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Bio-chemical alterations: markers for the identification of source of resistance in brassica germplasm against white rust disease

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Abstract

Rapeseed (*Brassica napus*) is affected by a devastating disease white rust (*Albugo candida*) which induces change in biochemistry of Plant. Pot experiment was conducted to study biochemical activities and basic association of resistance in germplasm. Fifteen brassica varieties/lines were cultivated in pots and kept in glasshouse. After inoculation, inoculated and uninoculated leaves were collected and enzyme activity was recorded for comparison. Basic trend of catalases (CAT), phenolic, and peroxidases (POD), super oxide dismutase (SOD) and Hydrogen peroxide showed increase in activity in inoculated plants of resistant variety (Faisal Canola) while decrease in protein activity was observed in inoculated leaves when compared to uninoculated. In resistant cultivars i.e Faisal Canola activity of SOD (0.123 to 1.961mg^{-1} Protein), POD (0.053 to 1.028µmol H_2O_2mg^{-1} Protein), CAT (0.214 to 1.912µmol H_2O_2mg^{-1} Protein), phenolic (0.123 to 1.961mg/mg of leaves) and H_2O_2 (0.448 to 1.714µmol g^{-1} fw) increased while reduction in protein activity was observed from (3.14 to 1.416mg/g of leaves). While in a comparison to resistant variety, a very rapid increase in above mentioned parameters was observed in susceptible variety (Toria Selection A). It was concluded that biochemical alterations provide strong factual evidence and effective marker for identification of resistant germplasm.

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Introduction

Rapeseed *(Brassica napus)* of family Brassicaceae is the second common traditional oilseed crop of Pakistan after cotton (Abbas *et al.,* 2009; Ali *et al.*, 2012). It has been cultivated in Asia, China, South Africa, Europe, and Canada since 2000 BC (Warwick *et al.*, 2006; Yao *et al.*, 2012). It is cultivated on an area of 25 million hectares with 46 million tons' productions worldwide (FAO, 2004). In Pakistan, it is cultivated on an area of 193.6 thousand hectares and its production of seed and oil content is 181 and 56 thousand tons respectively in 2015 (PODB, 2015). Its share in domestic production of oil in Pakistan is 17% (Abbas *et al.*, 2008). It has amino acids, ascorbic acid, tocopherols, phenolic, sugar, minerals and vegetable oil content. These compounds are important for the survival of plant. Brassica's oil is used in making vegetable oil and these compounds serve as antioxidant against major disease of human beings. They strengthen the body immune system (Jahangir *et al.*, 2009; Huber *et al.*, 2009; Rossetto *et al.*, 2013). Brassica is affected by white rust (*Albugo candida*) disease which is the most destructive disease in tropical and subtropical areas of Pakistan (Armstrong, 2007). It causes 20-90% yield losses throughout the world (Kumar and Kalha, 2005; Mishra *et al.,* 2009). This candidum (*candida*) specie of genus *Albugo* was first time reported by Gmelin (1792) while in Pakistan it is first reported from FATA and BAJUR Agency (Ahmed *et al.*, 1997). *Albugo candida* is an obligate parasite and can survive by means of producing oospores in infected plants parts and in soil for more than 20 years (Gupta *et al.*, 2004; Hina *et al.*, 2014). Optimum temperature for effective infection is 12-22°C with 60-90% relative humidity (RH) (Sullivan *et al.*, 2002). *Albugo candida* produces two types of symptoms. In the initial stage of infection, locally white pustules or blisters are formed on the aerial parts mainly on leaves and cotyledones. The upper area of the leaf becomes tan yellow. Small pinhead size, creamy white pustules on the abaxial surface and small pinhead size green island on the adaxial surface. Numerous pustules ultimately coalesce to form larger pustules. Often pustules appear in a circular arrangement around one big central pustule.

Mature pustules rupture to release a mass of white sporangia. In some cases, pustules may be surrounded by a yellow halo. On leaves, initial infection is on the under surface, but under humid conditions, both sides may produce white minute pustules. Pustules are also common on inflorescence and floral parts. Other symptoms also develop with two types of infection: local and systemic. Local infection is characterized by the formation of raised creamy white sporangial pustules on the under-surface of leaves and on tender shoots. The affected tissue turns necrotic and dies. Systemic infection is usually seen in young blossom and leaves. It stimulates hypertrophy and hyperplasia resulting in abnormal swelling and malformation of the affected organs. Floral organs turn green, become greatly enlarged and distorted, and seed formation is prevented (Saharan and Mehta, 2002; Mishra *et al.,* 2009).

The infection of *Albugo candida* brings different biochemical changes in plant. These changes in host are useful to understand the biochemistry and host pathogen-interaction and may be used to manage the disease. Plants have mechanisms to withstand against the pathogen by producing reactive oxygen species (ROS). But these antioxidant compounds are also toxic for plants membranes, so increased concentration of one enzyme in tissues is regulated by another one (Turkan and Demiral, 2009). Although, they are already present in resistant and susceptible germplasm but resistance is associated with the enzymes like Catalase, Peroxidase, Superoxide dismutase, protein and phenolic pathways. They act as defence signals transduction in plants. Higher or lower level of these compounds reduces the adherence of water or airborne inoculum, zoospore development and germ tube formation. These biochemical compounds are very important to investigate the resistance mechanism (Singh *et al.,* 1999; Meena *et al.,* 2008). *A. Candida* can change the quantity of protein (enzymes), phenol, ionic and sugar contents in brassica plants and this change may either be increased or decreased in the diseased leaves of crop. It induces change in host resistance in plants.

Plant–pathogen interactions cause a complex network of molecular and cytological events, which provide the host susceptibility or resistance. Peroxidase and other isoenzymes are positively co-related to initiating resistance response (Nawar and Kuti, 2003). That's why it was dire need to observe these alterations of different biochemical compounds with varying disease incidence. Although, there are several pathogens present on the surface of plant which can produce similar type of symptoms and simple symptomology study is not enough to identify source of resistance. Present study of biochemical changes is aimed to identify the source of resistance and aimed to analyse changes in concentrations of Catalases (CAT), Peroxidases (POD), Super oxide dismutase (SOD), Hydrogen peroxide (H_2O_2), total Phenolics and protein in uninoculated and inoculated leaves. Understanding the mechanism employed by plants to defend themselves against pathogens may lead to novel strategies to enhance disease resistance. Disease resistance depends upon the induction of defenses following exposure to organisms. Several types of biochemical changes above mentioned were found to be important in the investigation of resistance mechanism (Meena *et al.*, 2008). The aim of this research was to explore the possibility of biochemical changes for defense of oilseeds Brassica infected pathogen. Main objective of the present study was to explore the biochemical-markers for the identification of source of resistance in *Brassica* germplasm against *A. candida* pathogen.

Materials and methods

Establishment of Disease Screening Nursery

The experiment was performed in the glasshouse of Department of Plant Pathology, University of Agriculture Faisalabad. There were fifteen brassica cultivars named as Punjab Canola, Faisal Canola, Shirale, Dunkeld, Rainbow, AC-Exesl, Legend, Oscar, Cyclone, Con II, Con III, Bulbul 98, DGL, B.S.A, Toria selection "A" collected from Oilseed Research Institute of Ayub Agricultural Research Institute (AARI), Faisalabad. Plastic pots of 8cm diameter were filled soil, sand and farm yard manure with ratio of 1:1:1. Pots were cultured with untreated genotypes seeds in completely randomized design (CRD) and each genotype was replicated thrice. Pots were kept in tray of (90 x 60 x 27) and two healthy plants were maintained per pot. Another tray was established for inoculation of leaves to induce disease.

Inoculum preparation and Inoculation

Highly infected leaves were collected from susceptible variety (Toria Selection-A) showing severe disease symptoms. White Sporangial pustules were scraped into a Petri Dish (9 cm) from the downy surface of leaves. This scraped zoosporangial concentration of powder was weighed 40mg to have best level of inoculum using Precision Balance (Everrich™Model FA2004N china) and stored at below -10°C in refrigerator (PEL, PRGD-145). 60 mL Sterilized distilled water was mixed in this concentration and shaken vigorously with glass rod in 100 ml conical flask (Erlenmeyer flask, SB 19). Haemocytometer (Z359629 - Bright-Line™ Hemacytometer) was implied to adjust the spores concentration at $1.44x10^5$ zoosporangial suspension (Ranjan *et al.,* 2009). The culture was then incubated for about 4 hours at 15°C to obtain germination of sporangia to adjust suspension, which in the true sense served as the inoculum for inoculation. Fully expanded leaves of 25-30 day-old poted seedlings were used for inoculation. Inoculum was carefully applied by pipetting 10-µL droplets using fixed volume Micropipette (10-µL) (Kartell, Model 240234-0010 MiniFIX Micropipette) onto the abaxial surface of each leaf to avoid any inoculum runoff. After inoculation, Plants were then transferred to a greenhouse. Symptoms started to appear after 7 days of inoculation and for observations on the content of Catalases (CAT), Peroxidases (POD), Super oxide dismutase (SOD), Hydrogen peroxide (H_2O_2), total Phenolic and protein in inoculated leaves after infestation were collected. As a control, Uninoculated leaves were also collected and stored. All the cultural practices were performed to keep the plants in healthy condition.

Samples preparation for biochemical analysis

Inoculated and Uninoculated samples were collected in yellow bags (20x30cm) and stored. Stored frozen samples were washed with tap water and then with distilled water.

After air drying, 10g leaves were weighed and cut into uniform small pieces for grinding. Extraction buffer was prepared with the following chemicals i.e. 3.025mg K_2HPO_4, 3.025mg KH_2PO_4. Leaves were grinded with the extraction buffer in pestle and mortar and the mixture was placed in Eppendorf (®3800X) for further testing and centrifuged in "Table Top Centrifuge" if and when required at 4°C for 10-15 minutes. The supernatant was filtered and stored at 4°C in refrigerator (PEL, PRGD-145) and the residues were discarded.

SuperoxideI dismutase activity

SOD activity was measured based on its ability to inhibit the photoreduction of Nitrobluetetrazolium (Shahid *et al.,* 2012). The reaction solution was prepared with 50µl phosphate buffer of pH 7.8, 50µl NBT, 100µl methionine, 100µl Triton, 100µl riboflavin. Reaction mixture in test tubes was given UV treatment under fumes hood for 15 min. 50µl leaf extract, 1ml H_2O_2 and 100µl reaction mixture was taken in ELISA plate (UltraCruz® ELISA Plate, 96-wells) and Absorbance was measured by spectrophotometer (BEL©, Model L.24) at 560 nm (Shahid *et al.,* 2012).

Catalase activity

The catalase reaction solution was made up of 50µl phosphate buffer of pH 8.3, 50µl H_2O_2, and 100µl enzyme extract. The reaction was initiated by adding the leaf extract. 100µl samples was loaded on ELISA micro-plate (UltraCruz® ELISA Plate, 96-wells) and absorbance was measured at 240nm with the help of spectrophotometer (BEL©, Model L.24) (Shahid *et al.,* 2012).

Peroxidase activity

The Peroxidase (POD) reaction solution (3ml) was made by 50µl phosphate buffer of pH 7, 375µl guaiacol, 100µl H_2O_2, and 50µl leaf extract. 100-150µl samples was loaded on micro-plate (UltraCruz® ELISA Plate, 96-wells) and absorbance of reaction solution was recorded at 470nm through spectrophotometer (BEL©, Model L.24). One unit of catalase and Peroxidase activity was considered as an absorbance change of 0.01 unit's min^{-1} (Shahid *et al.,* 2012).

H_2O_2 Concentration

0.5g leaf sample with 0.1% Trichloroacetic acid (TCA) was grinded in grinder (Philips HL7756/00 750W Mixer Grinder) and centrifuged at 12,000rpm for 15min at 4°C. Supernatant (0.3mL) was mixed with 1.3mL potassium phosphate buffer of pH 7 and 1mL of 1M potassium iodide. It was incubated (Digital incubator) for 5 minutes and after incubation absorbance was measured at 390 nm with the help of spectrophotometer (BEL©, Model L.24). The amount of H_2O_2 was measured from a standard curve which was prepared from known concentrations of H_2O_2 and it will be indicated as $\mu mol \cdot g^{-1}$ FW (Velikova *et al.*, 2000).

Protein activity

40µL leaf extract with 160µL Bradford reagent was loaded on ELISA plate (UltraCruz® ELISA Plate, 96-wells) and absorbance was measured with the help of spectrophotometer (BEL©, Model L.24) at 595nm (Bradford, 1976).

Phenolic activity

Reaction mixture for phenolic (total phenolics) was made of 5ml of FC reagent mixed with 45ml of double distilled and 10g sodium carbonate mixed with water. 100µl leaf extract and 50µl FC reagent mixture was taken in eppendorf (®3800X) and shaken thoroughly. 150µl sample was loaded on micro plate (UltraCruz® ELISA Plate, 96-wells)) to observe the absorbance at 765nm in spectrophotometer (BEL©, Model L.24) (Shahid *et al.,* 2012).

Statistical analysis

All the experiments were performed in triplicates and data was recorded subject to Completely Randomized Design (CRD) as described by Steel *et al.*, (1997). Analysis of variance was recorded and Least significant difference (LSD) test was applied to determine the significant differences in mean ($P \leq 0.005$). All the statistical tests were performed by using SAS statistical software (SAS institute, 1990).

Results

Catalase (μ mol $H_2O_2 mg^{-1}$ Protein)

Generally, all the genotypes showed increase in catalase activity with few exceptions.

Faisal canola and Punjab canola were resistant varieties and they showed increase of (0.214 to 0.936) and (1.912 to 2.45) in uninoculated and inoculated leaves respectively. Shirale, Dunkeld, Rainbow CON II and Legend contain CAT ranged from (0.343, 0.966, 1.815, 1.718 and 1.877) to (1.077, 2.038, 3.250, 3.946 and 2.064) respectively in *brassica* leaves as there is considerable increase in catalase enzyme in such varieties. Its concentration in other susceptible varieties, i.e. Ac-Exsel, Oscar, DGL, CON III, and BSA, was 1.416, 3.487, 3.771, 2.563 and 2.697 in inoculated but in uninoculated leaves it contain 1.054, 2.613, 0.849, 1.558, and 1.672 respectively. Although there was significant increase in catalases enzyme activity but only DGL demonstrated sudden rapid increase. Cyclone, Bulbul 98 and Toria Selection-A were highly susceptible varieties which resulted in (4.137, 3.296 and 3.295) concentration of CAT in inoculated leaves as compared to the uninoculated leaves having 2.568, 0.587 and 0.969 respectively which depicts a prominent increase in activity. Drastic change was observed in Bulbul 98 and Toria Selection-A (Table 1).

Hydrogen peroxide (µ mol g^{-1} fw)

All the varieties showed increase in H_2O_2 content. In moderately resistant cultivars (Punjab Canola and Faisal Canola) its level was increased from (0.448, and 0.264) to (1.714, 0.925) respectively while in moderately susceptible lines i.e. Shirale, Dunkeld, Rainbow, CON II and Legend increased from (1.104, 2.884, 1.475, 1.567 and 3.094) to (2.162, 3.094, 1.971, 2.064 and 3.77) respectively comparing uninoculated leaves. Other susceptible varieties, i.e. Ac-Exsel, Oscar, DGL, CON III, and BSA, its quantity in inoculated was 1.842, 1.873, 6.864, 4.622 and 3.493 but uninoculated leaves containing (1.479, 0.308, 5.038, 1.702, and 2.034) which revealed slight increase in H_2O_2 activity in inoculated leaves. Prominent and sudden increase was recorded in highly susceptible varieties (Cyclone Bulbul 98 and Toria Selection A) which ranged from (3.513, 2.409 and 4.905) to (4.036, 3.729 and 5.729) respectively (Table 1).

Table 1. Comparison of mean values of catalase activity (µ mol $H_2O_2mg^{-1}$ Protein) and H_2O_2 (µ mol g^{-1} fw) in uninoculated and inoculated leaves of *Brassica*.

Varieties	Response	CAT (µ mol $H_2O_2mg^{-1}$ Protein)		H_2O_2 (µ mol g^{-1} fw)	
		Uninoculated	Inoculated	Uninoculated	Inoculated
Punjab Canola	MR	0.214 k	0.936 i	0.448 j	1.714 k
Faisal Canola	MR	1.912 b	2.451 f	0.264 k	0.925 l
Shirale	MS	0.343 j	1.017 i	1.104 i	2.162 h
Dunkled	MS	0.966 g	2.038 g	2.884 d	3.094 g
Rainbow	MS	1.815 c	3.25 d	1.475 h	1.971 hij
Ac-Exsel	S	1.054 f	1.416 h	1.479 h	1.842 jk
Legend	MS	1.877 bc	2.064 g	3.094 c	3.77 e
Oscar	S	2.613 a	3.487 c	0.308 jk	1.873 ijk
Cyclone	HS	2.568 a	4.137 a	3.513 b	4.036 d
CON lI	MS	1.718 d	3.946 ab	1.567 gh	2.064 hi
Bulble98	HS	0.587 i	3.296 cd	2.409 e	3.729 e
DGL	S	0.849 h	3.771 b	5.038 a	6.864 a
CON III	S	1.558 e	2.563 ef	1.702 g	4.622 c
BSA	S	1.672 d	2.697 e	2.034 f	3.493 f
Toria Selection A	HS	0.969 g	3.295 cd	4.905 a	5.729 b
LSD		0.062	0.203	0.164	0.207

*Means sharing common alphabets don't vary significantly as per the LSD test ($p \leq 0.05$).

M.R = Moderately Resistant, S = Susceptible, M.S = Moderately Susceptible, H.S = Highly Susceptible

if ($P \leq 0.05$) Significant

Peroxidase (µ mol $H_2O_2mg^{-1}$ Protein)

POD is specifically and relatively associated with resistance like Catalase and H_2O_2. Punjab Canola and Faisal Canola are resistant cultivars and POD level increased in theses from (0.053 and 0.133) to (1.028, 0.248) respectively. In moderately susceptible cultivars/lines i.e. Shirale, Dunkeld, Rainbow, Legend and CON II, its level climbed up from (0.028, 0.034,

0.122, 0.038 and 0.1014) to (1.272, 0.279, 1.207, 0.930 and 0.487) respectively. This was gradual change in concentration due to susceptibility. A rapid change in Ac-Exsel, Oscar, DGL, CON III, and BSA (susceptible cultivars) was observed in inoculated leaves demonstrating (1.023, 1.796, 0.315, 1.036, and 1.029) as compared to uninoculated leaves having (0.079, 0.046, 0.157 and 0.118) POD concentration respectively. Exceptionally oscar showed drastic decrease in POD content. Leaves of highly susceptible varieties (Cyclone, Bulbul 98 and Toria Selection A were resulted in concentration of (0.505, 0.875, and 1.154) and (0.077, 0.045 and 0.065) in inoculated and uninoculated leaves respectively It suggest that increased of this enzyme is regulating the increase of catalase and H_2O_2 (Table 2).

Superoxidase dismutase (mg^{-1} Protein)

Amount of SOD in moderately resistant cultivars (Punjab Canola and Faisal Canola) enhanced from (1.471 and 0.305) to (2.573 and 0.920) respectively while in moderately susceptible varieties/lines i.e. Shirale, Dunkeld, Rainbow, Legend and CON II, the amount of SOD ranged from (0.127, 1.658, 1.331, 1.0035 and 1.006) to (1.876, 2.139, 2.031, 2.247 and 3.909) respectively which depicts slight increase in resistant lines while gradually more than resistant. In Susceptible varieties, i.e. Ac-Exsel, Oscar, DGL, CON III, and BSA, the amount of SOD in inoculated leaves was (1.566, 3.558, 1.133, 2.561 and 4.389) as compared to uninoculated leaves (0.244, 2.491, 0.858, 1.577, 2.166) while in highly susceptible varieties (Cyclone, Bulbul 98 and Toria Selection A) was in higher amount (1.177), (4.006) and (3.596) while lesser amount of SOD in uninoculated leaves like (1.033), (2.567) and (3.045). This revealed that increase in SOD was regulating the concentration of POD, so it was greater increase in susceptible lines while slight increase in resistant lines (Table 2).

Table 2. Comparison of mean values of peroxidase (POD) activity (µ mol $H_2O_2mg^{-1}$ Protein) and SOD (mg^{-1} Protein) in uninoculated and inoculated leaves of brassica.

Varieties	Response	POD (µ mol $H_2O_2mg^{-1}$ Protein)		SOD (mg^{-1} Protein)	
		Uninoculated	Inoculated	Uninoculated	Inoculated
Punjab Canola	MR	0.053 gh	1.028 e	1.471 g	2.573 e
Faisal Canola	MR	0.133 c	0.248 l	0.305 k	0.92 l
Shirale	MS	0.028 i	1.272 b	0.127 m	1.876 i
Dunkled	MS	0.034 hi	0.279 k	1.658 e	2.139 g
Rainbow	MS	0.122 cd	1.207 c	1.331 h	2.031 h
Ac-Exsel	S	0.217 a	1.023 e	0.244 l	1.566 j
Legend	MS	0.038 hi	0.930 f	1.003 i	2.247 f
Oscar	S	0.079 ef	1.796 a	2.491 c	3.558 d
Cyclone	HS	0.077 ef	0.505 h	1.033 i	1.177 k
Con II	MS	0.101 de	0.487 i	1.006 i	3.909 c
Bulble98	HS	0.045 ghi	0.875 g	2.567 b	4.006 b
DGL	S	0.046 ghi	0.315 j	0.858 j	1.133 k
Con III	S	0.157 b	1.036 e	1.577 f	2.561 e
BSA	S	0.118 cd	1.029 e	2.166 d	4.389 a
Toria Selection A	HS	0.065 fg	1.154 d	3.045 a	3.596 d
LSD		0.023	0.017	0.046	0.095

*Means sharing common alphabets don't vary significantly as per the LSD test ($p \leq 0.05$).

M.R = Moderately Resistant, S = Susceptible, M.S = Moderately Susceptible, H.S = Highly Susceptible

Protein (mg/g of leaves)

Generally, it was observed that Protein activity was reduced in inoculated leaves of all varieties due to the invasion of pathogen which might destroyed the chlorophyll. Punjab Canola and Faisal Canola demonstrated reduced level from (3.41 and 2.683) to (1.416 and 2.07) respectively.

These were resistant lines and Protein activity was slightly decreased due to resistant genes. While in moderately susceptible cultivars (Shirale, Dunkeld, Rainbow, Legend and CON II) quantity of protein decreased from (2.333, 2.536, 3.623, 3.806 and 2.40) to (0.72, 1.046, 2.029, 2.043 and 1.823) respectively comparing to uninoculated.

In inoculated leaves of susceptible varieties (Ac-Exsel, Oscar, DGL, CON III, and BSA) protein was recorded (1.81, 0.32, 2.110, 1.78 and 1.73) as compared to uninoculated leaves of brassica (2.7, 2.916, 3.006, 2.160, and 2.433) respectively. In highly susceptible varieties (Cyclone, Bulbul 98 and Toria Selection A) protein activity was (2.31, 0.91 and 1.21) while uninoculated leaves have (3.176, 2.886 and 3.506) amount of protein. These values revealed (Table III).

Phenolic (mg/g of leaves)

Over all the response of phenolic activity was increased in inoculated leaves of all varieties as compared to the uninoculated leaves due to the interaction of pathogen with host. Phenolic activity in moderately resistant cultivars/lines (Punjab Canola and Faisal Canola) jumped from (0.123 and 0.154) to (1.961 and 1.043) respectively while in moderately susceptible cultivars (Shirale, Dunkeld, Rainbow, Legend and CON II) level of phenolic increased from (0.093, 0.276, 0.133, 0.235 and 0.175) to (1.66, 2.216, 1.055, 1.571 and 2.946) respectively. In inoculated leaves of susceptible varieties (Ac-Exsel, Oscar, DGL, CON III, and BSA) phenolic content was (1.293, 3.263, 1.733, 1.344 and 2.664) as compared to uninoculated leaves (0.142, 0.317, 0.234, 0.453 and 0.404) respectively. Data showed the smooth change in concentration. In highly susceptible varieties (Cyclone, Bulbul-98 and Toria Selection-A) phenolic level was (2.872, 1.605 and 3.045) while in uninoculated leaves have (0.165, 0.816 and 1.050) amount of protein (Table 3).

Table 3. Comparison of mean values of Protein (mg/g of leaves) activity (µ mol $H_2O_2mg^{-1}$ Protein) and Phenolics (mg/g of leaves) in uninoculated and inoculated leaves of brassica.

Varieties	Response	Protein (mg/g of leaves)		Phenolics (mg/g of leaves)	
		Uninoculated	Inoculated	Uninoculated	Inoculated
Punjab Canola	MR	3.410 bc	1.416 d	0.123 l	1.961 g
Faisal Canola	MR	2.683 ef	2.070 b	0.154 ij	1.043 m
Shirale	MS	2.333 gh	0.720 g	0.093 m	1.660 i
Dunkled	MS	2.536 fg	1.046 f	0.276 f	2.216 f
Rainbow	MS	3.623 ab	2.029 b	0.133 kl	1.055 m
Ac-Exsel	S	2.700 ef	1.810 c	0.142 jk	1.293 l
Legend	MS	3.806 a	2.043 b	0.235 g	1.571 j
Oscar	S	2.916 de	0.320 h	0.317 e	3.263 a
Cyclone	HS	3.176 cd	2.310 a	0.165 hi	2.872 d
Con II	MS	2.400 fgh	1.823 c	0.175 h	2.946 c
Bulble98	HS	2.886 de	0.910 f	0.816 b	1.605 j
DGL	S	3.006 d	2.110 b	0.234 g	1.733 h
Con III	S	2.160 h	1.780 c	0.453 c	1.344 k
BSA	S	2.433 fgh	1.730 c	0.404 d	2.664 e
Toria Selection A	HS	3.506 ab	1.210 e	1.050 a	3.045 b
LSD		0.302	0.153	0.016	0.034

* Mean values in a column sharing similar letters do not differ significantly as determined by the LSD test ($P \leq 0.05$).

M.R = Moderately Resistant, S = Susceptible, M.S = Moderately Susceptible, H.S = Highly Susceptible.

Discussion

Development of resistant varieties through mass selection breeding method is a time-consuming process. One of the best alternative solution is the screening of already available germplasm based on biochemical markers because these biochemical changes play an important role in the plant defence and induces resistance as the proteins form complex with fungus to inhibit it (Bansal *et al.*, 2005).

Generation of reactive oxygen species is first event towards the pathogen recognition. This term is used for radicals and other non-radicals but reactive species derived from oxygen which are responsible for resistance in plants. Cells contains oxygen radical detoxifying enzymes such as catalase, peroxidase, and superoxide dismutase at the sites of enhanced ROS production (Kuzniak and Sklodowska, 2001; Pnueli *et al.*, 2003).

H_2O_2 can function as a messenger in cellular communication demanding a finely tuned adjustment of its cellular concentration (Foyer and Noctor, 2000). It is required for cross linking of plant cell wall components as a part of structural defence response (Levine *et al.,* 1994). One compound regulates the level of other one in tissues as H_2O_2 is produced in a very high rate in susceptible but less in moderately resistant lines. As a defensive action on H_2O_2 the quantity of CAT which lead to increase in POD and SOD is increased to manipulate later and so is the way of regulating it.

Increase of Hydrogen peroxide (H_2O_2) trigger the hypersensitive cell death. An increased level of H_2O_2, stimulation of disease resistance in plants. Hydrogen peroxide act as signaling compound at normal level but when it exceeds from the normal level it become toxic which is very harmful for the plants (Mhamdi *et al*., 2010). However, this high level is reduced by scavenging via antioxidant enzymes such as CAT and POD, which prevent the most harmful effects of excess H_2O_2 in cells. Present study reveals that all the genotypes showed greater or less increase in following compounds which is similar to above mentioned facts in which resistant cultivars showed slight increase of SOD and POD while their amount was highly increased in susceptible to highly susceptible varieties which is indication of scavenging the higher production of CAT. Umasthun *et al*., (2012) reported that H_2O_2 is main radical produced in excess which is converted into water and oxygen by Catalase. Catalase (CAT) prevents the accumulation of H_2O_2 in the cells. Current research is in line with findings of (Abedi *et al*., (2010) who declared the increase in content of Catalase activity in resistant varieties and it breaks down the hydrogen peroxide into water and oxygen; in addition, Quiroga *et al*., (2000); Fahey *et al*., (2001) also reported enhanced level of this enzyme. Hahlbrock *et al*., (2003); Hagemeier *et al*., (2001); and Tan *et al*., (2004) conducted research on similar germplasm and their findings are true to this research. Similarly, enhanced catalase activity is supported by Tirmali and kolte, (2013) who observed increase Catalase activity in inoculated leaves of cucumber.

Peroxidase (POD) plays a significant role in defence response and its activity is associated with resistance induction in different species of plants. It is also involved in the breakdown of hydrogen peroxide. Increased activity of peroxidase leads to the production and accumulation of hydroxyproline rich glycoprotein into the cell wall. Peroxidase is involved in lignin polymerization. The enzyme has role in cell wall metabolism as well as in defence regulation and induction (Reuveni *et al*., 1997; Singh *et al*., 1999). In present research, increase in POD activity is observed in inoculated moderately resistant brassica cultivars of as compared to the uninoculated leaves against WR disease. This indicated that slight increase in such compound was due to infection which was regulated by defence mechanism of plant. In susceptible inoculated cultivars, POD was vigorously increased. Drastic increase in POD activity can be observed in a cultivar Cyclone. The current study is supported by the findings of Tirmali and Kolte, (2013) and Mahdy *et al.,* (2006), who observed the increase in POD activity in resistant cultivars of Cucumber.

Another phenomenon related to excessive production of Reactive Oxygen radicals is to initiate hypersensitive response (HR) via oxidative burst. HR is responsible for quick activation of defence mechanism of plant which restricts water and nutrients of Pathogen (Jones and Dangl, 2006; Glazebrook, 2005) and pathogen releases virulence factors which are recognized by the plant after that process. Singh *et al*., (2011) investigation is conformity to present research findings in which he studied different biochemical compounds and reported that maximum Peroxidase and invertase concentration was found in infected leaves. Amount of ascorbic acid oxidase reported to lowered with increase in infection. Another research conducted by Sharma *et al.,* (2006) demonstrated that the products of various reactions of peroxidases are known to stop the pathogen growth especially fungi in host in localized infection. Earlier studies also confirmed the role of peroxidases in resistance of crops like wheat against fungal pathogens. Several reports confirm an elevated level of anionic isoenzymes of peroxidase in basel zone of wheat roots infected with stinking smut (Khairullin *et al.,* 2000).

Superoxidase dismutase enzyme acts as a first line of defence against reactive oxygen species (ROS) and rapid induction of SOD leads to recognize the pathogen's a-virulence factors. In present study increase in SOD activity was observed in inoculated leaves of resistant varieties and these results were supported by Lebeda *et al.,* (2001); Hameed and Iqbal, (2014) as their results are exhibiting increased SOD activity. Its production in highly susceptible varieties was very high because of higher production of H_2O_2 which is toxic for plant.

In present research, reduction in protein activity was observed in inoculated susceptible lines but in highly susceptible varieties this reduction is rapid due to pathogen attacks on the plant it absorbs the protein and carbohydrates from the plant. In resistant varieties, there is less loss of protein as compared to the susceptible varieties because presence of protein blocks the active site by making a complex and inhibits the pathogenic enzyme (Freeman *et al.*, 2005). Another line of defence against infection is the very rapid synthesis of phenolic and their polymerization in the cell wall and altering the level of phenolic in plants has been demonstrated to change disease susceptibility. An increase in the activities of phenolic-related enzymes and the accumulation of phenolic was correlated with resistance to biotic stresses (Mohammadi and Kazemi, 2002). Present study showed the increase in phenolic contents in all varieties as it is supported by the findings of Mathpal *et al.,* (2011) in which he investigated the total phenolic contents of tolerant to susceptible mustard cultivars and stated that this content increases with the increase in disease incidence. As the infection rate increases concentration of total phenolics enhances in susceptible cultivars. So, it can be inferred from the present findings that it supports in resistance conferring in cultivars against pathogen. Similar trend is observed by Nawar and Kuti, (2003) and Ranjan *et al.,* (2009) as he studied the Phenols and polyphenol oxidase activity in different brassica varieties and reported the increase content of phenols with the progression of infection of fungus.

In addition, Mathpal *et al.,* (2011) showed that phenolic contents can be increased due to glycosidic esters formed by the enzymatic activity of host or pathogen or due to migration of phenols from uninfected tissues and such observations are in support present research. Such changes were exploring the new mechanism of resistance induction and development which can be incorporated in research studies to explore new varities/lines for future use and secure the farmer from heavy economic loss.

Conclusion

Enhanced biochemical contents like CAT, POD, SOD, Phenolic and H_2O_2 but decreased in protein content as compared to uninoculated leaves was observed. Such studies can be further incorporated in breeding programme to identify and develop resistant source. These changes can be used as effective disease controlling mechanism and resistance inducer.

Abbreviations:

Catalases (CAT), peroxidases (POD), super oxide dismutase (SOD)

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