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Influences of growth stages on peroxidase and phenol production in Indian mustard exposed to *Alternaria brassicicola* infections

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Abstract
Investigations were undertaken to understand the effect of growth stages on peroxidase and total phenol production in three *Brassica juncea* genotypes after inoculations with *Alternaria brassicicola*. From the study, it was observed that peroxidase showed significant differences for growth stages among the genotypes after 18 hours post inoculation only. Among the genotypes, BRRM-101 and Varuna showed high level of peroxidase activity in comparison to PM-25. However, it was higher in BRRM-101 than Varuna at both the growth stages i.e., 0.974 folds and 1.213 folds greater at 40 DAS and 55 DAS respectively. The phenol production presented significant differences between the growth stages among the genotypes at 24 hours post inoculation while it was significant among the genotype only at 18 hours post inoculation. At 24 hours post inoculation, the activity of total phenol in partial resistant genotypes BRRM-101 was found 0.870 folds higher than Varuna at 40 DAS but was reduced at 55 DAS. This study indicates that total phenol likely plays an important role in later stages of defense response producing more stable secondary metabolites and peroxidase being short lived acts in early stages of defense response and is likely to be involved in signaling and induction of stable responses during pathogenesis.

Keywords: *Alternaria brassicicola*, defence response, growth stages, peroxidase, phenol

Introduction
Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is one of the most important oilseed crops and shares 23.5% area and 24.2% production of total oilseeds in the country. Being the third largest producer (11.3%) of oilseed brassica in the world, it meets 57% of the domestic edible oil requirements. The major constraints in production are the biotic and abiotic stresses (Jat *et al*. 2019) [6]. Among the biotic stress due to Alternaria leaf spot infection yield losses up to 47% were reported (Kolte, 1985) [9]. Under delayed sown conditions as a result of delays in harvesting in paddy crop leads to exposure of the early growth stages of the crop to warmer conditions. The preference of *Alternaria brassicicola* to warmer incubation conditions may lead to dominance of this pathogen under warmer conditions (Sinha *et al*. 2021) [17]. Alternaria leaf spot attack induces various types of biochemical alterations in plant. These alterations form the basis of biochemical mechanisms for the host-pathogen interactions. Information regarding biochemical changes associated with pathogenesis can help to devise suitable management strategies. Induced biochemicals like peroxidases, phytoalexins, PR proteins, phenols, lignins are expressed during pathogenesis (Kushwaha *et al*. 2018) [11]. Expressions of plant peroxidase have been implicated in a variety of defense related processes including hypersensitive reaction, lignification, cross-linking of phenolics and glycoproteins, suberisation and phytoalexin production (Kushwaha *et al*. 2016; Baysal *et al*. 2003; Boudjeko *et al*. 2005) [10, 1, 2]. Genetic resistance in rapeseed-mustard against Alternaria blight is reported to be partial and may provide considerable advantage in disease management. The present study of biochemical changes at different stages of crop growth could help in identifying the mechanism of resistance against necrotrophic pathogen *A. brassicicola* and also help in exploring possible role of biochemical marker for identification of partially resistant source.

Materials and Methods
Test pathogen
The pathogen *Alternaria brassicicola* was isolated from diseased leaves of *Brassica juncea* from BAU, Sabour research farm. Pure culture of the pathogen was developed using hyphal tip method. After confirming pathogenicity, the virulent culture was maintained on slants at 4 ºC for further work.

Plant material for biochemical estimation

Based upon information on screening of fifteen genotypes of Indian mustard under late sown condition, three genotypes i.e., BRRM-101 (low disease severity), Varuna (high disease severity) and PM-25 (check variety for late sown condition) were selected for biochemical analysis (Naseem *et al*. 2023) [14]. Each selected genotype for biochemical analysis were planted in staggered dates of sowing (17th November, 2020 and 01st December, 2020) so as to coincide the different growth stages i.e., 40 DAS and 55 DAS for the biochemical assay at a single time point. Three rows of plant for each genotype at each of the date of sowing represented three replications. The experiment was laid in factorial Randomized Block Design (RBD). For biochemical estimations, single leaf of each selected genotype of each growth stages were artificially inoculated under detached leaf assay and sampled separately at 18 hours and 24 hours post inoculation. All the experiments were carried out in triplicates.

Method of inoculation

Detached leaf techniques of inoculation were used according to method described by Doullah *et al*. (2006) [4] for artificial inoculation followed by biochemical estimations. Upper 5th true leaf was collected from each genotype of 40 and 55 days old plants. After that, detached leaves were washed with distilled water to remove dust particle and excess moisture was dried using tissue paper, and placed on sterilized Petri plate (abaxial surface up) of size 9 cm having three layers of moistened blotter paper (previously sterilized) with distilled water to maintain high humidity. Then the concentration of conidial suspension 1×10^5 conidia mL^{-1} were taken and then drop inoculated on the detached leaf using sterilized syringe at four point of leaf and then Petri plates were wrapped with parafilm and marked and then incubate at 25 °C which were sampled separately at 18 and 24 hours post inoculation. After incubation, artificially inoculated leaves which contain only four bits of spotted area having size of 10 mm were for used for estimation of peroxidase and total phenol.

Biochemical estimation

Estimation of Peroxidase: Peroxidase activity was assayed by standard procedure given by Hammerschmidt and Kuc (1982) [5]. For this, only one gram of artificially inoculated leaf sample was collected from each growth stages of selected genotypes and extracted with 1 mL of 100 mM potassium phosphate buffer (pH-7) and centrifuged at 10,000 rpm for 15 min at 4 ºC. After that, the supernatant was separated and used for peroxidase test. The reaction mixture (3 mL) consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH-7) containing 10 mM hydrogen peroxide. The addition of 25 ml crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 470 nm. One unit of PO enzyme activity is defined as the increase in absorbance by one optical density (OD) at A_{470} per min.

Total Phenol estimation: Total phenol was estimated according to standard procedure given by Kofalvi and Nassuth (1955) [8]. For this, 0.5g of inoculated leaf sample of each growth stages of selected genotypes was collected and extracted with 1 mL of 50% methanol and kept in water bath at 80 ºC for 90 min, then the material was centrifuged at 14,000 rpm for 15 min at 4 ºC. After that, supernatant was separated and used for phenol test. The reaction mixture consists of 0.1 mL leaf extract, 0.9 mL distilled water, 0.5 mL of 1N Folin & Ciocalteu's Phenol Reagent and 1 mL of 20% sodium carbonate which was kept for 20 min and then reading was taken using spectrophotometer at 725 nm.

Statistical Analysis

The results were analysis using statistical package IBM SPSS Statistics for Windows, version 23.0 for Analysis of Variance (ANOVA) for the test of significance of mean at 1% level of significance ($p\leq0.01$). Here, absorbance in optical density (OD) is taken as relative measure of peroxidase and total phenol production in two different genotypes of Indian mustard when compared with susceptible check Varuna.

Results

The biochemical studies presented in (Table 2) showed that there is a significant change in peroxidase activity among genotypes and growth stages at 18 hours post inoculation period. However, the peroxidase activity was non-significant at 24 hours post inoculation. Differences in peroxidase activity at 18 hrs post inoculation indicates that these enzymes act as an indicator of early defense response to infections by *Alternaria brassisicola* on Indian mustard. Level of peroxidase in all three genotypes was almost showing same trends but slightly higher in early growth stages in comparison to later growth stages. This may indicate that at early growth stages i.e. at 40 days after sowing the enzymatic expression was more intense when compared to later growth stages. Among the genotypes, BRRM-101 (1.020, 0.820) and Varuna (1.047, 0.676) showed high level of peroxidase activity in comparison to PM-25 (0.286, 0.219) at both growth stages i.e., 40 DAS and 55 DAS respectively (Table 1).

It was observed from data (Table 2) that activity of total phenol was significantly influenced by genotypes, growth stages and its interaction after 24 hours post inoculation while it was only significant among genotypes after 18 hours inoculation period. This indicated that genotypes varying in phenolic production in response to infections may represent difference in their inherent capacity to resist infection by *A. brassicicola.*

It is evident from the Table 1, that at 18 hours post inoculation, the level of total phenol was higher in Varuna (0.606, 0.684) followed by BRRM-101 (0.466, 0.443) and PM-25 (0.622, 0.676) in both growth stages (40 DAS and 55 DAS). Table 1 indicated that at 24 hours post inoculation, total phenol was observed maximum in genotypes Varuna (0.626) followed by BRRM-101 (0.540) and PM-25 (0.408) at early growth stage (40 DAS) but at later growth stage (55 DAS) level of total phenol decreases in BRRM-101 genotype (0.470) while it increases in Varuna (0.822) and PM-25 (0.449).

Table 1: Effect of growth stages and genotypes on peroxidase (OD) and total phenol production (OD) in Indian mustard at 18 hours and 24 hours post inoculation infected by *A. brassicicola*.

		Post Inoculation Period			
		Peroxidase		Phenol	
Growth stages	**Genotype**	**18 h**	**24 h**	**18 h**	**24 h**
G1 (40 DAS)	Varuna	1.047±0.132*	0.590±0.135	0.606±0.079*	0.626±0.077
	BRRM-101	1.020±0.118	0.744±0.182	0.466±0.008	0.545±0.024
	PM-25	0.286±0.085	0.637±0.138	0.622±0.012	0.408±0.032
G2 (55 DAS)	Varuna	0.676±0.153	0.829±0.153	0.684±0.210	0.822±0.073
	BRRM-101	0.820±0.075	0.711±0.209	0.443±0.067	0.470±0.069
	PM-25	0.219±0.038	0.565±0.263	0.676±0.098	0.449±0.061
CD (0.01) Genotypes		0.216	NS	0.183	S
CD(0.01) Growth stages		0.177	NS	NS	S
CD(0.01) Growth stages × Genotypes		NS	NS	NS	0.14

*mean±SD (based on three replicates)

Table 2: Analysis of variance for effect of growth stages and genotypes on peroxidase production (OD) and total phenol production (OD) in Indian mustard infected by *A. brassicicola* at 18 hours and 24 hours post inoculation.

		Mean Sum of Squares			
		Peroxidase		Phenol	
Source of error	**Df**	**18 hpi**	**24 hpi**	**18 hpi**	**24 hpi**
Replication	2	0	0.001	0.008	0.006
Growth stage	1	0.204*	0.009	0.006	0.014*
Genotype	2	0.820*	0.028	0.074*	0.139*
Growth stage × Genotype	2	0.035	0.043	0.004	0.027*
Error	10	0.001	0.260	0.010	0.003
Total	18				
Corrected Total	17				

*****Significant difference at 1% probability ($p \leq 0.01$)

Discussion

The biochemical mechanism involved in plant disease resistance is a complex phenomenon which involves many key molecules like free oxygen radicle and peroxidase which is reported to be acting at an early stage of host signaling and defense response. Increase in peroxidase activity in host plants following pathogen invasion, has been reported by several other workers (Johnson and Lee, 1978) [7]. Instead of the amount of peroxidase it is the timing of their expression that is more important in limiting the pathogen attacks i.e., it acts as early defense response in plants to pathogen attack. Significant difference in peroxidase activity among the genotypes and growth stages were observed at 18 hours post inoculation which indicates that this enzyme might be differentially express in different genotypes in response to pathogen's attack at an early stage of pathogen's ingress. Whereas at 24 hours post inoculation no significant difference was observed for the test parameters. Among the genotypes at 18 hours post inoculation, BRRM-101 and Varuna showed high level of peroxidase activity in comparison to PM-25. However, it was higher in BRRM-101 than Varuna at both the growth stages i.e., 0.974 folds and 1.213 folds greater at 40 DAS and 55 DAS respectively. One of the supportive suggestions provided by Madadkhah *et al*. (2012) [13] who found that a rapid increase in peroxidase activity in both resistant and susceptible melon genotype were registered after inoculation with *F. oxysporum* race 1.

Reports suggest the increase in the level of peroxidases leads to generation and detoxification of reactive oxygen species, which are directly toxic to the pathogen and can also reduce the spread of the pathogen by increasing the cross linking and lignifications of the plant cell walls, suberization, downstream signaling molecules and act as one of the mechanisms of plant defense against various pathogens (Clark *et al*. 2000; Baysal *et al*. 2003; Kushwaha *et al*. 2016) [3, 1, 10]. An increase in peroxidase activity is often associated with an enhanced phenolic incorporation into the cell wall that helps in reinforcement of cell wall whose levels are naturally high in the resistant varieties of many crops (Saini *et al*. 1988; Onyeneho and Hettiarachchy, 1992) [15, 16]. Total phenol varied significantly among the genotypes after 18 hours post inoculation while significant effect of growth stages, genotypes and its interaction were observed at 24 hours post inoculation. This finding reveals that phenol may play a role in host defense, at later stages of pathogenesis. The susceptible genotypes showed higher total phenol at 18 hours post inoculation as well as 24 hours post inoculation than in BRRM-101. The activity of total phenol in BRRM-101 was 0.870 folds higher than Varuna at 40 DAS but it slightly reduced at 55 DAS. Here, the growth stages did not show any significant difference for total phenol. It was observed that since phenol are translated into other defense molecules leading to cell wall fortifications through production of secondary metabolites it is likely that total phenol were rapidly translated into other defense molecules in partial resistant genotype thereby resulting in lower total phenol levels in BRRM-101 than in susceptible genotype Varuna. Similar results on reduced total phenols in partially resistant lines of pea against rust pathogen were also observed as a result of rapid lignification of cell walls (Kushwaha *et al*. 2016) [10]

Conclusion

Our study revealed that peroxidase acts as an early stage of defense response and phenol acts as at a later stage of defense response during pathogenesis with *A. brassicicola* on Indian mustard. Higher level of peroxidase present in both growth stages (40 DAS and 55 DAS) at 18 hours post inoculation

were in support of the above conclusion. The level of total phenol was higher at 24 hours post inoculation in early growth stage (40 DAS) which is slightly reduced at later growth stages (55 DAS) in partial resistant genotype BRRM-101. This low level of phenol at later stages of growth shows that it is translated into other defense molecule. These changes like peroxidase and phenol at different growth stages may be involved to produce low disease severity in BRRM-101 genotype under infections by *A. brassicicola* under warmer conditions.

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