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Biochemical estimation of polyphenol and peroxidases in resistant sources against white rust disease of rapeseed mustard incited by *Albugo candida*

Pooja Upadhyay, AK Tewari and Himanshu Punetha

Abstract
White rust or white blister disease caused by oomycete fungi, *Albugo candia* is one of the major devastating disease of rapeseed mustard. Continuous emergence of new pathogenic races is responsible for breaking down of the resistance of the already existing resistance cultivars. Certain biochemical compounds can be responsible for initiating the resistance response during the defense process of plants. For this, alteration of peroxidases and polyphenol was evaluated in selected Brassica genotypes at 24 hrs, 48 hrs, 72 hrs, 5 days and 9 days after inoculation (DAI). At 24 hrs post inoculation, maximum increase of peroxidase activity was observed in Wester (10.235 μmol tetra-guaiacol min^{-1} mg^{-1} protein) while minimum in susceptible cultivar Varuna (8.624 μmol tetra-guaiacol min^{-1} mg^{-1} protein). At 48 hrs post inoculation, maximum increase of peroxidase activity was observed in Donskaja (10.313 μmol tetra-guaiacol min^{-1} mg^{-1} protein) and minimum in Varuna (9.823 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively. Similar trend was followed at 48 hrs, 72 hrs, 5 DAI and 9 DAI. In total polyphenol activity at 24 hrs post inoculation, Donskaja showed maximum increase (2.52 gallic acid equivalents mg^{-1} dry leaf) while minimum increase was observed in Wester (2.72 gallic acid equivalents mg^{-1} dry leaf). Similar results were obtained at 48 hrs, 72 hrs, 5 DAI and 9 DAI. These biochemical parameters peroxidases and polyphenol can be established as biochemical markers for the identification of resistant sources against white rust.

Keywords: White rust, biochemical estimation, peroxidase, polyphenol, resistance

1. Introduction
Oilseeds are the second most important component of Indian agricultural economy after cereal crops. Oilseeds include seven edible oilseeds, *viz.* groundnut, rapeseed-mustard, soybean, sunflower, sesame, safflower, niger and two non-edible oilseeds, *viz.* castor and linseed, Among different oilseed crops, rapeseed-mustard is one of the most economically vital agricultural commodities, which includes eight different species namely Indian mustard (*B. juncea*), black mustard (*B. nigra*), Ethiopian mustard (*B. carinata*), yellow sarson (*B. rapa var.* yellow sarson), brown sarson (*B. rapa var.* brown sarosn), gobhi sarson (*Brassica napus*), taramira (*Eureca sativa*), toria (*B. rapa var.* toria). The estimated area, production and yield of rapeseed-mustard in the world is 36.59 mha, 72.37 mt and 2134 kg/ha, respectively, during 2018-19 while in India rapeseed mustard consists an area of 6.12 mha, production of 9.26 mt and productivity of 13.4 kg/ha during the year 2018-19 (DRMR, 2020) [4]. Oilseed Brassica is attacked by a number of diseases and pests that limit its production and productivity adversely. Major fungal diseases that attack Brassica crop include Alternaria blight, White rust, Sclerotinia stem rot, Downey mildew, Powdery mildew etc. Among them, white rust disease caused by an obligate biotrophic fungus *Albugo candida* (Pers. ex Lev.) Kuntze, is one of the major threat in several Brassica species. Both vegetative and reproductive phases of plant are affected by this disease. Infection at vegetative phase results in the appearance of white pustules predominantly on abaxial surface of leaves. Systemic spread of pathogen can cause severe malformation in inflorescence through hypertrophy and hyperplasia resulting in staghead formation (Kolte., 1985; Punjabi *et al*., 2010) [6, 15].
Various protectant fungicides have been recommended for controlling white rust disease in rapeseed-mustard (Kolte and Tewari., 1980, and Kolte and Awasthi., 1987, Kalpana *et al.,* 2019) [7-8, 5]. But due to concern of health and environmental hazards, high cost of chemicals and problem of uniform distribution on different parts of the plant, farmers are usually reluctant to use these fungicides for the management of this disease.

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Disease resistance in plants is associated with the activation of a wide array of defense responses that slow down or withstand the infection at certain stage of host-pathogen interaction. Plants have evolved various pre-existing physical and chemical barriers, as well as inducible defense responses that interfere with pathogen establishment and cause oxidative stress in plants.

Due to oxidative stress cellular redox homeostasis of the cell is disrupted and resulting in generation of reactive oxygen species (ROS). These reactive oxygen species (ROS) play a central role in plant defense against various plant pathogens (Vanitha *et al.,* 2009) [20]. To protect cells from oxidative attack, plants have evolved different type of defense mechanisms constituted by enzymatic and non-enzymatic components. Enzymatic defense includes enzymes that are capable of removing, neutralizing, or scavenging free radicals produced during any kind of biotic and abiotic stresses.

Among different enzymatic and non-enzymatic compounds peroxidases and polyphenol play important role in activation of defense. These compounds express various biological activities towards variety of pathogens and are considered as biochemical markers for disease resistance (Schmelz *et al.,* 2011) [17]. Due to obligate parasitic nature of white rust pathogen very less information is available on biochemical

alteration in the resistant and susceptible genotypes. However, this information could be used as biochemical markers and be helpful in identifying the mechanism of induction of resistance in Brassica genotypes against this pathogen. This could also be utilized for the identification of resistance sources in different Brassica genotypes.

2. Material and Method
2.1 Selection of plant material
Planting material for conducting enzymatic assays were selected on the basis of cross interaction of *Albugo candida* pathogen and Brassica genotypes. For this purpose, 44 diverse *A. candida* isolates were collected from 17 states of India and cross inoculated with 32 Brassica genotypes consisting different Brassica species.

2.2 Collection of *A. candida* isolates
A total 44 diverse *A. candida* isolates were collected in the form of diseased leaf specimens, from 17 different states of India and that comprises of 10 Brassica species. The collected and purified samples were preserved at different temperature i.e. in refrigerator (4 °C and 10 °C) and in deep freezer (-20 °C) for further studies. The description of *A. candida* isolates collected during this study is given in the Table 1 and Fig. 1.
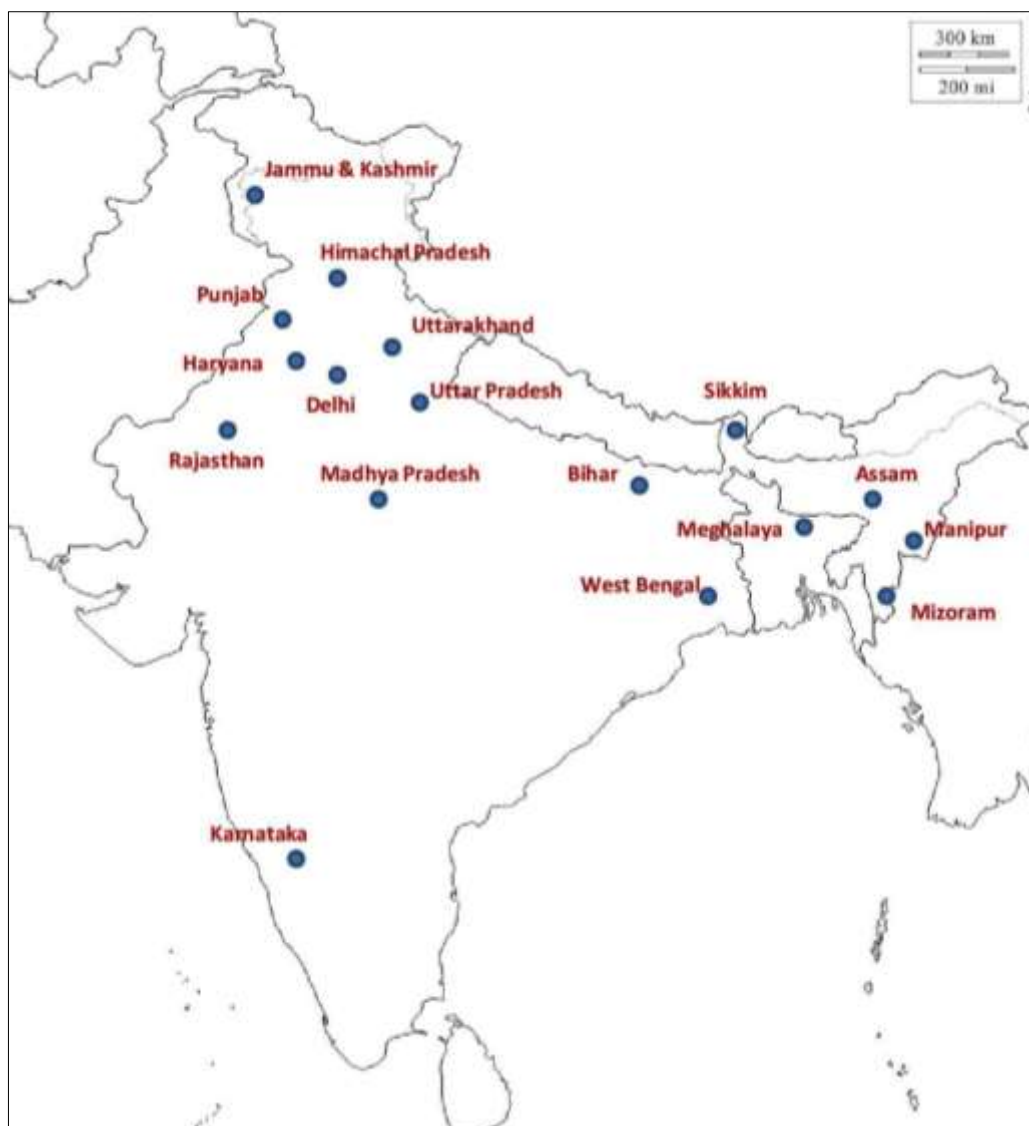
Fig 1: Collection and isolation of *A. candida* isolates from different states of India

Table 1: List of purified *A. candida* isolates collected from diverse places of India

S. No.	Name of the state	Name of the isolate	*Brassica* Species	Place of collection	Latitude & Longitude
1	Uttarakhand	Ac- *Bj*-Pnt	*B. juncea*	Pantnagar	29° 02' 60" N 79° 30' 59" E
2		Ac-*B.np*-Pnt	*B. napus*	Pantnagar	29° 02' 60" N 79° 30' 59" E
3		Ac-*B.ngr*-Pnt	*B. nigra*	Pantnagar	29° 02' 60" N 79° 30' 59" E
4		Ac- B.rai-Pnt	Banarasi rai	Pantnagar	29° 02' 60" N 79° 30' 59" E
5		Ac-Y.srs-Pnt	*B. rapa var. Yellow Sarson*	Pantnagar	29° 02' 60" N 79° 30' 59" E
6		Ac-bioysr-Pnt	*B. juncea* (Bioysr)	Pantnagar	29° 02' 60" N 79° 30' 59" E
7		Ac-*B.rug*-Pnt	*B. rugosa*	Pantnagar	29° 02' 60" N 79° 30' 59" E
8		Ac-*Bj*-Kran-Pnt	*B. juncea* (Kranti)	Pantnagar	29° 02' 60" N 79° 30' 59" E
9		Ac-O.rai-Pnt	Ornamental Rai	Pantnagar	29° 02' 60" N79° 30' 59" E
10		Ac-*Cir.arvs*-Pnt	*Cirsium arvense*	Pantnagar	29° 02' 60" N 79° 30' 59" E
11	Uttar Pradesh	Ac-*Bj*-Knp	*B. juncea*	Kanpur	26°26'59.6"N 80°19'54.8"E
12		Ac-*Bj*-Merut	*B. juncea*	Meerut	28° 59' 4" N 77° 42' 21" E
13		Ac-*Bj*-Faiz	*B. juncea*	Faizabad	26° 46' 12" N 82° 9' 0.00" E
14	New Delhi	Ac-*Bj*- Iari	*B. juncea* (Varuna)	IARI	28° 37' 55" N 77° 8' 19" E
15		Ac-*Bj*-Pbhr- Iari	*B. juncea* (PusaBahar)	IARI	28° 37' 55" N 77° 8' 19" E
16		Ac-bcs-Iari	*B. carinata* (BCS-1)	IARI	28° 37' 55" N 77° 8' 19" E
17		Ac- *Bj*-Uds	*B. juncea*	UDSC	28° 38' 41" N 77° 13' 0" E
18	Haryana	Ac- *Bj*-Hisr	*B. juncea*	Hisar	29° 9' 6.69" N 75° 43' 16" E
19		Ac- *Bj*-Sirs	*B. juncea*	Sirsa	29° 32' 11" N 75° 1' 31" E
20	Rajasthan	Ac- *Bj*-Alwr	*B. juncea*	Alwar	27° 33' 39" N 76° 37' 30" E
21		Ac- *Bj*-Bhart	*B. juncea*	Bharatpur	27° 13' 1" N 77° 29' 22" E
22		Ac- *Bj*-Sriganga	*B. juncea*	Sri ganganagar	29° 90' 94" N 73° 88' 00" E
23		Ac- *Bj*-Kotpt	*B. juncea*	Kotputli	27° 13' 1" N 77° 29' 22" E
24	Punjab	Ac- *Bj*-Ludh	*B. juncea*	Ludhiana	30° 42' 37" N 76° 11' 56" E
25		Ac- *Bj*-Abhr	*B. juncea*	Abhor	30° 08' 40" N 74° 11' 43" E
26	Madhya Pradesh	Ac- *Bj*-varu-Mor	*B. juncea* (Varuna)	Morena	26° 30' 0.0" N 78° 0' 0.0" E
27		Ac. *Bj*-mya. Mor	*B. juncea* (Maya)	Morena	26° 30' 0.0" N 78° 0' 0.0" E
28		Ac- *Bj*-vard-Mor	*B. juncea* (Vardan)	Morena	26° 30' 0.0" N 78° 0' 0.0" E
29		Ac- *Bj*-pb-Mor	*B. juncea* (Pusa Bold)	Morena	26° 30' 0.0" N 78° 0' 0.0" E
30	Himachal Pradesh	Ac- *Bj*-Simr	*B. juncea*	Sirmaur	30° 38' 24"N 77° 26' 24"E
31		Ac- *Bj*-Ponts	*B. juncea*	Ponta Sahib	30° 26'16"N 77°37' 26"E
32		Ac- *Bj*-Kngr	*B. juncea*	Kangra	32° 5' 59" N 76° 16' 8" E
33	Jammu & Kashmir	Ac- *Bj*-Chata	*B. juncea*	Chatha	32° 43' 58" N 74° 51' 51" E
34	Karnataka	Ac- *Bj*-Kanach	*B. juncea*	Kannachalli	12° 52' 15"N 76° 47' 18"E
35		Ac-*Bj*-Dharw	*B. juncea*	Dharwad	12° 72' 15"N 79° 39' 13"E
36		Ac- *Bj*-Bang	*B. juncea*	Bangaluru	12°97' 16" N 77° 59'46" E
37	Bihar	Ac- *Bj*-Samsti	*B. juncea*	Samastipur	25°51'39''N 85°46'56''E-
38	West Bengal	Ac- *Bj*-Kolk	*B. juncea*	Kolkata	22° 34' 21" N 88° 21' 50" E
39		Ac-Amr-Kolk	Amaranthus	Kolkata	22° 34' 21" N 88° 21' 50" E
40	Mizoram	Ac- *Bj*-Aizwl	*B. juncea*	Aizawal	23° 73' 07" N 92° 71' 73" E
41	Assam	Ac- *Bj*-Jorht	*B. juncea*	Jorhat	26° 45' 21" N 94° 12' 34" E
42	Meghalaya	Ac- *Bj*-Umi	*B. juncea*	Umiam	25° 39' 11" N 91° 53' 3.4" E
43	Manipur	Ac- *Bj*-Imphl	*B. juncea*	Imphal	24° 48' 50" N 93° 57'' 1" E
44	Sikkim	Ac- *Bj*-Mrchk	*B. juncea*	Marchak	27° 28' 97" N 88° 58' 87" E

2.3 Planting Material

A total 32 Brassica cultivar consisting 12 different Brassica species as listed in Table 2 were used for the study of host-pathogen interaction. These 32 Brassica genotypes were selected on the basis of their differential disease reaction under field conditions in two successive years. Seeds of theses cultivar were collected from Department of genetics and plant breeding, South campus, New Delhi, GBPUA&T, Pantnagar and different AICRP Rapeseed Mustard centres in India. Seeds were sown in 6 mm plastic pots containing sterilized soil and kept in glasshouse for germination. Three to four seedlings in each pot were maintained for pathogen inoculation.

Table 2: List of Brassica genotypes used for host pathogen interaction study

S. No.	*Brassica* spp./cultivar
1	*B. juncea* cv. Varuna
2	*B. juncea* cv. Pusa Bold
3	*B. juncea* cv. EC 399313
4	*B. juncea* cv. Heera
5	*B. juncea* cv. Donskaja
6	*B. napus* cv. Wester
7	*B. rapa* cv.Torch
8	*B. rapa* cv.Tobin
9	*B. rapa* cv.Candle
10	*B. juncea* cv. RL 1359
11	*B. rapa var. Yellow sarson* YSPB 24
12	*B. nigra* 2782
13	*B. nigra* cv. Sangam
14	*B. juncea* cv. Cutlass
15	*Eureca sativa*
16	*B. carinata* cv. DLSC 1
17	*B. rapa var. brown sarson* cv. BSH 1
18	*Sinapis alba*
19	*B. juncea* cv. NRCDR 515
20	*B. juncea* cv. RH 30
21	*B. rapa var.* Yellow Sarson cv. Ragini
22	*B. juncea* cv. Bioysr
23	*B. juncea* cv. EC 399301
24	*B. juncea* cv. Kranti
25	*B. rapa var. brown sarson* cv. Pusa Kalyani
26	*B. rapa var. toria* cv. TL 15
27	*B. rapa var. toria* cv. Bhawani
28	*B. rapa var. toria* cv. PT 303
29	*B. napus* cv. GSL 1
30	*B. carinata* cv. Kiran
31	*Raphanus sativus* (Local cultivar)
32	*B. olerecea* (Local cultivar)

2.4 Pathogen Inoculation

Fresh sporangial suspension of each pathogen isolate was prepared with single white rust pustule collected by sterile scalpel or blade in a glass vial containing 1-2 ml sterilized distilled cold water. The suspension was mixed properly and sporangial concentration in the suspension was maintained about 2.5×10^5 zoosporangia per ml by using haemocytometer. This suspension was kept overnight at 10 °C for the release of zoospores. Seedlings of each Brassica cultivar were cross inoculated with each pathogen suspension at true leaf stage (12-15 DAS) with 10μl spore suspension by using micropipette and ensured even inoculation of all the seedlings. After inoculation plants were transferred to plant propagator box which was kept in dark place for 72 hrs, with a relative humidity (RH) of more than 90 percent and temperature of 18±2 °C. After three days plants were taken out and kept in controlled environment of temperature (18±2 °C) and RH (more than 80%) in glasshouse for further observation.

Following observations were recorded 4-5 days after appearance of the disease in each Brassica genotypes against each *A. candida* isolate for the selection of Brassica genotypes for further enzymatic assays.

2.5 Percent disease index

The observations for disease index in each host against each isolate was recorded one week after appearance of disease, at true leaf stage, using 0-6 rating scale (Conn *et al.*, 1990) [3] as under.

Rating scale	Leaf area (%) covered by the pustules	Disease reaction (DR)
0	No symptom	Immune (I)
1	0-5	Highly resistant (HR)
2	5-10	Resistant (R)
3	10-20	Moderately resistant (MR)
4	20-35	Moderately susceptible (MS)
5	35-50	Susceptible (S)
6	More than 50	Highly susceptible (HS)

The percent disease index was calculated by using following formula:

$$\text{Disease index (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{Number of leaves examined x Maximum rating}} \times 100$$

2.6 Establishment of glasshouse experiment for the estimation of biochemical parameters

In a pot experiment seeds of selected Brassica genotypes collected from oilseed laboratory, deptt. Of plant pathology were used for the study. 10 seeds of each selected genotype were sown in each pot with three replications. Five seedlings of each selected genotype in each pot was inoculated with zoospores suspension of *A. candida* isolate at true leaf stage (15 DAS) by the method as described above. Control plants of each genotype were inoculated with water. All the plants were kept in plant propagator in glasshouse at 18±2 °C to maintain 80-90 percent RH for 72 hrs. After 72 hrs, plants were kept in glasshouse under controlled conditions at a temp. of 18±2 °C and humidity of 80-85%. Moisture on plant leaf surface was maintained by sprinkler/ hand sprayer during growth period of plant for the development of symptoms.

2.6.1 Collection of leaf samples

Leaf samples of each Brassica genotype (Inoculated and un-inoculated) were collected in butter paper bags at the time intervals of 24 hrs, 48 hrs, 72 hrs, 5 days and 9 days after inoculation. Collected samples were kept in ice box immediately after harvesting and stored at -20 °C at earliest for further analysis. The observations on percent disease index using 0-6 rating scale (Conn *et al.,* 1990) [3] were recorded 4-5 days after appearance of the disease for the comparison of induction of defense related compounds in relation to disease index in different inoculated and un-inoculated Brassica genotypes.

2.7 Biochemical estimation of defense related parameters
2.7.1 Preparation of enzyme extract

For preparation of enzyme extract, 200 mg sample was homogenized in liquid nitrogen. Further the homogenized powder was mixed with ice cold 1.5 ml of 50 mM Sodium phosphate buffer (pH-7.0) containing 2 mM EDTA, 5 mM β-mercaptoethanol (β-ME) and 4 percent PVP-40. The homogenate was centrifuged at 30000 rpm for 30 min at 4 °C and supernatant was stored in aliquots at -80 °C. The preparation of enzyme extract was same for all the enzymes except that for the ascorbate peroxidase for which the extraction buffer also contained 5 mM ascorbate.

2.7.2 Protein estimation

Protein was estimated by the method as described by Lowry *et al.* (1951) [9]. In this method Ttrichloroacetic acid (TCA) treated protein extract was prepared. For this 0.1 ml supernatant of enzyme extract was mixed with 0.1 ml of 20

percent Trichloroacetic acid (TCA) to precipitate the protein. Precipitated protein was incubated for 1-2 h at -20 °C followed by centrifugation at 10,000 rpm for 10 min at 4 °C and pellet was dissolved in 0.5 ml of 1N sodium hydroxide solution which was used for protein estimation. Quantification of protein content was done from the standard curve of Bovine Serum Albumin (BSA).

2.7.3 Peroxidase (POD) activity
POD activity was determined by the method described by Chance and Maehly (1955) [2] in which Solution A: Sodium phosphate buffer (100 mM, pH 7.0), Solution B: 10 mM H2O2 solution and Solution C: 20 mM guaiacols were freshly prepared. 1.5 ml Soluti2on A, 0.4 ml enzyme extract and 0.12 ml Solution B were taken in a cuvette. The increase in absorbance was recorded for 3 min at 1 min interval at 470 nm immediately after addition of 0.48 ml of solution C to the cuvette. The reaction mixture without enzyme served as blank. Quantification was done on the basis of extinction coefficient of the oxidation product, tetraguaiacol (26.6 mM^{-1} cm^{-1}).

2.7.4 Total polyphenol content
The total polyphenol content of plant samples was estimated by using Folin-Ciocalteu method Singleton and Rossi (1965) [18] with minor modifications. In this method appropriately diluted test sample (0.5ml) was reacted with 0.2 ml Folin-Ciocalteu reagent, and reaction was neutralized with saturated sodium carbonate (0.5 ml). Volume was made up with 10mL distilled water. Later the absorbance of the resulting blue colour was measured at 760 nm. Quantification was done on the basis of a standard curve with Gallic acid.

Statistical analysis
Data obtained on various traits under laboratory, glasshouse and field experiments were analyzed to estimate critical difference (CD 5%) among and within the population and coefficient of variation (CV%). Data were analyzed using ANOVA as per the design.

3. Results and Discussion
Disease response of 44 *A. candida* isolates on 32 Brassica genotypes was recorded by observing presence (+) and absence (-) of disease on each genotype (Table 4) and following observations were taken.

Observation
Percent Disease Index
On the basis of disease response of 44 *A. candida* isolates on 32 *Brassica* genotypes, percent disease index (PDI) was calculated for each isolate on each genotype one week after appearance of the disease at true leaf stage by using 0-6 rating scale (Conn, *et al.*, 1990) [3] and these genotypes were divided into 6 different groups on the basis of resistance and susceptibility.
Among those, Varuna showed maximum percent disease index raging 22.8-51.1 percent followed by Kranti (26.70-45.90%), RH 30 (28.60-45.60%), RL 1359 (22.6-40.2%) considered as highly susceptible genotypes. Pusa Bold (15.5-38.85%), EC 399301 (13.80-37.30%), Bhawani (16.2-33.5%), TL 15 (14.8-33.5%), Pusa Kalyani (17.6-32.8%), YSPB 24 (17.4-30.5%), PT 303 (12.8-30.2%), EC 399313 (11.8-27.6%) considered as susceptible genotypes. Cutlass (13.7-22.8%), Bn2782 (12.67-23.4%), BSH 1 (11.5-17.9%), Torch (6.9-19.6%), Tobin (4.8-17.9%), Ragini (8.9-18.4%), Heera (6.3-

16.6%), Bioysr (9.40-16.30%) considered as Moderately resistant. Candle (5.6-13.6%), *Sinapis alba* (4.9-11.8%), Sangam (4.9-11.3%), *E. sativa* (3.5-6.7%) considered as resistant genotype against the disease. Donskaja (2.2-4.6%), *B. oleracea* (3.7- 4.01%) considered as highly resistant. NRCDR 515, Wester, GSL 1, Kiran, DLSC 1, *Raphanus sativus* showing 0.00 percent disease index are considered immune or free from disease against all the *A. candida* isolates. Out of 6 groups, 08 genotypes were selected Eight Brassica genotypes were selected on the basis of different level of resistance *viz.* Highly susceptible: Varuna (*B. juncea*), Susceptible: Pusa Bold (*B. juncea*), Moderately resistant: Cutlass (*B. juncea*), Resistant: Candle (*B. rapa*), Highly resistant: Donskaja (*B. juncea*) and Immune: NRCDR 515 (*B. juncea*), Wester (*B. napus*), DLSC1 (*B. carinata*) and evaluated against white rust pathogen (*A. candida*) by inoculating Pantnagar isolate for the induction of defense related parameters.

3.1 Biochemical estimation of defense related parameters
Estimation of major anti-oxidative defensive compounds Peroxidases (POD) and polyphenol were evaluated after inoculation with *A. candida* pathogen in glasshouse under artificial epiphytotic conditions. Leaf samples for estimation were collected at different time intervals *viz.* 24 hrs, 48 hrs, 72 hrs, 5 days and 9 days after inoculation (DAI) and activity of biochemical compounds was calculated and compared with control (uninoculated plants).

3.1.1 Estimation of total protein content
Plant protein plays important role in plant defense in general or during biotic and Abiotic stresses by various mechanisms. During biotic stress or when plant is attacked by any pathogen, some plant protein start functioning by blocking the active sites and make a complex which inhibit the pathogenic and cellulolytic enzymes secreted by the pathogen. Some protein may function as structural barrier for pathogen entry by being the part of pre-existing defense barriers such as thionins, defensins or hydroxyproline-rich glycoproteins working in fortifying the plant cell wall to avoid pathogen entry. In the present study, during the evaluation of alteration in protein content against *A. candida* infection, both factors *viz.* varietal difference (Factor-A) and time interval (Factor-B) showed alteration in protein concentration (mg gm^{-1}) over the course of infection period. A continuous decrease in protein content was observed in pathogen inoculated plants as compared to control plant up to a period of 5 DAI post inoculation then increased in inoculated plant as compared to healthy plants in all the genotypes i.e. 9 DAI.
At 24 hrs post inoculation maximum decrease in protein content was observed in Wester (57.23 & 53.67 mg/g dry wt) in control and inoculated plant respectively, followed by DLSC-1 (55.92 & 52.88 mg/ g dry wt), NRCDR 515 (55.02 & 52.46 mg/g dry wt) and Donskaja (56.13 & 53.87 mg/g dry wt), while minimum decrease of protein content was observed in Pusa Bold (56.12 & 54.77 mg/g dry wt) followed by Varuna (56.17 & 54.68 mg/g wt) in control and inoculated plant respectively. At 48 hrs post inoculation maximum decrease in protein content was observed in DLSC-1 (58.85 & 54.88 mg/g dry wt) in control and infected plant respectively, followed by Wester (57.91 & 54.14 mg/g dry wt), Donskaja (57.23 & 53.65 mg/g dry wt) and NRCDR 515 (55.13 & 51.79 mg/g dry wt) while minimum reduction in protein content was observed in Varuna 57.89 & 54.88 mg/g dry wt), followed by Pusa Bold (56.46 & 54.23 mg/g dry wt).

However, Cutlass and Candle were at par with each other in protein content.

At 72 hrs post inoculation with maximum decrease in protein content was observed in Wester (62.77 & 56.96 mg/g dry wt), followed by Donskaja (58.99 & 54.36 mg/g dry wt), DLSC-1 (60.43 & 55.80 mg/g dry wt) and NDCDR 515 (58.91 & 56.62 mg/g dry wt) while minimum decrease in protein content was observed in Varuna (58.13 & 54.46 mg/g dry wt), followed by Pusa Bold (58.26 & 54.83 mg/gm dry wt). At 5 DAI, maximum decease was observed in Wester (56.80 & 52.78 mg/g dry wt) and minimum in Pusa bold (55.67 & 53.57 mg/g dry wt) in control and inoculated plant respectively. At 9 DAI increase in protein content was observed in all the inoculated plants as compared to control plants (Table 3, Fig.2). The protein content was estimated on basis of the standard curve for BSA.

The present findings were supported by Mishra *et al.* (2009) [13] who observed decreased protein content in white rust infected *B. juncea* cultivars (Varuna, Kranti, EC-399296, EC-399299, EC-399313 and EC-399301) at cotyledonary and true leaf stages at 14 DAI and 21 DAI respectively as compared to control plants with progress of disease. Sanjeev *et al.* (2017) [16] studied biochemical alteration on resistant and susceptible cultivars of *Brassica juncea*, infected with *Albugo candida* at 14, 56, 84 days after sowing and observed that protein content was lower in resistant genotypes EC 399296 and EC 399301, as compared to susceptible cultivar in both healthy and infected plants. Similar results were obtained by Asif *et al.* (2018) [1] who observed decrease in protein content in resistant Brassica cultivar, Faisal canola as compare to susceptible cultivar when attacked by *A. candida* pathogen.

Table 3: Total protein content in Brassica genotypes against *A. candida* inoculated with *A. candida* under glasshouse

Brassica genotype	Total protein content (mg/gm dry weight)									
	Healthy Plant					Inoculated Plant				
	24h	48h	72h	5d	9d	24h	48h	72h	5d	9d
Varuna	56.172 (48.53)	57.892 (49.34)	58.132 (49.67)	56.264 (48.58)	49.342 (44.60)	54.682 (47.66)	54.882 (47.78)	54.463 (47.54)	53.78 (47.15)	50.114 (45.05
Donskaja	56.131 (47.93)	57.23 (48.56)	58.99 (49.00)	55.952 (48.40)	49.01 (43.96)	53.873 (47.78)	53.65 (46.91)	54.364 (46.18)	52.09 (46.91)	49.162 (44.44)
NRCDR-515	55.021 (48.15)	55.134 (48.51)	58.912 (49.53)	53.912 (47.23)	48.342 (53.74)	52.464 (46.39)	51.79 (46.00)	56.623 (47.63)	50.12 (45.05)	49.982 (44.40)
Wester	57.230 (47.91)	57.912 (48.68)	62.768 (52.38)	56.80 (48.32)	46.134 (42.75)	53.67 (46.83)	54.143 (47.36)	56.962 (48.98)	52.78 (46.58)	47.23 (43.40)
Cutlass	56.292 (48.59)	54.954 (47.82)	59.05 (51.95)	56.153 (49.09)	45.65 (42.49)	54.09 (47.32)	52.714 (46.54)	55.064 (47.88)	53.12 (46.77)	46.846 (43.17)
Pusa Bold	56.123 (8.50)	56.463 (49.09)	58.262 (51.95)	55.674 (48.81)	47.98 (43.82)	54.77 (47.41)	54.233 (47.41)	54.832 (47.75)	53.57 (47.03)	48.134 (43.91)
Candle	56.124 (48.22)	54.89 (47.79)	56.234 (50.17)	55.252 (47.99)	46.07 (42.73)	54.114 (47.34)	52.314 (46.31)	52.69 (46.52)	52.21 (46.25)	47.214 (43.39)
DLSC-1	55.921 (48.38)	58.852 (50.08)	60.432 (52.77)	57.746 (46.72)	48.463 (44.10)	52.882 (46.63)	54.88 (47.78	55.802 (48.31	53.68 (45.31)	49.282 (44.57)
CD 5%										
Genotype (A)	1.01 (0.97)					0.57 (0.42)				
Time interval (B)	0.80 (0.67)					0.45 (0.37)				
AXB	2.26 (1.98)					1.27 (0.96)				
CV	8.17 (9.13)					7.27 (8.19)				

*Values in paranthesis are angular transformed value
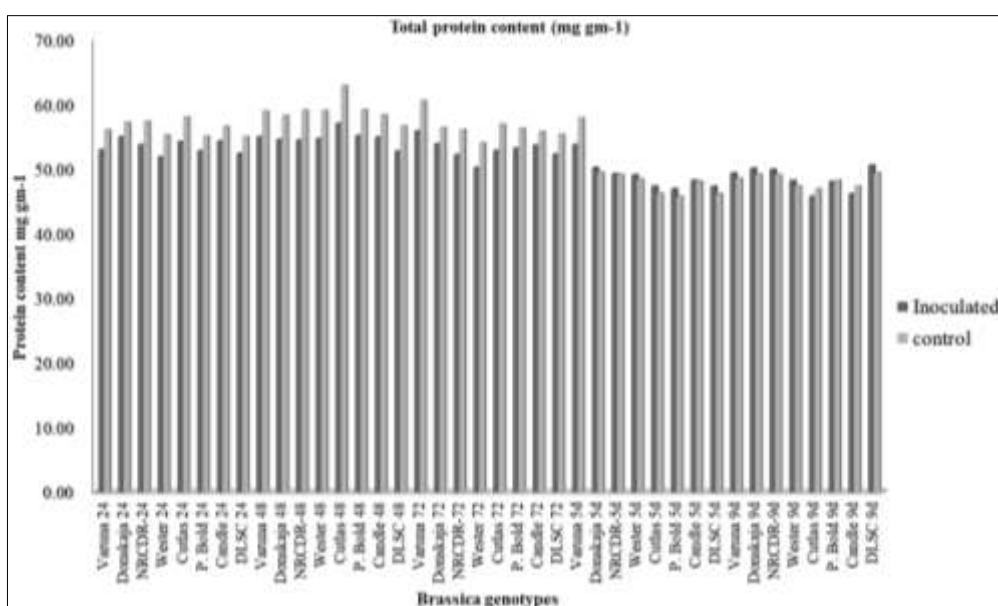
Fig 2: Total Protein content in Brassica genotypes inoculated with *A. candida* in glasshouse at different time intervals

3.1.2 Estimation of total Peroxidase (POD) activity

Peroxidases enzyme draws first line of biochemical defense against plant pathogens by its involvement in cell wall lignifications, suberification, polymerization of hydroxyl proline rich glycoprotein activities. In the present study during the evaluation of total peroxidase enzyme concentration, both factors, *viz.* varietal difference (Factor-A) and time interval (Factor-B) showed significant influence towards change in total peroxidase (POD) activity over the course of infection period. Significant increase in the peroxidase activity was observed in all the inoculated genotypes as compared to control plants at 24 hrs post inoculation. However, Wester showed maximum increase in the total peroxidase activity (10.235 & 11.310 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively, followed by Donskaja (9.234 & 10.120 μmol tetra-guaiacol min^{-1} mg^{-1} protein), DLSC1 (10.624 & 11.480 μmol tetra-guaiacol min^{-1} mg^{-1} protein) and NRCDR 515 (9.376 & 10.001 μmol tetra-guaiacol min^{-1} mg^{-1} protein), while minimum increase of peroxidase activity was observed in Varuna (8.624 & 8.940 μmol tetra-guaiacol min^{-1} mg^{-1} protein), followed by Pusa Bold (8.624 & 8.970 μmol tetra-guaiacol min^{-1} mg^{-1} protein), Cutlass (9.634 & 10.071 μmol tetra-guaiacol min^{-1} mg^{-1} protein) and Candle (9.745 & 10.210 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively.

Significant increase in peroxidase activity was observed in all the genotypes at 48 hrs post inoculation. Donskaja showed maximum increase in the peroxidase activity (10.313 & 11.952 μmol tetra-guaiacol min^{-1} mg^{-1} protein), followed by Wester (11.562 & 13.110 μmol tetra-guaiacol min^{-1} mg^{-1} protein), DLSC 1 (11.712 & 12.974 μmol tetra-guaiacol min^{-1} mg^{-1} protein), NRCDR 515 (10.734 & 11.716 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively. While minimum increase of peroxidase activity was observed in Varuna (9.823 & 10.260 μmol tetra-guaiacol min^{-1} mg^{-1} protein), followed by Pusa Bold (9.515 & 10.010 μmol tetra-guaiacol min^{-1} mg^{-1} protein), Cutlass (10.461 & 11.141 μmol tetra-guaiacol min^{-1} mg^{-1} protein) and Candle (10.356 & 11.212 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively. Similar results were observed at 72 hrs post inoculation which showed a significantly higher increase in peroxidase activity in all the genotypes with increase in time interval. At 5 and 9DAI, total peroxidase activity decreased in both inoculated as well as in control plants.

At 5DAI maximum increase in the peroxidase activity was observed in Donskaja (10.414 & 11.853 μmol tetra-guaiacol min^{-1} mg^{-1} protein), followed by Wester (11.164 & 12.581 μmol tetra-guaiacol min^{-1} mg^{-1} protein), while minimum increase in Varuna (9.234 & 9.645 μmol tetra-guaiacol min^{-1} mg^{-1} protein), followed by Pusa Bold (9.812 & 10.342 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively.

Similar trend was followed at 9DAI with maximum peroxidase activity in Donskaja (8.154 & 8.780 μmol tetra-guaiacol min^{-1} mg^{-1} protein) and minimum activity in Varuna (7.616 & 7.731 μmol tetra-guaiacol min^{-1} mg^{-1} protein) in control and inoculated plant respectively. In the present study continuous increase in peroxidase activity was observed in all the genotypes up to 72 hrs, thereafter activity decreased in control as well as in inoculated plants. (Table 4, Fig 3).

The results obtained in the present study were similar with the findings of many other researchers. Mathpal *et al.* (2011) [12] and reported increased activity of peroxidase enzyme tolerant brassica cultivar PAB 953 as compared to susceptible cultivars Varuna when inoculated with *Alternaria brassicae*. Mallick *et al.* (2015) [11] reported increased activity of peroxidase enzyme in resistant Brassica cultivar *Sinapis alba,* when attacked by *Alternaria brassicae* pathogen. Asif *et al.* (2018) [1] reported increased peroxidases activity in resistant Brassica cultivar Faizal canola against *Albugo candida* pathogen. Syed (2020) [19] reported increased peroxidase activity in tolerant Brassica cultivar PAB 112 and *Sinapis alba* as compare to susceptible cultivar when inoculated with *Alternaria brassicae.* Thus in the present study, peroxidases concentration is positively correlated with disease resistance against *A. candida* pathogen. Peroxidase catalyses the oxidation of substrates like phenol and its derivates, by H_2O_2 and are associated with plant defense by synthesis of phenolic compounds effective against pathogens. It also participates in the production of ethylene, which increases frequently in pathogenesis process and trigger the ISR and increases defense against the pathogen (Nakano *et al.,* 1981) [14].

Table 4: Total Peroxidase (POD) activity in Brassica genotypes inoculated with *A. candida* under glasshouse

Brassica genotype	Peroxidase (POD) activity (μmol tetra-guaiacol min^{-1} mg^{-1} protein)									
	Healthy plant					Inoculated plant				
	24h	48h	72h	5d	9d	24h	48h	72h	5d	9d
Varuna	8.624 (17.07)	9.823 (17.68)	10.581 (17.82)	9.234 (18.65)	7.616 (18.08)	8.940 (17.39)	10.260 (18.54)	11.523 (18.43)	9.645 (19.64)	7.732 (18.50)
Donskaja	9.234 (17.07)	10.313 (18.18)	11.523 (19.01)	10.414 (18.26)	8.154 (18.72)	10.120 (17.42)	11.952 (18.63)	14.465 (19.80)	11.853 (18.67)	8.780 (20.22)
NRCDR-515	9.376 (19.12)	10.734 (19.87)	11.245 (18.86)	10.205 (17.96)	8.015 (18.76)	10.001 (20.01)	11.716 (21.22)	13.332 (19.49)	11.220 (18.44)	8.452 (19.56)
Wester	10.235 (20.01)	11.562 (18.97)	12.113 (19.83)	11.164 (19.59)	9.103 (20.36)	11.310 (21.10)	13.110 (19.84)	14.801 (22.34)	12.581 (21.41)	9.872 (22.62)
Cutlass	9.634 (19.49)	10.461 (19.09)	11.142 (19.56)	10.123 (20.59)	8.012 (17.68)	10.071 (21.01)	11.141 (20.12)	12.850 (20.97)	11.014 (22.60)	8.320 (18.08)
Pusa Bold	8.624 (18.82)	9.515 (18.62)	10.704 (19.51)	9.812 (18.54)	7.761 (18.25)	8.970 (20.13)	10.010 (19.56)	11.840 (20.77)	10.342 (19.38)	7.980 (18.75)
Candle	9.745 (19.28)	10.356 (19.97)	11.223 (16.01)	10.912 (16.59)	8.885 (16.44)	10.210 (19.94)	11.212 (21.12)	12.812 (16.14)	11.640 (17.23)	9.201 (16.89)
DLSC-1	10.624 (17.55)	11.712 (16.44)	12.376 (16.17)	11.675 (17.34)	9.564 (18.01)	11.480 (18.31)	12.974 (16.76)	14.782 (16.40)	12.996 (17.65)	10.152 (18.57)
CD 5%										

Genotype (A)	0.185 (0.152)				0.169 (0.132)			
Days (B)	0.146 (0.129)				0.134 (0.121)			
AXB	0.413 (0.387)				0.378 (0.219)			
CV	5.13 (6.25)				6.62 (7.16)			

* Values in parenthesis is angular transformed value
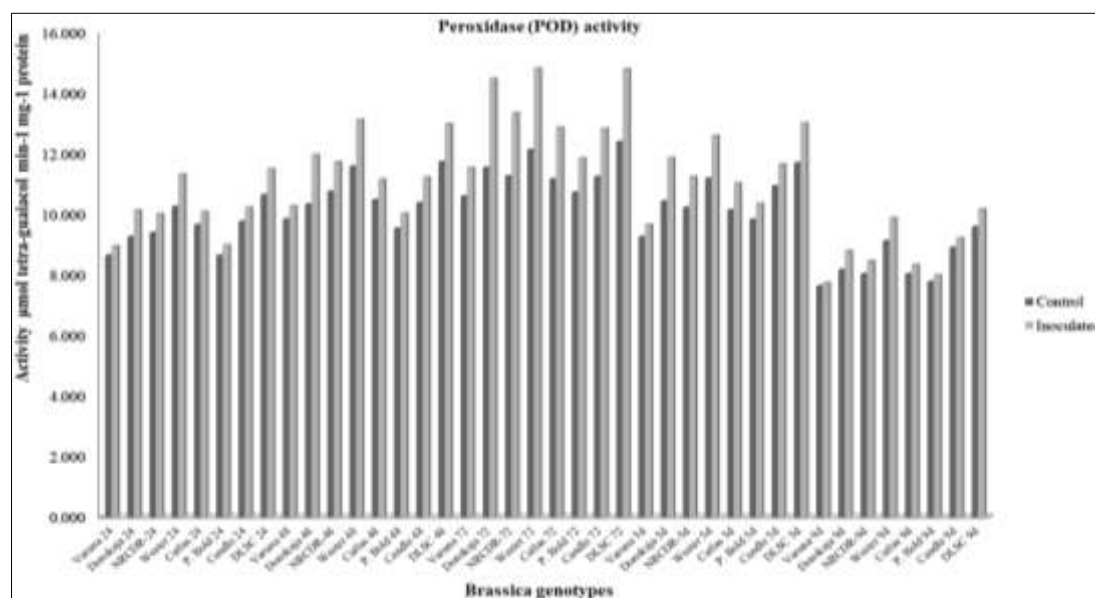
Fig 3: Total peroxidases activity in Brassica genotypes inoculated with *A. candida* in glasshouse at different time intervals

3.1.3 Estimation of total Polyphenol content

Polyphenol has an initial role in defense process where damage of cell membrane due to pathogen invasion cause releases of chlorogenic acid and other phenolic compounds. It catalyzes the oxidation of phenolic compounds into free radicals that react with biological molecules of the pathogen and creates an unfavorable environment and restricts the growth and development of the pathogen. In the present study, during evaluation of total polyphenol content, both factors *viz.* varietal difference (Factor-A) and time interval (Factor-B) showed significant influence towards change in total Polyphenol concentration over the course of infection period.

Significant increase in the Polyphenol content was observed in all the genotypes at 24 hrs post inoculation with Donskaja showing maximum increase (2.52 & 2.78 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively, followed by NRCDR 515 (2.42 & 2.64 gallic acid equivalents mg^{-1} dry leaf,) Candle (2.54 & 2.69 gallic acid equivalents mg^{-1} dry leaf) and Cutlass (2.63 & 2.78 gallic acid equivalents mg^{-1} dry leaf), while minimum increase in polyphenol concentration was observed in Wester (2.72 & 2.83 gallic acid equivalents mg^{-1} dry leaf) followed by DLSC-1 (2.76 & 2.743 gallic acid equivalents mg^{-1} dry leaf), Pusa bold (2.612 & 2.90 gallic acid equivalents mg^{-1} dry leaf) and Varuna (2.762 & 2.923 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively.

Similar significant increase in total Polyphenol content was observed in all the genotypes at 48 hrs post inoculation. Donskaja showed maximum increase (2.64 & 3.01 gallic acid equivalents mg^{-1} dry leaf), followed by NRCDR 515 (2.50 & 2.82 gallic acid equivalents mg^{-1} dry leaf), Candle (2.66 & 2.92 gallic acid equivalents mg^{-1} dry leaf) and Cutlass (2.75 & 2.99 gallic acid equivalents mg^{-1} dry leaf), while minimum increase was observed in Wester (2.82 & 2.98 gallic acid equivalents mg^{-1} dry leaf), followed by DLSC-1 (2.79 & 2.95

gallic acid equivalents mg^{-1} dry leaf), Pusa bold (2.70 & 2.91 gallic acid equivalents mg^{-1} dry leaf) and Varuna (2.80 & 3.03 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively.

Similar results were observed at 72 hrs post inoculation which showed a significantly higher increase in the total Polyphenol content in all the genotypes. Donskaja showed maximum increase of polyphenol content (2.70 & 3.34 gallic acid equivalents mg^{-1} dry leaf) followed by NRCDR 515 (2.60 & 3.12 gallic acid equivalents mg^{-1} dry leaf), Candle (2.73 & 3.12 gallic acid equivalents mg^{-1} dry leaf) and Cutlass (2.81 & 3.20 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively while minimum increase in Wester (2.91 & 3.13 gallic acid equivalents mg^{-1} dry leaf), followed by DLSC-1 (2.80 & 3.03 gallic acid equivalents mg^{-1} dry leaf), Pusa Bold (2.86 & 3.08 gallic acid equivalents mg^{-1} dry leaf) and Varuna (2.95 & 3.19 gallic acid equivalents mg^{-1} dry leaf) was observed in control and inoculated plant respectively.

With increase in time interval, at 5 DAI, total Polyphenol content increases in all the genotypes with maximum increase of polyphenol content in Donskaja (2.90 & 3.45 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively, followed by NRCDR 515 (2.70 & 3.15 gallic acid equivalents mg^{-1} dry leaf), while minimum increase in Wester (3.01 & 3.21 gallic acid equivalents mg^{-1} dry leaf), followed by DLSC-1 (2.93 & 3.11 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plants respectively.

At 9 DAI Polyphenol content decreases as compare to previous time intervals, with maximum polyphenol content in Donskaja (2.70 & 2.96 gallic acid equivalents mg^{-1} dry leaf) which is 8.75 percent and minimum in Wester (2.83 & 2.90 gallic acid equivalents mg^{-1} dry leaf) in control and inoculated plant respectively. The total polyphenol content was estimated from the standard curve of gallic acid. Table and graphical representation of the above mentioned results have been made

in Table 5, Fig. 4.
Resistance to white rust pathogen has been observed to be connected with the leaf enzymes polyphenol oxidase, which is involved in phenolic pathway and facilitate the formation of fatty acid chains on leaf surface to restrict pathogen penetration in the host plant. Since *Brassica napus* and *B. carinata* have naturally more epicuticular wax on leaf surface as compared to *B. rapa* and *B. juncea* so less polyphenol content was observed in such genotypes. Higher content of phenolic compounds on leaf surface has been connected with resistance to white rust as higher fatty acids deposition form a physical obstruction as a hydrophobic layer to decrease deposition of water borne inoculums and decline in sporangial germination and germ tube formation. The results obtained here are similar to the findings of other researchers eg. Maitra and Samajpati (1982) [10] reported increased polyphenol

content in resistant cultivar of cauliflower when infected by *Alternaria brassicae*. Mishra *et al.* (2009) [13] who observed increased phenol content in resistant Brassica cultivars EC-399296, EC-399299 as compare to susceptible cultivar with progress of disease. Similarly, Asif *et al.* (2018) [1] reported increased polyphenol content in resistant Brassica cultivar *viz.* Faisal canola and Punjab canola, as compare to susceptible Brassica genotypes when attacked by *A. candida*. Syed (2020) [19] reported increased polyphenol content in tolerant Brassica cultivar PAB 112 and *Sinapis alba* as compare to susceptible cultivar when inoculated with *Alternaria brassicae*. In the present study a positive correlation is obtained between amount of polyphenol content and resistance against *A. candida* pathogen infection. Thus, increased content of polyphenol concentration can be an indicator of activation of defense system in Brassica genotypes against the pathogen.

Table 5: Total polyphenol content in Brassica genotypes inoculated with *A. candida* in glasshouse

Brassica genotypes	Total Polyphenol (Gallic acid equivalents mg⁻¹ dry leaf)									
	Healthy Plant					Inoculated Plant				
	24h	48h	72h	5d	9d	24h	48h	72h	5d	9d
Varuna	2.762 (9.63)	2.812 (9.67)	2.952 (9.39)	3.101 (9.09)	2.912 (9.55)	2.923 (10.01)	3.033 (9.94)	3.193 (9.84)	3.390 (9.67)	3.022 (9.97)
Donskaja	2.521 (9.14)	2.642 (9.28)	2.701 (9.56)	2.902 (9.47)	2.723 (9.16)	2.782 (9.60)	3.012 (9.52)	3.343 (9.80)	3.450 (9.69)	2.961 (9.45)
NRCDR	2.421 (9.80)	2.523 (10.14)	2.603 (9.99)	2.713 (9.63)	2.612 (9.45)	2.643 (10.23)	2.82 (10.60)	3.122 (10.30)	3.151 (10.17)	2.80 (9.12)
Wester	2.723 (9.61)	2.820 (9.45)	2.913 (9.63)	3.012 (9.80)	2.832 (9.80)	2.832 (9.89)	2.982 (10.53)	3.134 (10.10)	3.211 (10.30)	2.901 (10.19)
Cutlass	2.630 (9.80)	2.751 (9.80)	2.812 (9.45)	2.987 (9.28)	2.762 (9.80)	2.782 (10.32)	2.989 (10.15)	3.212 (9.90)	3.212 (9.44)	2.831 (9.99)
Pusa bold	2.612 (8.94)	2.703 (9.33)	2.862 (9.55)	2.921 (9.35)	2.601 (9.45)	2.743(9.35)	2.912 (9.61)	3.085 (9.80)	3.161 (9.99)	2.690 (9.82)
Candle	2.545 (9.45)	2.663 (9.28)	2.734 (9.63)	2.802 (9.63)	2.645 (9.80)	2.694 (10.16)	2.923 (10.17)	3.125 (10.30)	3.123 (10.02)	2.7322 (10.70)
DLSC-1	2.762 (9.68)	2.790 (9.27)	2.801 (9.28)	2.932 (9.45)	2.723 (9.45)	2.891 (9.80)	2.952 (9.51)	3.03 (9.63)	3.110 (9.68)	2.782 (9.60)
CD 5%										
Genotype (A)	0.087 (0.063)					0.096 (0.087)				
Days (B)	0.069 (0.057)					0.076 (0.062)				
AXB	0.194 (0.148)					0.215 (0.192)				
CV	5.98 (8.61)					7.78 (9.66)				

* Values in parenthesis is angular transformed value
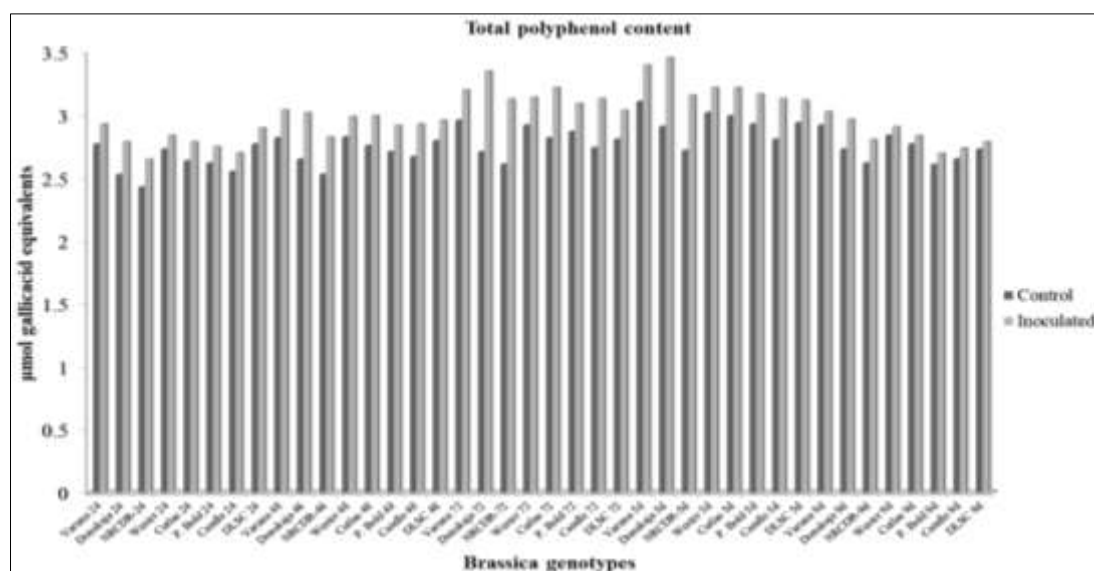
Fig 4: Total polyphenol content in Brassica genotypes inoculated with *A. candida* in glasshouse at different time intervals

Present study revealed that infection of obligate pathogen *A. candida* causes different biochemical changes in host Brassica genotypes, which affect the biochemistry and host pathogen interaction during the invasion of pathogen. As generation of reactive oxygen species (ROS) is the first event which takes place during pathogen recognition by host plant so for mitigating the effect of harmful ROS, plant cells have oxygen radical detoxifying enzymes which are produced at the site of ROS production. This enhanced ROS lead to the production of H_2O_2 which is toxic to plant cells if released in excess amount. Although, H_2O_2 acts as a messenger in cellular communication during defense process if present in optimum and desired concentration but its excess is harmful for cell activities. Thus enzymatic and non-enzymatic defense related compounds tend to release to balance the amount of H_2O_2 in cell so that it acts only in the facilitation of defense process rather than functioning against the plant cells. In response of this biochemical alteration, enzymatic and non-enzymatic defense compounds eg. peroxidases, and, polyphenol along with protein content alter their amount to withstand the pathogen infection. Thus, biochemical parameters can be considered as biochemical markers to identify the activation of defense process in Brassica genotypes during *A. candida* infection.

This study revealed that all the immune Brassica genotypes *viz*. Wester, DLSC 1, NRCDR 515, highly resistant genotype Donskaja, resistant genotype Candle showed higher increase in peroxidases (POD) activity with probably more breaking down of H_2O_2 and oxygen radicals into water and oxygen, while moderately resistant genotype Cutlass, susceptible genotype Pusa bold and highly susceptible genotype Varuna showed lesser amount of these anti-oxidative enzymes.

In the present study decrease in protein content was observed with course of infection period with maximum decrease in Immune Brassica genotypes (Wester, DLSC 1, NRCDR 515) followed by highly resistant (Donskaja), resistant (Candle) and moderately resistant (Cutlass) while minimum decrease in protein content was observed with highly susceptible (Varuna) and susceptible (Pusa bold) genotypes. Less protein content in immune, highly resistant, resistant and moderately resistant genotypes is an indication of secretion of more anti-oxidative enzymes in these genotypes as enzymes are the part of protein and during the infection process some protein content get converted into enzymes for facilitating the defense process. Thus for the secretion of more anti-oxidative enzymes during pathogen infection major protein content get converted into enzymes and amount of total protein content decreases accordingly. In this way amount of protein content is negatively correlated with resistance against *A. candida* pathogen.

4. Conclusion
The present study revealed that defense related compounds peroxidases, polyphenol, and protein content can be used as biochemical markers in the identification of resistant sources against *A. candida.* Though the clear defense mechanism adopted by these compounds against *A. candida* is not well known but it is speculated that increasing content of Peroxidase and Polyphenol can be an estimation of activated defense mechanism against *A. candida* pathogen and can be used as biochemical markers for identifying resistant genotypes against the pathogen under glasshouse conditions. As development of resistant cultivars through conventional breeding is a time-consuming process thus biochemical markers can be an alternative for screening of already existing resistant germplasm for the identification of resistant sources and that will again be an eco-friendly method of disease management.

5. References
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