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Screening of Defense Response against *Alternaria brassicae* (Berk.) Sacc. causing Leaf Spot in Indian Mustard (*Brassica juncea* L.)

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ABSTRACT
The genetic biodiversity of a species rely on its genotype and metabolic activities in normal as well as in stress conditions. In terms of biotic stress, a biochemical investigation was accomplished for evaluation of defense responses against Alternaria brassicae in the different varieties of Indian mustard. A. brassicae (Berk.) Sacc. is the most destructive pathogen of mustard. It causes a highly destructive disease, Alternaria blight (leaf spot) in mustard and other crucifers. Regulation of defense mechanism against pathogen attack is majorly associated with reactive oxygen species that are produced by plant cellular metabolism. This screening revealed the activity of several enzymatic and non-enzymatic antioxidants that scavenge the 'oxidative-stress'. After infection, the ROS scavenging enzymes i.e. SOD, CAT, APX, GPOX and other antioxidative metabolites viz., total phenol, protein, proline, and chlorophyll content, were elevated in some of the genotypes which can be concluded as tolerant to biotic stress. However, in most genotypes, these antioxidants were found less active as these can be said as susceptible to the pathogen. Among all genotypes, RH-749 was found promising germplasm which showed higher activity in most of the biochemical parameters.
Keywords : *Sustenance, antioxidants, scavenging enzymes, oxidative stress, germplasm.*

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INTRODUCTION

Indian mustard (*Brassica juncea* L. 2n=36) is the key edible and economically important crop of the Brassicaceae family, having 113 known varieties released by Directorate of Rapeseed-Mustard Research till 2018. It is an annual, dicot and *Rabi* season oilseed crop also known as *Sarson*, requires a relatively cool temperature, a wet growing season and a dry harvest time duration. Following China and Canada, India is the third-largest producer country in the world with nearly 13 percent of total production. In India, it accounts for 69.37 lakh ha (22.2%) of the area and 82.5 lakh MT (22.65%) of the production among all oilseed crops, whereas Government of India pre-estimated in advance 84 lakh MT and productivity are reported about 1145kg/ha [1,2]. However, despite the rapid spread of the crop, a disheartening trend is that productivity has dropped, due to several biotic and abiotic factors resulting lowest yield level of our country in the world. Disease susceptibility is the major threat among all biotic factors that limit the yield. Among all major diseases, leaf spot caused by *Alternaria brassicae* (Berk.) Sacc. is one of the most widespread and destructive diseases of mustard resulting in significant yield losses ranging from 15% to 71% in productivity and 14% to 36% in oil content. The pathogen is highly influenced by weather, the highest recorded disease in moist seasons and regions with relatively high rainfall [3]. It was first reported by Dey [4] at Kanpur, U.P. that caused severe yield losses. For understanding the impact of this disease, it is important to study the physio-biochemical characters of the host plant which fluctuates during stress conditions. Among these biochemical parameters, reactive oxygen species (e.g. $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$,$^1O_2$) are most important, short-lived, highly reactive and partially reduced or activated forms of atmospheric oxygen (O_2) produced as by-products of aerobic metabolism

causing progressive oxidative damage and ultimately cell death [5]. It has been reported that plants have developed enzymatic and non-enzymatic systems for scavenging or regulating the ROS to maintain sustainability during environmental stress [6]. Higher activities of enzymatic and non-enzymatic antioxidants have been observed in mustard varieties resistant/tolerant to Alternaria blight and the function of these metabolites may require the activity of deterrence, poisoning or equivalence to physical defense mechanisms [7-9]. So, the introduction of cultivars having higher antioxidants and cell-protecting enzymes could be a reliable strategy to control this disease. However, several other approaches such as induction of drought stress and bio-agent, have been reported as management of this disease [10]. In this study, the biochemical defense responses of Indian mustards were screened against Alternaria blight infection compared to normal conditions.

MATERIAL AND METHODS

Pathogen culture. A strain of *Alternaria brassicae* (Berk.) Sacc. was isolated from infected mustard leaves of Varuna grown in the agriculture farm of Rajiv Gandhi South Campus, Mirzapur and identified based on its conidial morphology with the help of microscope and host specific pathogenicity. The isolates were inoculated on PDA medium (pH 5.6) and incubated at 25 ^{0}C in the BOD chamber for 14 days. The culture was purified and maintained by single spore method [11].

Inoculation of pathogen suspension on plants. The seeds of 10 varieties of mustard (*Brassica juncea* L.) were procured from the Department of Genetics and Plant Breeding, Banaras Hindu University, Varanasi, India. The seeds were surface sterilized with. 0.2% mercuric chloride ($HgCl_2$) and sown in treated soil containing FYM and sand (1:2:1). The seedlings were grown for 30 days with the application of water when required. The concentration of pathogen suspension was adjusted using a microscope to 30-50 conidia per microscopic field under 10X magnification. The conidial suspension was used for the inoculation of plants [3]. Lower leaf samples of healthy and infected plants were freshly collected from each variety for three consecutive days and washed twice with sterilized water. These samples: control without infection, 24 hours after infection, 48 hours after infection and 72 hours after infection were analyzed for following biochemical estimations on the same day when the samples were collected [12].

Biochemical analysis. Enzyme extract for superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and catalase were prepared by fresh amount of leaf samples (0.2 g) grinding in chilled condition with 2 ml extraction buffer (0.1 M phosphate buffer, pH 7.4, containing 0.5mM EDTA in case of SOD, CAT, and GPOX, and 0.5mM EDTA and 1mM ascorbic acid in case of APX). The crude extract was centrifuged at 15,000 g and 4 ^{0}C for 20 min and the supernatant was used for enzyme assay. The protein content (mg/g FW) was measured by the method of Lowry *et al*. (1951) [13]. The assay of the SOD, CAT, APX and GPOX were performed following the work of Kapadia *et al*. [12].

Enzyme extract for polyphenol oxidaseactivity was prepared from 0.1 g leaf sample homogenized in 1 ml of 0.1 M sodium phosphate buffer, pH 6.8. It was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme activity (µM/min/g protein) [14]. Phenol content (mg/g FW) was estimated by using Malick and Singh method [15]. Estimation of proline content (µM/g FW) was performed following the method of Bates *et al.* [16]. Chlorophyll and carotenoid content (mg/g FW) were estimated following the work of Sumanta *et al*. [17].

Statistical Analysis. All the biochemical parameters were analysed in three replications. The data obtained by biochemical constituents and enzymes determination were subjected to a simple completely randomized design (CRD) for study in the significance of various data.

RESULTS AND DISCUSSION

ROS scavenging enzymes. Reactive oxygen species (ROS) are aerobic toxic by-products, removed through antioxidants and antioxidative enzymes. Radical scavenger enzymes superoxide dismutase, catalase, ascorbate peroxidase, etc. keep under control the production of reactive oxygen species in affected but surviving nearby cells. However, by the host cell's hypersensitive response, the presence of active oxygen species also affects the membranes and cells of the advancing pathogen either directly or indirectly [18]. The current study has revealed the fluctuations of different antioxidants related to ROS.

The vast fluctuations in SOD activity among all genotypes were observed with different time duration (fig-1A). In 72 HAI, RH-749 genotype showed about 20 times greater SOD activity (12.41 unit/g protein) than in GIRIRAJ (0.61unit g-1 protein) while NRCHB-101 showed higher activity 15.57 unit/g protein and least in ASHIRWAD (1.65 unit/g protein) in 48 HAI. In 24 HAI, enzyme activity was higher in DIBYA (14.72 unit/g protein) while genotype ASHIRWAD (2.88 unit/g protein) had the lowest activity. In the control condition, higher enzyme activity was measured in RH-749 (10.42 unit/g protein) and lowest in JD-6 (2.34 unit/g protein). Upon biotic stress, a primary ROS superoxide anion ($O_2^{\bullet-}$) is formed that is dismutated into H_2O_2 with the help of SOD [5] in the mitochondria, chloroplast and cytosol of plant cells

[12]. Increased activity of this enzyme is correlated to tolerance in mustard plants under *A. brassicae* inoculated condition [10] and against abiotic stress [19]. In this study, RH-749 could be considered as defense potential genotype against *A. brassicae* attack.

Catalase activity was recorded maximum (16.79µM/min/g protein) in KRANTI nearly like RH-749 (16.22µM/min/g protein) while minimum (3.13µM/min/g protein) in NRCHB-101 at 72 HAI (fig-1B). In 48 HAI, GIRIRAJ (10.28µM/min/g protein) showed higher activity almost like RH-749 (9.81µM/min/g protein) while KRANTI (2.76µM/min/g protein) had least activity. Maximum catalase activity (19.45µM/min/g protein) was observed in DIBYA and lowest (1.95µM/min/g protein) was in MAYA in 24 HAI, while in control plants, DIBYA (13.21µM/min/g protein) showed maximum activity and minimum observed in GIRIRAJ (2.86µM/min/g protein).In plant cells, H_2O_2 is generated by electron transport chain system and photorespiration but enhanced under stress condition that is mainly decomposed into H_2O by catalase [5]. Elevation of catalase activity has reported in transgenic and resistant varieties [20] to Alternaria blight disease [21]. So, in 72 HAI, the elevated level of catalase in KRANTI nearly like RH-749 indicates that these germplasms have great potential to detoxify H_2O_2 under *A. brassicae* inoculated condition.

The highest APX activity was recorded in RH-749 (136.45µM/min/g protein) and KRANTI (131.03µM/min/g protein) while minimum (16.18µM/min/g protein) in JD-6 in 72 HAI. RH-8812 (111.42µM/min/g protein) had higher while GIRIRAJ (11.67µM/min/g protein) had the lowest enzyme activity in 48 HAI. In 24 HAI, maximum activity was observed in RH-749 (111.44µM/min/g protein) and minimum in NRCHB-101 (24.17µM/min/g protein). In a control condition, DIBYA (124.28µM/min/g protein) showed maximum activity while GIRIRAJ (41.93µM/min/g protein) had the lowest activity (fig-1C). APX actively detoxifies the excess H_2O_2 in the cytosol and organelles of tolerant variety than the sensitive one [20] [22]. Thus, higher activity of RH-749 and KRANTI in 72 HAI indicate that these genotypes might be actively involved in the excavation of excess H_2O_2 under stress conditions.

GPOX activity varied in all genotypes (fig-1D). In 72 HAI, PM-30 (59.96µM/min/g protein) andRH-749 (58.91µM/min/g protein) showed maximum enzyme activity while lowest was recorded in JD-6 (8.17µM/min/g protein). PM-30 (119.45µM/min/g protein) again showed the highest activity in 48 and 24 HAI while the lowest in JD-6 (4.15µM/min/g protein) and DIBYA (54.88µM/min/g protein) in 48 and 24 HAI, respectively. Under control condition, KRANTI (158.28µM/min/g protein) and PM-30 (155.13µM/min/g protein) showed maximum GPOX activity while minimal activity recorded in JD-6 (49.43µM/min/g protein). GPOX oxidizes pyrogallol and guaiacol with the utilization of H_2O_2 producing H_2O that is related to several important metabolisms in plant cells under biotic and abiotic stress [5]. In the subsequent infection phase, enhanced activity of GPOX has reported in transgenic genotypes than wild *B. juncea* lines leading to induced resistance against Alternaria blight disease [21]. In this experiment, GPOX activity decreased with disease progression in all genotypes, however, PM-30 and RH-749 would be involved in the stimulation of tolerance against this disease than the rest varieties.

With the progress of the infection, PPO showed an overall decreasing trend concerning their control condition at 48 HAI and 72 HAI. However, in 72 HAI some varieties displayed a slightly increase viz. KRANTI (0.25µM/min/g protein), ASHIRWAD (0.24µM/min/g protein), DIBYA (0.23µM/min/g protein), GIRIRAJ (0.20µM/min/g protein) and RH-749 (0.19µM/min/g protein) while least activity recorded in NRCHB-101(0.13µM/min/g protein) (fig-2A). PPO, a phenolic compound is poisonous to pathogens, often generates and accumulates at a faster rate after infection, particularly in a resistant plant variety compared to a susceptible one [18]. A clear increasing trend of PPO has reported with the infection progress in the *A. brassicola*-Brassiceae pathosystem [23] and in mustard genotypes treated with bioagent Trichoderma spp., followed by *Alternaria brassicae* inoculation compared to bioassay control [22]. So, KRANTI may be concluded as a tolerant line over all genotypes.

Antioxidant metabolites. A gradual increase of total phenol content was recorded in the infected genotypes as compared to control plants. A maximum phenol content was found in GIRIRAJ (85.92 mg/g FW), followed by RH-749 (79.72 mg/g FW) in 72 HAI while minimum in JD-6 (62.53 mg/g FW). In 48 HAI, NRCHB-101 (93.78 mg/g FW) had maximum content while minimum in DIBYA (64.41 mg/g FW). GIRIRAJ (99.09 mg/g FW) had found to have higher content and lowest in ASHIRWAD (67.22 mg/g FW) in 24 HAI. The highest content observed in PM-30 (63.78 mg/g FW) and lowest in NRCHB-101(40.34 mg/g FW) in control plants (fig-2B). The plant cells at the infection site die quickly in high concentrations of toxic phenolic compounds [18]. It accumulates at a higher rate in the infected sites of the plant than the healthy one [8]. The post-infection increase in phenolic contents could be due to their release by the enzymatic activity of host or pathogen [24]. In this test, any specific trend has not seen that could depict the resistance and susceptibility in all genotypes.

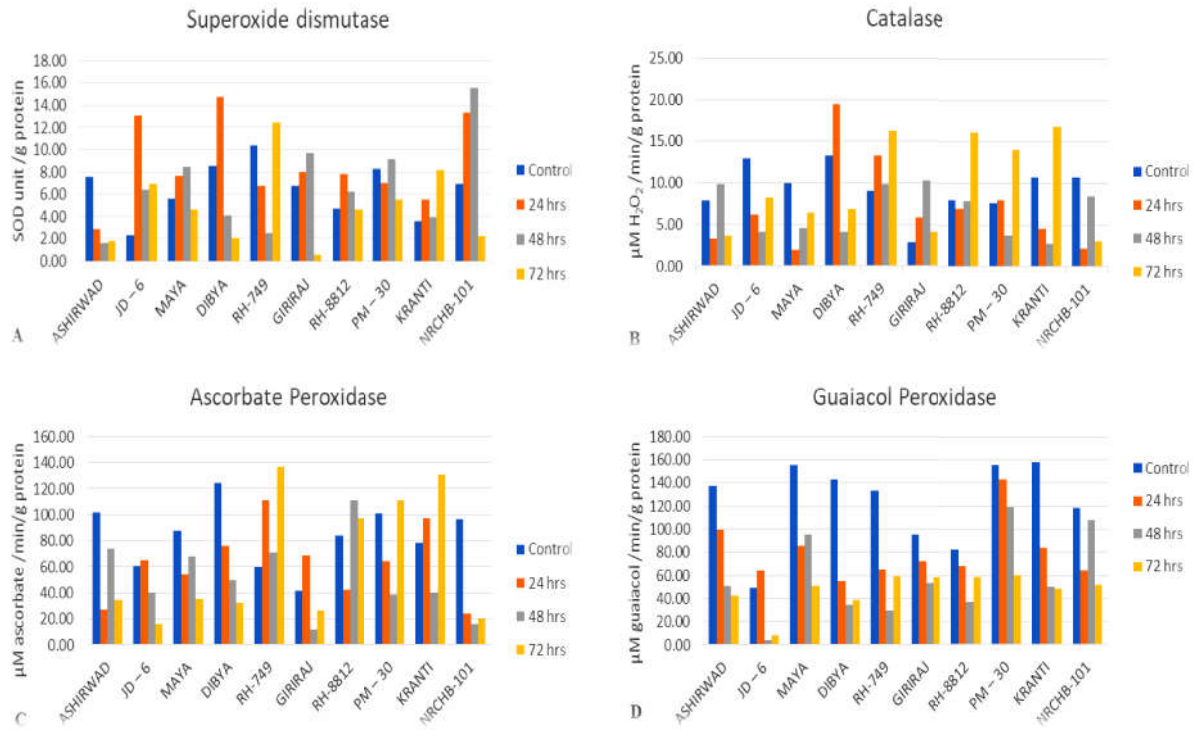
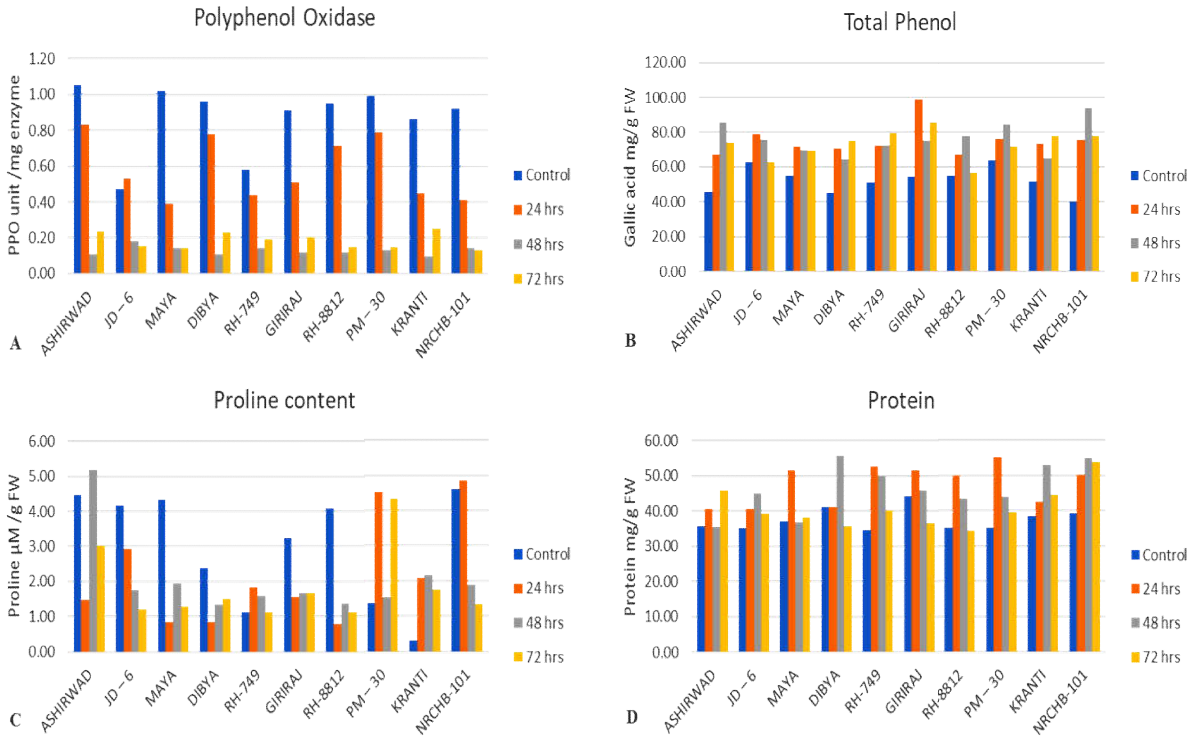


Figure 1. Changes in ROS scavenging enzymes activities in the leaves of Indian mustard after infection with *Alternaria brassicae* (Berk.) Sacc. at different time intervals. (A) Superoxide dismutase; (B) catalase; (C) ascorbate peroxidase; (D) guaiacol peroxidase.



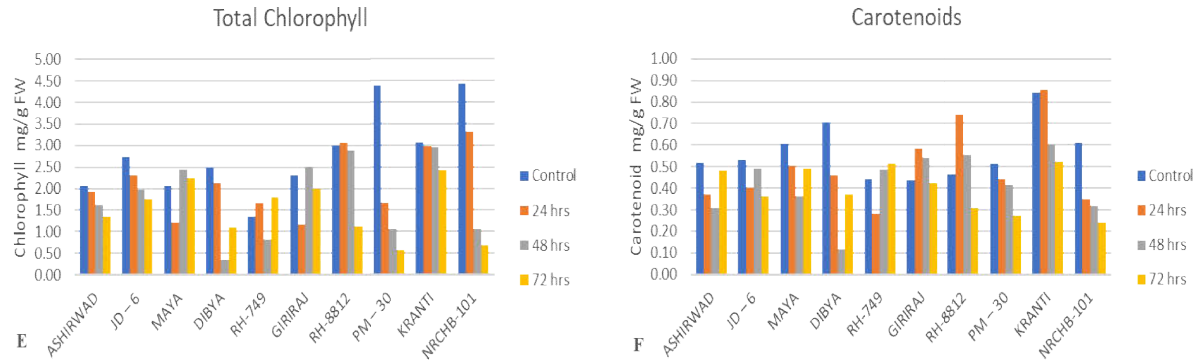


Figure 2. Changes in antioxidant metabolites in the leaves of Indian mustard after infection with *Alternaria brassicae* (Berk.) Sacc. at different time intervals. (A) Polyphenol oxidase; (B) total phenol; (C) proline content; (D) protein content; (E) total chlorophyll and (F) carotenoids.

A significant change observed in proline content in 10 varieties of mustard at all stages after infection (fig-2C). Mostly, the proline content declined at 72 HAI to all genotypes but PM-30 (4.33 µM/g FW) showed a significant increase while minimal content was observed in RH-8812 (1.11 µM/g FW). ASHIRWAD (5.18 µM/g FW) showed the highest content while RH-8812 and DIBYA had the lowest (5.18 µM/g FW) in 48 HAI. Higher proline content was seen in NRCHB-101 (4.86 µM/g FW) and PM-30 (4.51 µM/g FW) while RH-8812 (0.78 µM/g FW) had the lowest one in 24 HAI. NRCHB-101 (4.61 µM/g FW) showed a higher amount of proline in the control line while KRANTI (0.34 µM/g FW) found with the least content. The fungal infection triggers cellular protein denaturation and proteolysis, leading to the increased free amino acid level of host tissues. It also stimulates cell-protecting biosynthesis along with proteins engaged in antioxidant manufacturing [7]. Proline content increases after induction of bioagent to stimulate the resistance against Alternaria blight in Indian mustard [22]. So, in this estimation, higher proline accumulation was found in PM-30 that depicts *A. brassicae* had triggered proteolysis along with cell-protecting antioxidant generation in this genotype most than the rest varieties, while in RH-8812 production of antioxidant was least.

A consequent increase in protein content was obtained with all stages after infection with regards to the control condition (fig-2D). However, at 72 HAI, NRCHB-101 (53.72 mg/g FW) showed higher content while RH-8812 (34.41) had the lowest. DIBYA (55.61 mg/g FW) and NRCHB-101 (55.03 mg/g FW) showed increased content while decreased in ASHIRWAD (35.43 mg/g FW) at 48 HAI. In 24 HAI, PM-30 (55.18 mg/g FW) showed maximum content while ASHIRWAD (40.51 mg/g FW) showed the minimum. GIRIRAJ (44.29 mg/g FW) showed higher content in the control condition while RH-749 (34.70 mg/g FW) had the lowest. *A. brassicae* causes an increase in total protein content in Indian mustard. The higher enhancement of protein was seen in leaf spot resistant variety but less in susceptible lines [7]. So, here we can consider NRCHB-101 has involved in metabolic processes related to resistance against leaf spot disease and RH-8812 has less involved in resistance metabolism.

Maximum chlorophyll content was observed in KRANTI (2.43 mg/g FW) and minimum in PM-30 (0.58 mg/g FW) at 72 HAI. In 48 HAI, KRANTI (2.96 mg/g FW) again recorded as maximum chlorophyll content and DIBYA (0.35 mg/g FW) as the lowest. NRCHB-101(3.32 mg/g FW) showed a higher amount of chlorophyll and GIRIRAJ (1.16 mg/g FW) had the least amount in 24 HAI. However, in control, NRCHB-101 (4.43 mg/g FW) had higher content andRH-749 (1.36 mg/g FW) had the lowest content (fig-2E). Chlorophyll concentration was noticed to be decreased in this research due to disease caused by *A. brassicae*. So, KRANTI may have more pace of photosynthesis in 72 HAI compared to rest 9 genotypes. In the control condition, NRCHB-101 and PM-30 may have more rates of photosynthesis rather than the rest genotypes.), demonstrated that the reduction in chlorophyll concentration is regarded to be a symptom of the oxidative stress situation that is caused by a decreased rate of photosynthesis [25] (Barka and El-Matty, 2008). A greater chlorophyll level in *B. alba* (resistant) while decreasing content in susceptible mustard genotypes against *A. brassicae* has also reported [8].

The carotenoids level in mustard lines was all over decreased at 72 HAI, however, KRANTI (0.52 mg/g FW) and RH-749 (0.52 mg/g FW) showed higher content of carotenoids while least in NRCHB-101 (0.24 mg/g FW). In 48 HAI, maximum content was seen in KRANTI (0.60 mg/g FW) while a minimum in DIBYA (0.12 mg/g FW). The higher content was observed in KRANTI (0.86 mg/g FW&0.84 mg/g FW) again in 24 HAI and control while lower in RH-749 (0.28 mg/g FW& 0.44mg/g FW), respectively (fig-2F). Carotenoids function as accessory pigments in light-harvesting in chloroplasts, but they play a more

significant function in detoxifying active oxygen and triplet chlorophyll generated by the light because of exciting photosynthetic interactions. Higher carotenoid content has been correlated with the regulation of 'oxidative-stress' in Indian mustard [26]. So, KRANTI and RH-749 might have greater potential to play a significant role in degrading active oxygen species after infection rather than other varieties.

CONCLUSION

As in stress condition, ROS level enhances that results in oxidation of proteins, lipid peroxidation, DNA damage, enzyme inhibition and ultimately triggers programmed cell death pathways. As the alteration in all biochemical aspects was observed with respective responses in the host plant against a pathogen, thus, based on their functions, a fluctuation can be induced through relevant approaches in these biochemical parameters for regulating the stress condition. However, variety RH-749 followed by KRANTI showed comparatively satisfactory involvement in most of the parameters, so, it can be considered as a promising cultivar in the disease-prone region while NRCHB-101 was found most susceptible against *Alternaria* blight. Although the field experiment must be evaluated for the confirmation of the respective genotype's tolerance level. This biochemical study could be extended at the molecular level to visualize and understand the mechanism of regulatory molecules of oxidative stress.

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