was found to be less in PJ-1430 than SU-1080. The induction of enzymatic antioxidants (catalase, ascorbate peroxidase, superoxide dismutase, glutathione reductase) was found in roots and leaves of plants with *M. phaseolina* inoculation. Our results indicate that the cultivar PJ-1430 is found to be tolerant to *M. phaseolina* stress, associated with higher antioxidative activity and thus less oxidative damage. SU-1080 experienced maximum damage in terms of reduced antioxidant activity. Better coordination of antioxidants protected PJ-1430 from *M. phaseolina* induced damage, whereas lesser antioxidants activity in SU-1080 resulted in maximum damage.

**Keywords:** SOD, CAT, APX, GR, MDA, *S. bicolor*

1. Introduction

*Macrophomina phaseolina*, a global devastating necrotrophic fungal pathogen, infects more than 500 plant hosts [1, 2, 3]. Charcoal rot of sorghum caused by *M. phaseolina*, soil and seed-borne disease of sorghum, is endemic to tropical and temperate regions of the world [4]. The disease severity may be enhanced by hot and dry environment [5] and may cause up to 100% yield losses [6]. Increased incidence of the pathogen on diverse crop species has been reported worldwide, highlighting the importance of this disease to crop production in drought prone regions [7].

*Macrophomina phaseolina* infects plants on almost all growth stages and are instigated by seed, soil and plant residues [8]. Plant defoliation and wilting occurs due to formation of red to brown lesions on roots and stems with production of dark mycelia and black microsclerotia [9]. 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 sorghum are due to drought and are aggravated by charcoal rot disease which usually appears at grain maturity stage and causes severe lodging. Sorghum charcoal rot is characterized by lodging of plants as they approach maturity. *M. phaseolina* induces the production of reactive oxygen species (ROS) including superoxide radical, hydrogen peroxide and hydroxyl radical which may result 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 *M. phaseolina* induced inhibitory effects of ROS, plants possess the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and glutathione reductase [10]. Among, antioxidant enzymes, superoxide dismutase constitutes the primary step of cellular defense and dismutate O$_2^-$ to H$_2$O$_2$ and O$_2$. Further, the accumulation of H$_2$O$_2$ is restricted through the action of catalase or by ascorbate glutathione cycle, where ascorbate peroxidase is converted to H$_2$O. Finally, glutathione reductase catalyzes the NADPH-dependent reduction of oxidized GSSG to the reduced GSG. It has been reported that *M. phaseolina* induced damage in cereals is found to be associated with the ability of antioxidant machinery to scavenge *M. phaseolina* induced production of excessive ROS [11]. The current study was undertaken to see the role of antioxidants machinery in two cultivars of sorghum against the charcoal rot pathogen *M. phaseolina* and to examine the correlation in susceptible...
and resistant cultivars and *M. phaseolina* induced oxidative damage. This study also explores the possibility of using these factors as components of resistance with phenotypic parameters for the efficient selection of charcoal rot resistant cultivars of sorghum. Selection of resistant cultivars in different species against *M. phaseolina* has proven to play a crucial role in breeding programmes.

2. Materials and methods:

2.1 Experimental design and plant growth

Sorghum seeds were obtained from Agricultural Research Institute Durgapura, Jaipur, Rajasthan. Selected healthy seeds were sterilized with 1% sodium hypochlorite solution (w/v) for 10 min, thoroughly washed with double distilled water for 1 h. For sowing, seeds were placed in pots of 15 cm diameter containing 1 kg sterilized soil and soilrite (3:1) in water for 1 h. For sowing, seeds were placed in pots of 15 cm (w/v) for 10 min, thoroughly washed with double distilled

2.2 Estimation of Lipid peroxidation

Malondialdehyde (MDA) was measured by the method of [13]. The extent of lipid peroxidation was evaluated by the thioarbituric acid reaction. 0.4 gram of frozen plant tissue (root and leaf) was homogenized in 0.1% trichloroacetic acid (1:10, w/v) and centrifuged at 10,000 rpm for 15 min. The supernatant was used for enzyme assay. One ml of the supernatant was incubated with 4 ml of 0.5% thioarbituric acid in 20% trichloroacetic acid at 95°C for 30 min and then cooled in ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was read at 532 nm and corrected for the non-specific absorbance recorded at 600 nm. The concentration of thioarbituric acid reactive substances was calculated as malondialdehyde equivalent using the extinction coefficient (155 mM⁻¹ cm⁻¹).

2.3 Antioxidant enzyme assays

2.3.1 Enzyme extraction

The enzyme extractions were carried out at 4°C. 0.25 gm of frozen plant tissues (roots and leaves) were ground to a fine powder in liquid nitrogen in a mortar with a pestle and extracted at a ratio 1:10 (w/v) fresh weight to extraction buffer (100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTT and 5% (w/v) insoluble PVPP). The homogenate was centrifuged at 10000 rpm for 30 min and the supernatant kept stored in separate aliquots at -80°C to be used for SOD, CAT, APX and GR analysis.

2.3.2 Determination of SOD

The activity of SOD was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) [14]. The 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. The 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 photoreduction of NBT (production of blue formazan) was measured at 560 nm and an inhibition curve was made against various volumes of the extract. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT at 560 nm.

2.3.3 Determination of catalase activity

The activity of CAT was determined by the method of [15] with slight modification by monitoring the disappearance of H₂O₂ at 240 nm. 3 ml H₂O₂-phosphate buffer was taken in cuvette and 100 µl enzyme extract was added. The absorbance was taken in the UV-VIS-2250 Shimadzu spectrophotometer at 240 nm. The absorbance was allowed to decrease from 0.45 to 0.4 from the time required in seconds (Δt) against diluted phosphate buffer as blank. Δt should be less than 60 seconds. The activity was calculated by using extinction coefficient 0.036 mM⁻¹cm⁻¹. One unit of the enzyme is the amount necessary to decompose 1 mmol of H₂O₂ per min at 25°C.

2.3.4 Determination of APX activity

The activity of APX was determined according to [16]. 3 ml of 50 mM phosphate buffer (pH 7) containing 5 mM ascorbate was taken and 100µl of enzyme extract was added to it. 2.5 µl H₂O₂ was added and the reaction was determined spectrophotometrically at 290 nm. One unit of the enzyme is the amount necessary to decompose 1 µmol of ascorbate per min at 25°C. APX activity was calculated by using extinction coefficient 2.8 mM⁻¹cm⁻¹.

2.3.5 Determination of glutathione reductase

GR activity was assayed spectrophotometrically at 30°C as described by [17] in a reaction mixture consisting of 3 ml 100 mM potassium phosphate buffer (pH 7.5) containing 1mM 5,5′-dithiobis(2-nitrobenzoic acid), 1mM oxidized glutathione and 0.1mM NADPH. The reaction was initiated by the addition of 50 µl of plant extract. The rate reduction of oxidized glutathione was followed by monitoring the increase in absorbance at 412 nm over 2 min. GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH. The activity of GR was calculated by using extinction coefficient 6.2 mM⁻¹cm⁻¹. One unit of enzyme was the amount necessary to decompose 1 mmol of NADPH per min at 25°C.

2.4 Statistical analysis

For statistical validity, each treatment was made in 5 replicates for estimating enzyme activity. The data were expressed as mean ± standard deviation (SD) and were analyzed by ANOVA using SPSS software. Significance of differences was checked with the Tukey’s post-hoc test. Statistical significance was set at p<0.05.

3. Results & Discussion

3.1 Quantitative determination of MDA content in roots and leaves of *S. bicolor* infected with *M. phaseolina*

The MDA content (a measure of lipid peroxidation) in the root and leaf samples was determined in cultivar PJ-1430 and SU-1080 in *S. bicolor* plants inoculated with *M. phaseolina* and expressed in terms of MDA content µmol.gfw⁻¹. The effect of *M. phaseolina* on MDA content in root and leaf samples is presented in (Fig. 1-2). The MDA content increased in both root and leaves in time dependent manner. An exposure of *M. phaseolina* in soil in 15 day-old *S. bicolor* plants, resulted in increase in MDA content, in PJ-1430 the increase in MDA content in root is
CAT expression was higher in cultivar PJ-1430 as compared to SU-1080 at 168 h (Fig. 2). Similarly, an increase in SOD activity in leaves of infected sorghum plants with increasing time of infection. Levels of MDA gradually increased in leaves of infected sorghum plants with increasing time of infection. When plants are subjected to pathogen attack, the equilibrium between productions and scavenging of ROS is broken, resulting in oxidative damage of proteins, DNA and lipids. MDA is the marker for lipid peroxidation released from cellular membranes of tissues and are formed by the reaction of ROS (H₂O₂ and O₂⁻) with lipid molecules [18]. Thus, lipid peroxidation in sesamum plants has been proved to be induced by pathogens, and the subsequent products have been shown to possess antimicrobial properties and signalling function [19].

An exposure of M. phaseolina in soil in 15 day-old S. bicolor plants, resulted in increase in MDA content, in PJ-1430 the increase in MDA content in leaf was 0.54 µmol gfw⁻¹ to 1.41µmol gfw⁻¹ which was 2.3 folds higher than control plants and 0.41 to 1.82 µmol gfw⁻¹ which was 4.4 folds higher in SU-1080 at 168 h (Fig. 2). Similarly, an increase in the MDA content in leaves of Citrus aurantifolia inoculated with Xanthomonas citri subsp. Citri [20].

### 3.2 Catalase activity in leaves and root of S. bicolor infected with M. phaseolina

The catalase activity of 15 days old plants was analysed at 0, 2, 4, 24, 48, 72, 96, 120, 144 and 168 h after mycelia inoculation and expressed as CAT activity (µmol H₂O₂/min mg⁻¹ protein).

The highest catalase activity in roots was obtained at 96 h and 72 h with 9.93 and 4.63 µmol H₂O₂/min mg⁻¹ protein after pathogen inoculation for cultivar PJ-1430 and SU-1080, respectively, which was 176.6% and 113% higher in inoculated plants as compared to the control plants (Fig. 3). The catalase activity was 2.8 and 2 fold higher in cultivar PJ-1430 and SU-1080, respectively, in pathogen inoculated plants as compared to the catalase activity at 0 h.

Our findings are similar with the earlier studies who reported that the enhanced catalase activity was observed in resistant variety of Capsicum annuum against the pathogen Phytophthora capsici L. in comparison to the susceptible one [21]. Similarly, induction of CAT has also been observed in nodulated soybean roots colonized by Glomus mosseae [22]. The CAT activity in leaves (Fig. 4) was 6.54 µmol H₂O₂/min mg⁻¹ protein and 5.28 µmol H₂O₂/min mg⁻¹ protein in cultivar PJ-1430 and SU-1080 which was 2.5 and 1.7 fold higher than the control at 0 h. Similarly, infection by Fusarium oxysporum significantly increased CAT activity in leaves of tomato plants at different stages of growth as compared with non-infected control plants [23].

Overall, on comparing the leaves and roots of sorghum plants the CAT activity was higher in roots as compared to leaves. Our study indicates that the antioxidant enzyme CAT is actively involved in imparting resistance to charcoal rot disease of sorghum. It was observed that upon inoculation CAT expression was higher in cultivar PJ-1430 as compared to SU-1080. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to sorghum against M. phaseolina.

### 3.3 Superoxide dismutase (SOD) activity in root and leaves of S. bicolor infected with M. phaseolina

The effect of M. phaseolina on SOD activity in both the cultivars of S. bicolor is shown in (Fig. 5-6). The SOD activity was higher in root and leaf samples as compared to control after pathogen elicitation. The SOD activity was determined in terms of SOD activity NBT reduced mg⁻¹ protein.

The maximum SOD activity obtained at 96 h in root 6.17 NBT reduced mg⁻¹protein and 3.77 NBT reduced mg⁻¹protein which was 3 and 2.5 fold higher in the pathogen inoculated plants of cultivar PJ-1430 and SU-1080, respectively, as compared to control (Fig. 5).

Our results are similar to the results reported by [24] who observed induced SOD activity in roots of Pinus massoniana after infection with Pisolithus tinctorius. Similarly, significant increase in roots of chickpea plant following Fusarium oxysporum interaction [25]. Further, [26] reported the differential induction of superoxide dismutase in downy mildew-resistant and susceptible genotypes of pearl millet (Pennisetum glaucum) on inoculation with Sclerospora graminicola. Gossypium hirsutum varieties resistant to verticillium wilt (Verticillium dahliae) showed increased SOD activity, while susceptible ones showed slightly decreased activity as compared to resistant varieties [27]. The potential accumulation of H₂O₂ by SOD activity may have several important effects on the host-pathogen interaction. Hence, an increase in SOD activity was observed in resistant genotypes after inoculation. Upon inoculation in resistant genotypes H₂O₂ accumulates due to SOD activity. This may inhibit the growth and viability of the pathogen, directly suppressing attempted invasion. This in turn may impart resistance to sorghum against root rot disease in resistant genotypes.

15 days old sorghum leaves indicates a higher SOD activity in pathogen inoculated plants compared to control plants. The maximum SOD activity in pathogen inoculated sorghum plants occurs at 96 h and 72 h which was 2 and 1.4 folds higher in cultivar PJ-1430 and SU-1080, respectively, as compared to 0 h inoculated plants (Fig. 6).

However, with further increase in inoculation time beyond 96 h, no further increase in the SOD activity is observed, rather a gradual decrease up to 168 h was observed (Fig. 5-6). Further the SOD activity is higher in PJ-1430 as compared to cultivar SU-1080. This is in agreement with results reported by [28] that in SOD activity in leaves of barley plants increased in pathogen (Blumeria graminis) inoculated plants than the control ones. Similarly, increase in the SOD activity in leaves of Nicotiana benthamiana plant infected with Pepper mild mottle virus [29].

Superoxide dismutase (SOD) plays an important role in antioxidant defense system. It is known to catalyze the production of hydrogen peroxide (H₂O₂) by scavenging superoxide radicals (O²⁻). The production of reactive oxygen species (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. The SOD, catalyzing the dismutation reaction of the free radicals to the hydrogen peroxide (H₂O₂), 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 [30].

3.4 Ascorbate peroxidase (APX) activity in leaves and root of S. bicolor infected with M. phaseolina

The APX activity of 15 days old plants are given in Fig. 7. The activity was expressed in terms of APX activity µmol Ascorbate/min mg⁻¹ protein.

APX activity in the inoculated plants of both the cultivars was significantly higher than that of the control plants. In cultivar PJ-1430, APX activity was higher than in cultivar SU-1080 at the all-time point investigated, in root samples. The level of APX reached its maximum (4.35 µmol Ascorbate/min mg⁻¹ protein) at 72 h in the inoculated PJ-1430 cultivar, which was higher than that of SU-1080 (2.52 µmol Ascorbate/min mg⁻¹ protein) at this time point (Fig. 5). Similarly, the increased production of APX in tomato root inoculated with F. oxysporum counteracted the higher MDA and H₂O₂ levels and prevents oxidative damage of root tissues [31]. Ascorbate peroxidase might be responsible for the fine modulation of ROS for signaling [32].

The APX activity in leaves of inoculated plants was (2.99 µmol Ascorbate/min mg⁻¹ protein) and (1.80 µmol Ascorbate/min mg⁻¹ protein) at 72 h which was 2 and 1.3 folds higher in PJ-1430 and SU-1080 respectively than the control plants. After that a continuous decline was observed in the activity in both the cultivars but the decline was more pronounced in SU-1080 (Fig. 8).

Similar to APX, glutathione reductase is also involved in scavenging H₂O₂ and protecting cells in higher plants [33]. It is assumed that the APX activity was maintained at a higher level in the leaves of the resistant sorghum plants than in susceptible plants during M. phaseolina infection process.

APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants [35]. Among peroxidases, ascorbate peroxidases are well known for their role in H₂O₂ detoxification in plant [36]. Infection of sweet orange leaves by Xanthomonas axopodonis subsp citri showed increase in activity of ascorbate peroxidase activity [37]. The change in antioxidant enzyme activity in tomato leaves infected by Fusarium was reported [31].

3.5 Glutathione reductase (GR) activity in roots and leaves of S. bicolor infected with M. phaseolina

The GR activity of 15 days old plants are given in Fig. 9-10. The activity was expressed in terms of GR activity µmol NADPH/min mg⁻¹ protein.

S. bicolor cultivars PJ-1430 and SU-1080 showed a differential response in GR activity with the increase in the time interval of pathogen inoculation as compared to control. In cultivar PJ-1430, GR activity increased gradually up to 96 h, after that the GR activity gets reduced but was still higher than control. On the other hand in cultivar SU-1080, the GR activity gets induced and reaches to maximum at 72 h and then decreased gradually till 168 h. GR activity reached its maximum to 3.18 µmol NADPH/min mg⁻¹ protein in PJ-1430 whereas the cv. SU-1080 leveled up to 2.76 µmol NADPH/min mg⁻¹ in root samples of S. bicolor (Fig. 9). The results are in agreement with [25] who reported the induction of GR activity in Cicer arietinum on inoculation with Fusarium oxysporum. GR activity significantly increased in the roots of chickpea plant. Increased activity of GR, a H₂O₂ detoxifying enzyme, observed in our experiments, is likely to have been induced by elevated levels of H₂O₂, as a response to oxidative stress. Similarly, the same pattern of induction of GR was observed in plant roots colonized with Piriformospora indica and Fusarium verticillioides-Hordeum vulgare interactions and it was suggested that the higher GR activity maintains an enhanced level of reduced glutathione which is involved in maintaining antioxidant capacity [38].

Similarly, the GR activity in leaves of both cultivars was increased at 96 h and then a gradual decline was observed. GR activity was highest in PJ-1430 2.84 µmol NADPH/min mg⁻¹ and in the susceptible cultivar the value was 2.07 µmol NADPH/min mg⁻¹ which was 2.4 and 1.5 folds higher than the control in both the cultivars respectively (Fig. 10). Similar results were reported by [39] who reported the increase in the GR activity in wheat plants inoculated with Pyricularia oryzae at 96 h post inoculation.

Further, the GR activity in leaves of maize plants increased upto 2 folds in maize plants inoculated with Piriformospora indica [40]. Similarly, the GR activity was measured in the two barley cultivars, resistant and sensitive against B. graminis which leads to an increase in the GR activity in the tolerant cultivar than the sensitive one [41]. Inoculation of tomato leaves with Botrytis cinerea led to markedly increase in the GR activity [42]. Overall, on comparing the leaves and roots of sorghum plants the GR activity was found to be higher in roots as compared to leaves. Similar results were reported by [25] who reported the induction of GR activity in Cicer arietinum on inoculation with Fusarium oxysporum.
4. Figures

Fig. 1: Changes in the MDA content in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants root samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

Fig. 2: Changes in the MDA content in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants leaf samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

Fig. 3: Changes in the CAT activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants root samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).
**Fig. 4:** Changes in the CAT activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants leaf samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

**Fig. 5:** Changes in the SOD activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants root samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

**Fig. 6:** Changes in the SOD activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants leaf samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).
Fig. 7: Changes in the APX activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants root samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

Fig. 8: Changes in the APX activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants leaf samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).

Fig. 9: Changes in the GR activity in control and pathogen (*Macrophomina phaseolina*) inoculated 15 days old *Sorghum bicolor* plants root samples of cultivar viz. PJ-1430 (a) and SU-1080 (b).
5. Conclusions:
MDA content increased with the progression of the infection time reflecting enhancement of lipid peroxidation. As a result of infection plants have evolved antioxidative machinery to combat oxidative stress. The activities of SOD, CAT, APX and GR increased to combat the effect of lipid peroxidation in sorghum. The induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD), glutathione reductase (GR) 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 *S. bicolor* appear to be important biochemical constituents in imparting resistance to *M. phaseolina* charcoal root rot disease.

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7. References


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