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ANTIOXIDANT RESPONSE OF *BRASSICA* PLANTS IN PROTECTION AGAINST *ALTERNARIA BRASSICICOLA*

SANA MUNIR[1], MUHAMMAD KAMRAN QURESHI[1*] AND AHMAD NAEEM SHAHZAD[2]

[1]Department of Plant Breeding and Genetics, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan
[2]Department of Agronomy, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan
[*]Corresponding author's email: m.k.qureshi81@gmail.com

Abstract

In current study eleven *Brassica* genotypes, with diverse range of response to *Alternaria* black spot disease (ABSD), were evaluated to assess the role of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in disease resistance. Post-infectional POD and SOD activities increased the resistance in genotypes. Genotype BC9 accumulated highest POD and SOD activity, while lowest was observed in susceptible genotype BC1, which showed maximum ABSD incidence. The significant ($p \leq 0.01$) negative correlation was found between pre- and post-infectional POD, SOD activities and percent disease index (%DI). In contrast, post-infectional CAT activity was lower than pre-infectional CAT activity except in genotype BC3. So, there was non-significant correlation between pre- and post-infection CAT activity and % DI. It can be proposed that CAT may have not significant role in disease resistance and it is predicted that such CAT activity is due to its enzyme inhibition by ABSD. Correlative evidence established the role of POD and SOD in black spot disease resistance in *Brassica*. The SOD and POD activities can be utilized as biochemical markers in screening of *Brassica* germplasm to differentiate resistant genotypes against ABSD.

Key words: Alternaria black spot disease, Catalase, percent disease index, Peroxidase, Superoxide dismutase.

Introduction

Brassica species (*B. campestris, B. juncea*) are extensively grown as vegetable and oilseed crop in the world (Ali *et al.,* 2015). Several pathogens attack *Brassica*, cause infection pertaining to disease and interrupt all normal physiological processes thus disturbing plant growth and development. Among diseases, *Alternaria* blight, also known as *Alternaria* black spot, is the most devastating *Brassica* disease triggered by *Alternaria brassisicola* (Kumar *et al.,* 2014; Javaid *et al.,* 2018), which results in yield reduction of oilseed crops and other members of *Brassicaceae* family. *Alternaria* black spot disease (ABSD) causes upto 15% to 70% *Brassica* yield reduction world-widely, via infecting seedlings and seeds (Nowicki *et al.,* 2012; Kumar *et al.,* 2014). Symptoms of disease development comprises formation of brown to black necrotic spots on leaves and black to greyish lesions on stems and siliques. Severely infected siliques became shrunk, dried, open prematurely and finally drop off (Kumar *et al.,* 2014).

Plant diseases could be managed via effective methods i.e., plant resistance (Akhtar *et al.,* 2007). Activation of signaling cascades mainly antioxidant system (Goud & Kachole, 2012) induced by host-pathogen interaction (Parihar *et al.,* 2012) made genotypes dependent for varied responses from susceptibility to resistance. Signaling cascades involve defense mechanism, which consists of array of cellular and biochemical variations occuring in response to pathogen infection in plants (Jones & Dangl, 2006). In host pathogen interaction, accretion of reactive oxygen species (ROS) occurs and such overproduction of ROS results in cell death, which enhances plant susceptibility (Torres *et al.,* 2006). Enzymatic antioxidants are involved in the elimination of different types of lethal ROS (Barna *et al.,* 2012). In short, plant activates its defense mechanism against *Alternaria* diseases via defense-linked enzymes i.e. CAT, POD and SOD (Parihar *et al.,* 2012; Taheri *et al.,* 2014; Mallick *et al.,* 2017).

SOD enzyme catalyzes the dismutation of O_2^- to H_2O_2 and O_2; acts as first line of defense against ROS and oxidative stress (Alscher *et al.,* 2002). The POD enzyme oxidizes several phenolic molecules. Phenolics are robust non-enzymatic antioxidants because of presence of its phneolic hydrogen, in addition to enzymatic-H_2O_2 scavenging system (Sharma *et al.,* 2012). Some phenolics comprises of lignin, which are oxidized by POD using H_2O_2. POD and SOD act as primary defensive enzymes in rice against *A. alternata* (Taheri *et al.,* 2014). Cell utilizes CAT to decompose H_2O_2 into less reactive oxygen and H_2O molecule thus the cell escapes from cessation (Parihar *et al.,* 2012). Overproduction of H_2O_2 is scavenged by the activity of CAT and POD (Hameed *et al.,* 2008). These enzymes also play important role in cell defense (Hameed *et al.,* 2009). Augmented activity of POD and SOD is often correlated with plant resistance against necrotrophic fungi belonging to genus *Alternaria* (Taheri *et al.,* 2014). Several findings support the fact that POD and SOD activity has defensive role in plant disease tolerance against *Alternaria* species (Taheri *et al.,* 2014; Mallick e*t al.,* 2015). Such bonding between biochemical parameters and disease resistance can be utilized as selection tool for resistant plants against these fungal diseases (Tyagi *et al.,* 2008).

Enzymatic antioxidants have significant role in plant development and in response to stress. They play an integral part in cellular resistant against fungal diseases (Mallick *et al.,* 2015). In current study, objectives were to find (i) biochemical indices for the identification of resistant genotypes (ii) role of enzymatic antioxidants i.e. POD, CAT and SOD in disease resistance in *Brassica* genotypes against ABSD and to (iii) assess resistant abilities of *Brassica* genotypes against *Alternaria* blight. This study delivers the correlative evidence for role of POD, CAT and SOD in black spot disease resistance in *Brassica*.

Materials and Methods

Plant and fungal material: Eleven diverse genotypes of *Brassica* with varying response to ABSD were used in the current experiment. Nine genotypes were from *B. campestris* (BC1, BC2, BC3, BC4, BC5, BC6, BC7, BC8 and BC9) and two from *B. juncea* (BJ1 and BJ2) (Table 1). Experiment was carried out at the Department of Plant Breeding and Genetics, Bahauddin Zakariya University, Multan, Pakistan using randomized complete block design with three replications.

A. brassicicola pathogen was isolated from naturally infected *Brassica* plants grown at experimental farms of FAST, BZU, Multan. *A. brassicicola* was grown on potato dextrose agar medium. After identification and purification of *A. brassicicola*; inoculum was prepared from 15 days old culture via potato dextrose broth media. Spore density was maintained at 10^7/L. Three plants (seven weeks old) from each replication were inoculated via broth suspension by cut-scissor method and enclosed with the help of polyethylene bags for three-four days. Plants were daily examined and disease progression was assessed one to two weeks after inoculum. Disease scoring was calculated using the rating system based on 0-10 as described by Doullah's (2006). Disease index was calculated using Hameed *et al.,* (2010) method.

Table 1. Codes representing *Brassica* genotypes and its specification.

Codes	Genotypes	Genus	Species	Origin
BC1	EC-001333	*Brassica*	*campestris*	USA
BC2	EC-001347	*Brassica*	*campestris*	USA
BC3	EC-001354	*Brassica*	*campestris*	USA
BC4	EC-001368	*Brassica*	*campestris*	USA
BC5	EC-001418	*Brassica*	*campestris*	Pakistan
BC6	EC-001483	*Brassica*	*campestris*	Pakistan
BC7	EC-001490	*Brassica*	*campestris*	Pakistan
BC8	EC-025018	*Brassica*	*campestris*	Pakistan
BC9	EC-025047	*Brassica*	*campestris*	Pakistan
BJ1	EC-001358	*Brassica*	*juncea*	USA
BJ2	EC-001495	*Brassica*	*juncea*	Pakistan

Estimation of enzyme activity: For enzyme activity, leaves samples were collected before and after inoculum. For pre-infection, leaves samples were collected before application of inoculum. For post-infection, leaves samples were collected after 7 days of inoculum when symptoms were evident. Leaf samples (0.5g) were grounded in 50mM phosphate buffer and centrifuged at 12000 rpm for 20 minutes. The supernatant was used to estimate CAT, SOD and POD activity.

POD activity was calculated using Chance & Maehly, (1955) method. POD activity assay mixture contains 50 mM phosphate buffer, 40mM H_2O_2, 20mM guaiacol and 0.1ml enzyme extract. Change in absorbance was recorded at 470 nm after every 20 sec for 2 minutes. POD activity was expressed as U/ min/ mg protein.

CAT activity was assayed by H_2O_2 decomposition. Reaction mixture contained 50 mM phosphate buffer, 5.9 mM H_2O_2 and 0.1ml enzyme extract. Absorbance change was observed for 2 min at 240 nm after every 20 seconds using spectrophotometer. CAT activity was expressed as µmol of H_2O_2 /min/ mg protein (Chance & Maehly, 1955).

SOD activity was measured via photochemical reduction of NBT (nitro blue tetrazolium) at 560nm. Reaction mixture was prepared by adding 50mM phosphate buffer, 75mM EDTA, 13mM methionine, 1.3 µM riboflavin, 50 µM NBT and 50 µl enzyme extract. SOD activity was measured as SOD U/min/mg protein (Giannopolitis & Ries, 1977). The readings were taken using Spectrophotometer (Agilent Cary 60 UV). Enzymes activities were estimated on protein base while protein was assessed via Bradford assay (1976).

Biometrical analysis: Statistical calculations comprising of t-test for comparing mean of antioxidants enzyme before and after inoculum was performed using SPSS v20 while all graphical presentation and correlation coefficient (R^2) were performed and calculated using computer software Microsoft Excel 2011.

Results

Brassica genotypes showed a varying range of response from susceptible to resistant against ABSD. Among eleven genotypes, genotype BC9 was extremely rated as resistant (DSI = 20) followed by genotype BC3 (DSI=30) while genotypes BC6 (DSI = 32.5), BJ1 (DSI = 36) and BJ2 (DSI = 40) were found as moderately resistant. Hence, genotypes having DSI (>40) were recorded as susceptible including genotypes BC5 (DSI = 43.9), BC7 (DSI = 53.3), BC4 (DSI = 55), BC8 (DSI = 60), BC2 (DSI = 60) and BC1 (DSI = 95; Fig. 1).

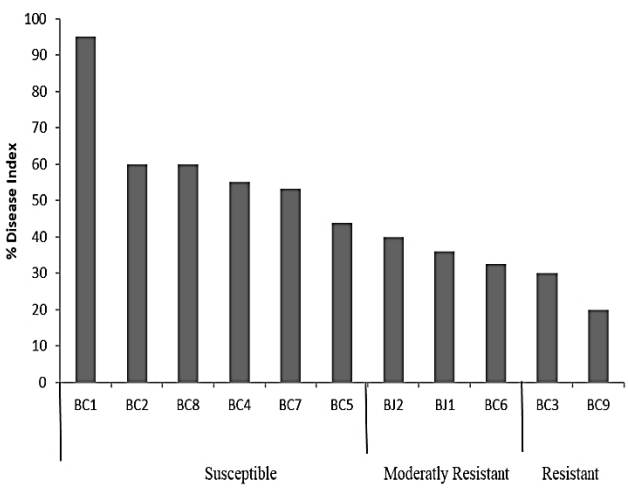

Fig. 1. Disease index in *Brassica* genotypes against ABSD.

In current study, remarkable range of pre- and post-infectional POD activity was observed in *Brassica* genotypes against ABSD (Fig. 2). Post-infectional POD activity was increased with the increase of resistance level in genotypes. Lowest POD activity was observed in susceptible genotype BC1, while resistant genotype BC9 showed highest and significant ($p \leq 0.01$) POD activity in response to ABSD followed by BC3.Correlation between POD activity and % DI is shown in Fig. 3. Significant and negative correlation was found between pre- and post-infectional POD activity and % DI showing that genotypes with high susceptibility risk to *A. brassicicola* showed less POD activity and vice versa. Hence, POD activity was reciprocal to disease index.

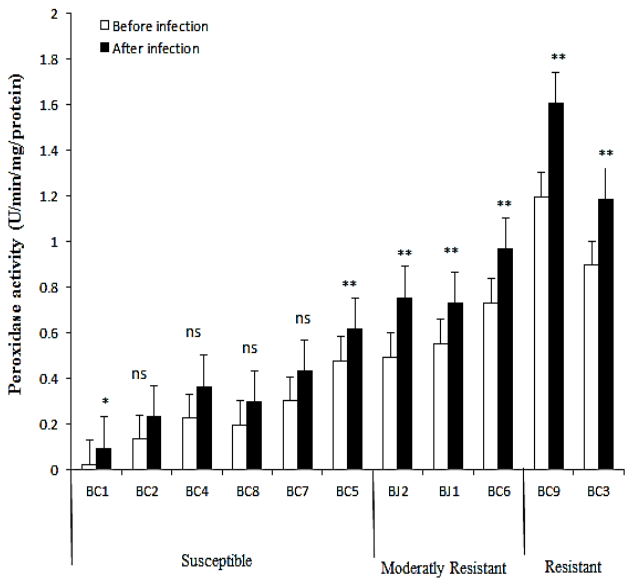

Fig. 2. POD activity in *Brassica* genotypes before and after infection with *A. brassicicola.*
Each bar represents mean ± SE of three replicates.*, **, show significant difference between treatments of pre- and post-infection in *Brassica* at p≤0.05 and p≤0.01, level of significance, respectively.

Pre- and post-infection SOD activity varied among *Brassica* genotypes against ABSD (Fig. 4). Significant correlation was found between SOD activity and % DI. However, correlation was negative as in previous cases of POD, representing that genotypes with higher SOD activity showed more resistance on exposure to ABSD (Fig. 5). Highest SOD activity was observed in resistant genotype BC9 followed by BC3 after inoculation. An increase in SOD activity was observed in all genotypes after infection with *A. brassicicola.* In resistant and moderately resistant genotypes, SOD activity increased in comparison to susceptible genotypes depicting that they have role in disease resistance against ABSD. It was also observed that SOD activity also increased in susceptible genotypes but their magnitude is less as compared to resistant genotypes.

The CAT activity does not vary distinctly among genotypes in response to *A. brassicicola.* A slight change in level of CAT activity was detected in all *Brassica* genotypes except BC3, which exhibited highest CAT activity level (Fig. 6). Significant reduction in CAT activity (p≤0.05) was observed in genotypes BJ2 and BC4 after inoculation. Post-infectional CAT activity was lower in resistant genotypes except BC3 contrast to pre-infectional CAT activity. Overall, CAT showed undefined pattern of its activity. Simultaneously, correlative evidence depicted that correlation was non-significant between CAT activity and % DI (Fig. 7).

Hence, increased activities of defensive enzymes have direct correlation in disease resistance against pathogen in plants. Alike, in current study, POD and SOD levels enhanced significantly in all *Brassica* genotypes when inoculated with *A. brassicicola*. The highest activity was found in BC9 resistant genotype and lowest in susceptible genotype BC1.

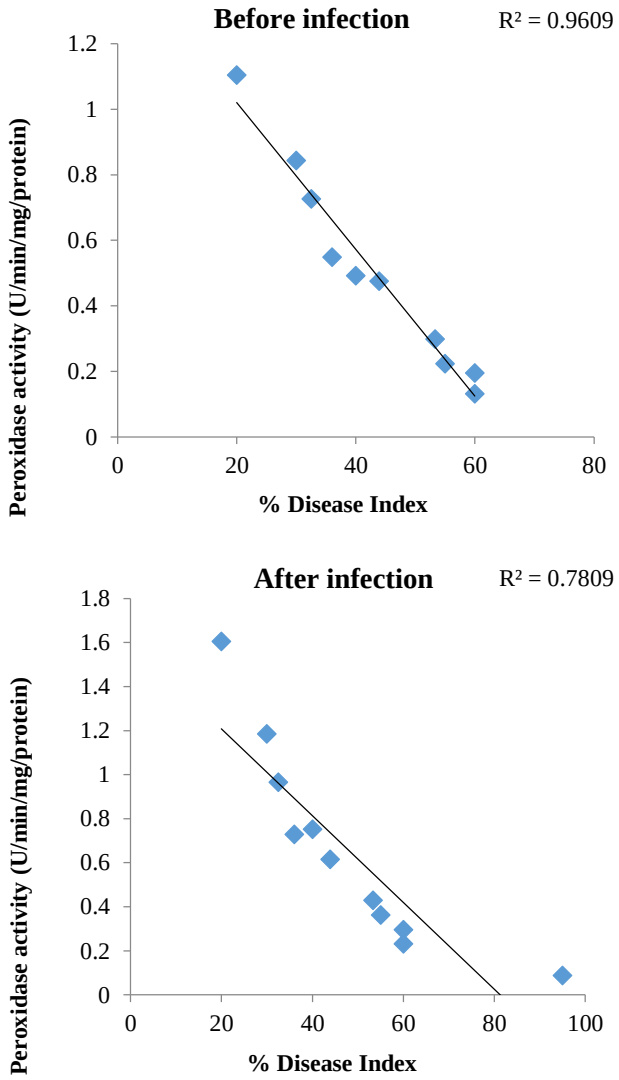

Fig. 3. Correlation between % DI and POD in different *Brassica* genotypes before and after infection with *A. brassicicola.*

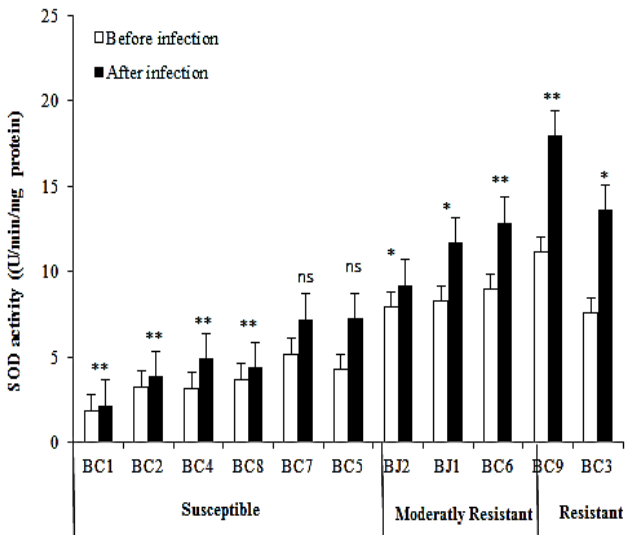

Fig. 4. SOD activity in *Brassica* genotypes pre and post infection with *A. brassicicola.* Each bar represents mean ± SE of three replicates. *, **, show significant difference between treatments of pre- and post-infection in *Brassica* at p≤0.05 and p≤0.01, level of significance, respectively.

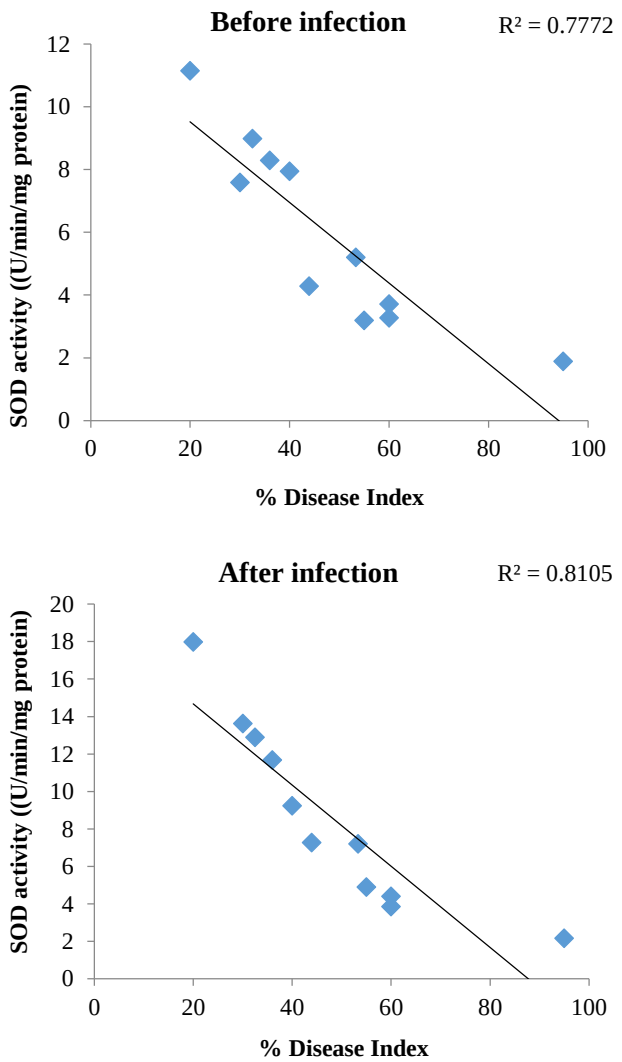

Fig. 5. Correlation between % DI and SOD in different *Brassica* genotypes before and after infection with *A. brassicicola*.

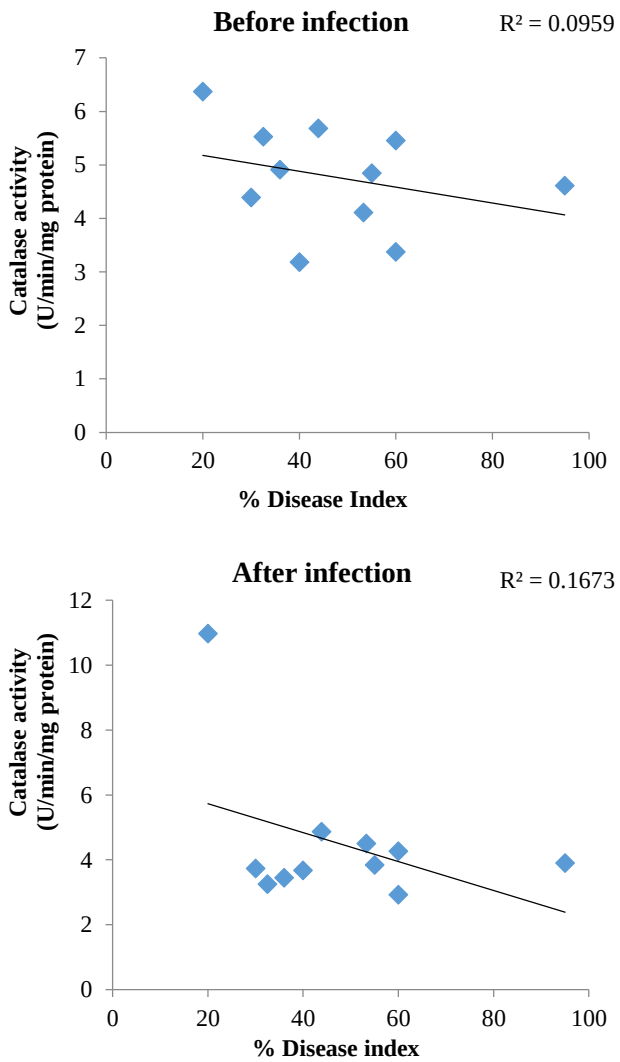

Fig. 7. Correlation between % DI and CAT in different *Brassica* genotypes before and after infection with *A. brassicicola.*

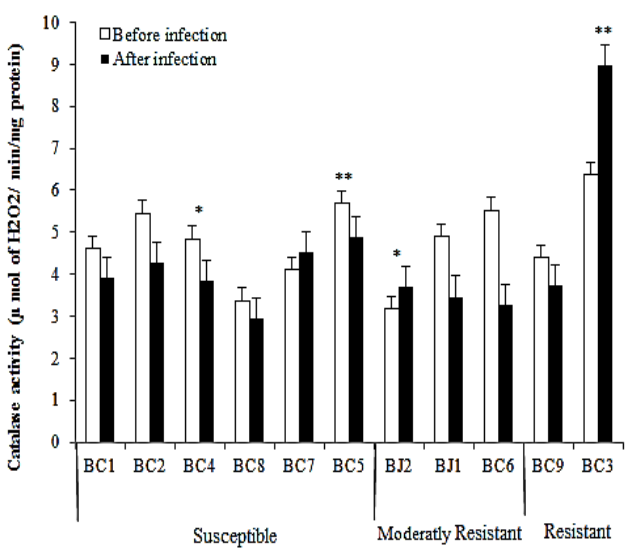

Fig. 6. CAT activity in *Brassica* genotypes before and after infection with *A. brassicicola*

Each bar represents mean ± SE of three replicates. *, **, show significant difference between treatments of pre- and post-infection in Brassica at p≤0.05 and p≤0.01, level of significance, respectively.

Discussion

Enzymatic antioxidants are important biochemical markers in plant species against biotic stress. Previously, rise in enzymatic antioxidants activity was involved in sustainability in plant growth and development under stress and acted as an indicator of disease resistance (Hameed *et al.,* 2010; Aftab *et al.,* 2015). Higher enzyme activity might be linked with recognition of phytopathogen via host receptors. Further, this recognition in plant hosts activates signaling cascades and transduction of these signals results in increasing activities of these antioxidant related defensive enzymes (Solino *et al.,* 2016).

These antioxidants scavenge excessive ROS produced during host pathogen interaction. In defense mode, higher antioxidants enzymes activities have been reported in plants against biotic stress and thus protect host cells from pathogen invasion (Mallick *et al.,* 2015). Besides, increasing biosynthesis of antioxidants and scavenging enzymes as defense mechanism, other biochemical became active, which directly or indirectly play role in host plant sustainability under biotic stress.

Under stress, induction of antioxidants enzymes such as POD, CAT and SOD is the utmost common apparatus for scavenging ROS (Mittler, 2002).

Plants PODs have been concerned with range of defense-linked mechanisms comprising cross-linking of phenolics, lignification, phytoalexin production and hypersensitive response (Parihar *et al.,* 2012). In plants, higher POD activity could be associated with infection (Gurjar *et al.,* 2015). POD has a dual part in disease resistance. They oxidize phenols to quinones, which are toxic to the pathogen and catalyze the polymerization of monolignols during cell lignification; an early response in hypersensitive cell death (Brisson *et al.,* 1994). The POD enzyme oxidized the phenolic molecules exerting toxic effect on phytopathogen, simultaneously playing their part in cell degeneration of tissues (necrosis) as resistance apparatus of plant (Mallick *et al.,* 2011, 2014). In current study, an increase in POD activity was observed in *Brassica* genotypes against ABSD. Highest POD activity was observed in BC9 which showed highest resistance against ABSD (Fig. 2). Previously, higher POD activity was reported with resistance to *A. brassicae* infection in Chinese cabbage (Rosta *et al.,* 2002); *A. triticina* in wheat (Tyagi *et al.,* 2008); *Alternaria* leaf blight of tomato (Hameed *et al.,* 2010) and against *Alternaria* blight in *B. juncea* (Parihar *et al.,* 2012). Thus, plants with high resistance showed high POD activity.

The plant capability to overcome oxidative damage partially relies on initiation of SOD activity and on regulation of other antioxidants (Alscher *et al.,* 2002). In current study, SOD activity increased significantly in *Brassica* genotypes against ABSD (Fig. 4). Enhanced ROS production acts as substrate that leads to an increase in SOD activity via enhancing SOD encoding genes expression. Results showed that highest post-infectional SOD activity was observed in BC9 followed by BC3, which might lead to resistance against ABSD. However, BC1 showed minimum SOD activity followed by BC2 that might be associated with lower potential of these genotypes to scavenge O_2^- after infection with *A. brassicicola*. SOD enzyme has protective action against oxidative stress under biotic stress and has higher activity in resistant genotypes (Mallick *et al.,* 2014,). SOD enzyme acts as defense mechanism by scavenging superoxide (Mallick *et al.,* 2011) and singlet oxygen (Mallick *et al.,* 2014). In host-pathogen interaction, antioxidant enzymes activity has direct correlation with host resistance mechanism (Mallick *et al.,* 2015). Co-regulation of SOD and POD under stress has been reported earlier (Abedi & Pakniyat, 2010). Such findings are similar to current findings that SOD and POD showed simultaneous increase and decrease in *Brassica* genotypes.

The present study indicated that CAT activity decreased in moderately resistant to resistant genotypes except BC3 on exposure to ABSD (Fig. 6). Reports on CAT activity under various stresses vary (Wilson *et al.,* 2014) and such undefined pattern has been previously reported (Kumar *et al.,* 2009; Abedi & Pakniyat, 2010). Similarly, CAT activity decreases in resistant genotypes against *Alternaria carthami* in safflower; *Alternaria blight* in *B. juncea* and root rot in cowpea (Chandra *et al.,* 2001; Parihar *et al.,* 2012; Mahadik & Mali, 2018). CAT activity was higher in susceptible genotypes, which accounted extreme disease severity in comparison to resistant genotypes by proposing that CAT might have not significant role against potato blight and tobacco mosaic virus (Mehdy, 1994) and *Alternaria* blight in *B. juncea* (Parihar *et al.,* 2012). This proposes that CAT activity was unaltered in *Brassica* genotypes due to its enzyme inhibition by ABSD or might not acted as H_2O_2 scavenging enzyme. Similar findings were obtained earlier that stress inhibited the CAT activity (Khedr *et al.,* 2003; Parihar *et al.,* 2012) or CAT was unable to operate as H_2O_2 scavenging enzyme (Mahadik & Mali, 2018). It may also be because of photo-inhibition of enzyme or linked with enzyme degradation initiated by peroxisomal proteases (Abedi & Pakniyat, 2010). Nevertheless, biological functions of post infectional CAT activity with *A. brassicicola* in plants have been challenging to verify and are not yet resolved.

Conclusion

Results confirmed that antioxidant defense ability and increase in its activity is mainly dependent on type of plant genotype under biotic stress. Different *Brassica* genotypes responded differently to POD, CAT and SOD activity in response to ABSD. The SOD and POD activities increases in genotypes which showed lower ABSD incidence in *Brassica*. These biochemical markers can be utilized as selection tool for breeding material used to develop resistant cultivars against ABSD in *Brassica*. Genotypes BC9 and BC3 exhibited higher antioxidants activities and showed resistance towards oxidative stress induced by ABSD, which can be utilized as resistant genotype for cultivation.

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