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Biochemical defense mechanism in rapeseed-mustard genotypes against Alternaria blight disease

P. Mathpal[1], H. Punetha[1], A.K. Tewari and S. Agrawal[1]

[1]*Department of Biochemistry, College of Basic Science and Humanities*
[2]*Department of Plant Pathology, College of Agriculture, GBPUAT, Pantnagar 263 145*
[*]*Corresponding author: punetha_hp@rediffmail.com*

Abstract

The present study deals with biochemical defense response in rapeseed-mustard genotypes infected with *Alternaria brassicae* (Berk.) Sacc. causing blight at different growth stage. Three genotypes viz. *Brassica juncea* cv. Varuna (susceptible), *B. juncea* cv. PAB-9534 (moderately resistant) and *B. alba* (tolerant) were selected. The results revealed that all the genotypes showed variable disease severity. It was observed that in all stages of pathogen infection, disease severity and characteristic symptoms were more prominent in susceptible genotype than the other two. The biochemical analysis of leaves of different varieties of mustard revealed that total phenol, o-dihydroxy phenol, total sugar, reducing sugar, chlorophyll content and flavonol contents were observed to be more in resistant genotype (*B. alba*) than others. With progress of infection total phenol, o-dihydroxy phenol and protein content increased in all three genotypes while the chlorophyll, total sugar, reducing sugar and flavonol content decreased. The results indicated that factors conditioning the host response to *A. brassicae* might be the outcome of complex biochemical changes operated in host genotypes.

Key words: *Alternaria brassicae, Brassica juncea, B.alba, biochemical changes, defence mechanism*

Introduction

Oilseed crops play an important role in agriculture economy of India which constitutes the second largest agriculture product next to food grains in the country. Indian mustard (*B. juncca*) is a major crop of oilseed Brassica, which fulfils nearly 29.2% of national oilseed crop outputs (Nashaat *et al*., 2007). Among the foliar blight disease complex, leaf blight caused by *A. brassicae* is the major yield and quantity reducing factors of mustard. Depending upon severity, the yield losses have been estimated to range from 35 to 46% (Kolte *et al*., 1987). Alternaria blight disease caused by *A. brassicae* has been reported from all the continents of the world affects most cruciferous crops and is one among the important diseases of rapeseed mustard causing severe yield losses with no proven source of transferable resistance in any of the hosts (Meena *et al*., 2010). *A. brassicae* is a necrotrophic pathogen which produce lesion on leaves, stem and siliquae affecting seed quantity as well as quality by reducing oil content, seed size and seed colour. This disease may cause significant losses in both temperate and tropical *Brassica* crops.

Although resistance to Alternaria blight has not been reported in any cultivated *Brassica* species, but a high degree of resistance against the pathogen has been reported in *B. alba* (Conn *et al*., 1988). Resistance to Alternaria blight in mustard has been found to be associated with leaf enzymes associated with the phenolic pathway, higher leaf sugar contents (Singh *et al*., 1999) and high deposits of leaf epicuticular wax forming a hydrophobic coating to reduce the adherence of water-borne inoculum, conidia germination and germtube formation (Saharan, 1992). The species *B. napus*, *B. carinata* and *B. alba* have relatively more epicuticular wax than *B. rapa* and *B. juncea* and tend to be less sensitive to Alternaria blight (Conn *et al.*, 1984; Tewari, 1986). Understanding the mechanism employed by plants to defend them-

selves against pathogens may lead to novel strategies to enhance disease resistance in crop plants. Disease resistance depends upon the induction of defenses following exposure to organisms. Several types of biochemical changes which include the level o f phenolics was found to be important in the investigation of resistance mechanism (Arora & Wagle, 1985 and Meena *et al*., 2008). The aim of this research was to explore the possibility of biochemical changes for defense and symptomological changes in the three genotypes of oilseeds Brassica infected by *A. brassicae* under natural conditions at different stages of Alternaria blight disease development.

Materials and Methods

The analysis of biochemical parameters were carried out in the Department of Biochemistry and symptomatology was studied at Crop Research Centre, GBPUA&T, Pantnagar. Healthy and diseased leaves of three genotypes were collected from the field at 60, 80 and 100 days after sowing (DAS). The leaf samples were thoroughly washed with tap water followed by distilled water. Leaf samples were dried at 50-60 ºC for 2-3 days in a hot air oven to remove any moisture present on it and ground in mortar and pestle to make the powder. The leaf powder was stored in sealed vials and was used for the analysis of biochemical parameters. The experiment was laid out in a randomized block design (RBD) with three replications. Critical differences were calculated at 5% probability level of significance.

Study of symptoms

Symptoms produced by *A. brassicae on B. juncea* (Varuna and PAB-9534) and *B. alba* were observed at 60, 80 and 100 DAS. The sample of 10 leaves from each replication of each treatment were selected randomly and observations on disease severity, number of spots per leaf, size of spot, formation of concentric rings, number of concentric rings per spot, colour of spot and yellow halo formation around the spot was determined visually.

Biochemical stidies

Determination of total phenols

Total phenols were estimated following the methods described by Bray and Thorpe (1954). A sample of 0.5 g from each replicate sample was ground in 10-times volume of 80 per cent ethanol in mortar and pestle and centrifuged at 10,000 rpm for 20 min. A sample of 20 µl was taken for total phenols, estimated colorimetrically with Folin-Ciocalteu reagent. The absorbance was taken at 650 nm against a reagent blank and catechol was used as standard.

Determination of o-dihydroxy phenols

o-dihydroxy phenols were estimated using the method described by Mahadevan and Sridhar (1986). For the estimation, 200 µl of aliquot was used along with Arnow's reagent. The absorbance was measured at 515 nm using catechol as standard.

Determination of flavonols

The flavonols were estimated colorimetrically at 500 nm, based on the method described by Swain and Hills (1959) by using phloroglucinol as standard.

Determination of total soluble proteins

The total soluble proteins from the leaves were analyzed based on the method described by Bradford (1976). The sample of 1 g from each replicate was ground in pre-chilled mortar and pestle by using pestle and then centrifuged at 10,000 rpm at 4ºC for 20 minutes. The absorbance was recorded at 595 nm, BSA was used as standard.

Determination of chlorophyll content

The chlorophyll content was estimated (Hiscox and Israelstam, 1979) by adding 10 ml of DMSO (Dimethyl sulphoxide) to 50 mg finely chopped fresh leaves. The test tubes were then heated at constant temperature of 65 ºC for 3 hrs in an oven or water bath. The content was shaken once or twice during incubation period. After 3 hrs, absorbance was taken at 645 nm & 663nm The blank taken was of pure DMSO.

Estimation of total sugar

Total sugar was extracted from healthy and diseased leaves with 80% ethanol and estimated using anthrone (Sadasivam and Manickam, 1992). Total sugars were measured as glucose equivalent after comparing with the standard curve prepared from standard glucose and expressed as mg g-1 fresh weight of tissue at 630 nm.

Estimation of reducing sugar

The reducing sugars were estimated based on 'the protocol described by Somogyi (1952). The absorbance of blue colour was read at 620 nm and standard curve was prepared by using glucose.

Results and discussion

In the present study various pathological symptoms were observed to be more prominent in case of susceptible variety followed by moderately resistant and least in resistant (Table1).The maximum disease severity was observed in Varuna (64 %) followed by PAB-9534 (30%) and least in *B. alba* (5%) at 80 DAS. The colour of lesions caused by *A. brassicae* varies from brown/ dark brown to black on different species of mustard. It was some what dark brown in Varuna at all stages of growth surrounded by black bordered area around the spots. In *B. alba* the colour of spot was not much dark at any stage where as PAB-9534 has combination of dark and light brown colour. Larger sizes of spots were observed in susceptible genotype as compared to resistant genotype and it was increased with the age of plant and with increase in disease severity in all the three genotypes. The size of spots was observed to be larger in Varuna (4.3mm, 5.5mm and 5.8 mm at 60, 80 and100 DAS respectively) in all stages and it was increased with increase in plant age and disease severity. Size of spot indicated that it could be used as a parameter for the study of host-pathogen interaction. The similar study was reported by Kadian and Saharan (1983) and observed that larger lesions developed on susceptible genotypes than resistant *Brassica* species. Maximum number of spots was observed in Varuna (265 at 80 DAS) and was significantly higher as compared to PAB-9534 (134) and *B. alba* (22). The number of spots per leaf increased from 60 to 100 days after sowing. Number of lesions per unit had been extensively used to evaluate the resistant in different crops with respect to many foliar pathogens and in case of rapeseed-mustard against *A. brassicae* (Saharan and Kadian, 1983). The highest numbers of concentric rings were observed in spots of Varuna were 5, 8 and 9 at 60, 80 and 100 DAS respectively as compared to other genotypes at all stages of growth and it was increased with age of plant and increased disease severity. The typical concentric rings that developed in Alternaria leaf spots (usually in cases of susceptible reaction) are due to successive spread of fungal invasion across the tissue barrier of the host (Mondal *et al.*, 2007). The necrotic area was surrounded by yellow halo zone which was very clear in case of Varuna than the other two genotypes at all stages of growth. In *B. alba,* the formation of yellow halo was not much prominent at any stage as compared to Varuna followed by PAB-9534.The very prominent formation of concentric rings in the lesions and a zone of yellow halo around the lesions is justify by the work reported by Kadian and Saharan (1983). The shape of spot is mainly rounded in all three genotypes.

Plants have developed an arsenal of defence mechanisms to protect themselves against pathogen attacks. Infection by *A. brassicae* was associated with a marked increase in the phenolic contents of diseased tissues. A gradual increase in total phenolic contents was noticed with the progress of disease development. The resistant genotype *B. alba,* had highest amount of total phenols in healthy and infected leaves in all stages of progressive growth of plant and it was observed to be 7.20 mg/g at 80 DAS in infected stage and least in Varuna in all stages. The total phenols were higher in infected leaves than healthy. The post inflectional increase in phenolic contents could be due to their release from glycosidic esters by the enzymatic activity of host or pathogen (Noveroske et al., 1964), or due to migration of phenols from non-infected tissues (Farkas and Kiraly, 1962). Similar trends were also observed for the contents of o-dihydroxy phenols. The o-dihydroxy phenol contents was highest in *B. alba* in all stages of growth in healthy

Table 1: Symptomological changes observed in *B. juncea* (Varuna and PAB-9534) and *S. alba* during *A. brassicae* disease development

Pathological parameters	Name of Genotypes (60DAS)			CD 5%	Name of Genotypes (80 DAS)			CD 5%	Name of Genotypes (100 DAS)			CD 5%
	Varuna	PAB 9534	*S. alba*		Varuna	PAB 9534	*S. alba*		Varuna	PAB 9534	*S. alba*	
Disease severity	3.45	0.83	0.70	0.18	64	30	5	1.30	78	55	32	1.30
No. of spots	41.0	8.00	7.00	2.14	265.0	134.0	22.00	4.28	287.0	240.0	50.00	3.70
Size of spots	4.30	2.40	2.10	0.13	5.50	3.60	2.30	0.17	5.80	3.50	2.70	0.21
No. of concentric rings	5.00	3.00	0	0.65	8.00	5.00	0	1.30	9.00	6.00	3.00	2.34
Colour of spots	Light and Dark Brown	Brown	Very light brown	-	Light, dark Brown or Black	Dark Brown	Dark Brown	-	Dark Brown or Black	Brown	Light Brown	-
Yellow Halo formation	Yes	Rare	No	-	Yes	Yes	Yes	-	Yes	Yes	Yes	-
Shape of spots	Round	Round	Round	-	Irregular and Round	Oval and Round	Round	-	Rectangular, Round, Irregular	Oval and Round	Oval and Round	-

Table 2. Total Phenol, o-dihydroxy phenol and Flavonol contents in the healthy (H) and infected (I) leaf samples of *B. juncea* and *S. alba*

Genotype	Total phenol (mg/g of dry leaf powder)						o-dihydroxy phenol (mg/g of dry leaf powder)						Flavonol content (mg/g of dry leaf powder)					
	60DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS	
	H*	I*	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I
Varuna	3.920	4.200	4.180	5.400	4.500	6.190	0.180	0.240	0.220	0.280	0.260	0.470	0.149	0.122	0.132	0.106	0.108	0.080
PAB-9534	5.030	5.650	5.270	6.500	5.910	7.900	0.230	0.290	0.250	0.360	0.320	1.010	0.185	0.161	0.173	0.148	0.130	0.110
S. alba	5.280	6.400	5.550	7.200	6.210	8.600	0.260	0.350	0.310	0.450	0.360	1.250	0.212	0.190	0.194	0.176	0.176	0.156
CD (5%)	0.047	0.038	0.039	0.119	0.060	0.136	0.052	0.037	0.098	0.122	0.021	0.203	0.008	0.006	0.009	0.006	0.008	0.006

and infected leaves (0.450mg/g at 80 DAS) and it was least in Varuna at all stages (0.280 mg/g at 80DAS) and it was observed to be increased with progress of infection and with increase in plant age. Several workers have implicated o-dihydroxy phenol concentration as a resistance factor because they become highly reactive upon oxidation and may form substances toxic to pathogens or inactivate enzymes including hydrolytic enzymes produced by plant pathogenic fungi (Patil and Dimond, 1967). The oxidation of o-dihydroxy phenols in resistant plant varieties may stimulate the active defence reaction, while such reactions may be less strong in susceptible genotypes (Ravise and Trique, 1972).

The flavonols content was observed to be less in infected leaves than healthy and was found to be highest in *B. alba* at all stages of growth (0.176 mg/g at 80DAS in infected stage) and was least in Varuna (0.122 mg/g at 80DAS). The amount of flavonols decreased with the progress of infection and plant age (Table 2). These results are confirmatory with the findings of Saharan *et al*., (2000), who reported that flavonols content was decreased with the advancement of age in cluster bean infected with Alternaria blight. However less significant role of flavonols was reported in plant defense as compared to total phenols and ortho-dihydroxy phenols (Atwal *et al*., 2003).

The concentration of total soluble protein increased in leaves with progress of disease of Alternaria blight. It was observed that Varuna (20.80 mg/g at 80 DAS in infected stage), contained higher amount of protein content as compared to PAB-9543(18.40) and *B. alba* (16.30). The protein content was highest at100 DAS in both healthy and infected stages and it was higher in healthy leaves as compared to infected leaves. With the increase in infection and plant age, the protein content was increased in all genotypes. During host-pathogen interaction, amino acids act as a substrate for the pathogen (Titarenko *et al*., 1993) or they may have a fungistatic effect through their involvement in metabolic reactions associated with disease resistance (Misra, 2008). Onifade and Agboola (2003) also observed similar trend in increase in protein content due to fungal infection in coconut and postulated that proliferation of microorganism synthesize several enzyme proteins and sometimes cause rearrangement of nutritional composition of substrate due to formation of several degradation products thereby increasing its protein content. (Table 3)

The total sugar and reducing sugar was highest in *B. alba* (8.50mg/100g and 0.064mg/100g at 80DAS in infected stage) and least in Varuna. After infection, sugar level decreased in all the genotypes. With the increase in infection and plant age, total sugars and reducing sugars were found to be decreased in both the healthy and infected genotypes (Table 3). These results are supported by the findings of Jaypal and Mahadevan (1968), who reported that post-infectional decrease in sugar levels may be caused by rapid hydrolysis of sugars during pathogenesis through enzymes secreted by the pathogen. The invading pathogens may utilize the sugar leading to decrease in its content**.** The co-existence of free-sugars and phenols results in glycosylation of phenols by sugars, forming phenolic glycosides, which are more soluble in cell sap, thus provide more efficiency in the resistance expression (Walker, 1975).

There was higher chlorophyll content in resistant genotypes and it was decreased following infection. The highest amount total chlorophyll was observed in *B. alba* (1.68mg/g at 80DAS in infected stage) and it was decreased with increase in age of plant in all genotypes. The highest amount of chlorophyll 'a' was observed in *S. alba* (1.37 mg at 80DAS). With progress of infection, chlorophyll 'a' level decreased in all genotypes. The chlorophyll 'b' was also found to be maximum in *B. alba* (0.309mg) and it was decreased with increase in age of plant in all three genotypes (Table 4). Various plant pathogens are known to produce toxic metabolites, which may destroy the chloroplast resulting into decrease of chlorophyll pigments. Borah *et al.* (1978) suggested that the reduction in content of chlorophyll 'a' and 'b' might be due to inhibition of synthesis rather than degradation of pre-existing pigments. The high degree of resistance in *B. alba* to *A. brassicae* is due to higher content of chlorophyll and reduced size of stomata in

Table 3. Protein, Total sugar and Reducing Sugar contents in the healthy (H) and infected (I) leaf samples of *B. juncea* and *S. alba*

Genotype	Protein content (mg/g)						Total sugar content (mg/100mg of dry leaf powder)						Reducing sugar content (mg/100mg of dry leaf powder)					
	60DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS	
	H*	I*	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I
Varuna	21.10	20.00	21.90	20.80	22.40	21.50	10.80	7.20	9.62	6.60	8.90	6.00	0.053	0.038	0.035	0.020	0.030	0.012
PAB-9534	18.50	18.00	19.20	18.40	19.70	19.10	12.00	7.83	10.40	7.30	9.90	6.69	0.071	0.053	0.058	0.047	0.045	0.036
S. alba	16.20	16.00	16.80	16.30	17.30	17.00	12.90	10.13	10.60	8.50	10.30	7.40	0.080	0.065	0.072	0.064	0.061	0.049
CD (5%)	0.028	0.145	0.052	0.064	0.141	0.034	1.235	0.718	0.513	0.597	0.130	0.382	0.008	0.014	0.016	0.006	0.007	0.007

Table 4. Total Chlorophyll, Chlorophyll ‘a’ and Chlorophyll ‘b’ contents in the healthy (H) and infected (I) leaf samples of *B. juncea* and *S. alba*

Genotype	Total Chlorophyll content (mg/g of fresh tissue)						Chlorophyll ‘a’ content (mg/g of fresh tissue)						Chlorophyll ‘b’ content content (mg/g of fresh tissue)					
	60DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS		60 DAS		80 DAS		100 DAS	
	H*	I*	H	I	H	I	H	I	H	I	H	I	H	I	H	I	H	I
Varuna	2.05	1.8	1.87	1.4	1.78	0.58	1.85	1.69	1.71	1.27	1.66	0.56	0.19	0.11	0.16	0.135	0.11	0.023
PAB-9534	1.69	1.3	1.55	0.87	1.33	0.36	1.49	1.31	1.40	0.68	1.28	0.35	0.20	0.13	0.15	0.186	0.09	0.016
S. alba	2.14	1.9	2.09	1.68	1.84	0.76	1.93	1.79	1.91	1.37	1.82	0.72	0.21	0.14	0.18	0.309	0.13	0.040
CD (5%)	0.05	0.23	0.16	0.13	0.04	0.04	0.13	0.14	0.07	0.10	0.06	0.03	0.010	0.022	0.009	0.035	0.007	0.005

H and I = Indicate healthy and infected stages of a plant

comparison to susceptible cultivars (Awasthi, 1988). The decrease in chlorophyll a/b ratio is considered to be a symptom of oxidative stress condition this decrease in the ratio after virus infection might be due to the generation of reactive oxygen species (ROS) causing damage to chlorophyll a. This indicates that plant failed to capture the light and so photosynthesis might be decreased or stopped (Barka and El-Matty, 2008).

Based on the present findings, it may be concluded that high level of total phenols, o-dihydroxy phenols, flavonols contents and high sugar contents of *B. alba* appeared to be the important biochemical constituents which may impart resistance against Alternaria leaf blight infection. Post infectional response of the metabolites under consideration in the susceptible genotype seems to be associated with its susceptible response and symptom expression. Further studies in this direction may provide information regarding host-pathogen interaction which can be utilized for resistance breeding for the development of desirable trait by incorporating resistance in promising but susceptible genotype of mustard.

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