Elucidation of biochemical alterations in citrus cultivars due to brown spot disease

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ABSTRACT

Biochemical compounds are key components in plant-pathogen interaction. Plants become susceptible to various pathogenic microbes due to alteration in these antioxidant compounds. These compounds are help the plants to combat with pathogens. So, study of alterations in biomolecules will be helpful for growers and researchers to develop better preventive measures against brown spot of citrus. For this purpose, experiments were conducted to observe alterations in biochemical compounds infected with brown spot of citrus through artificial inoculation of pathogen. After two years screening of thirty citrus species, six varieties (Susceptible and resistant) were selected. Leaves of resistant (Eruka lemon, Mayer lemon and Citron) and susceptible (Washington navel, Valentia late and Kinnow) cultivars of inoculated and un-inoculated group were collected and analyzed through Nested Structured Design for alterations in biomolecules of citrus leaves. Significant variations ($p \leq 0.05$) were present in biochemical enzymes of resistant susceptible types. Susceptible types of plant indicated 2.64,3.31, 1.02, 2.38, 1.74, 4.20 and 0.77, resistant types of plants showed 3.42, 3.72,1.53,2.04,2.51,3.27 and 0.63 µg/g of total phenolic contents, total soluble sugars, SOD, POD, total soluble proteins, Catalases and H_2O_2 respectively. In case of group, Un-inoculated plants of citrus expressed 4.30,4.17, 2.16,1.26,3.30, 2.28, 0.27 while inoculated plants showed 1.76, 2.86, 0.39,3.16,0.94,5.19 and 1.14 µg/g of these compounds. It is concluded from the current findings that alterations in biochemical compounds can serve as effective marker for the documentation of resistant germplasm towards brown spot of citrus.

Keywords: Pathogen, Catalases, Biomolecules, Significant variations, Resistant germplasm, Valentia late

INTRODUCTION

Citrus fruit is full of nutrition and is grown all over the tropical and sub-tropical areas of the world and has promising position across the globe including Pakistan (Iftikhar *et al*., 2020). Globally, citrus is cultivated on an area of 13900 thousand hectare with annual production of 194.4 million tons while Pakistan produces 2.46 million tons of citrus with area of 206 thousand hectare (FAOSTAT, 2020; Mushtaq *et al.,* 2022). However, the production of citrus is affected by various biotic and abiotic factors. Among biotic stresses, brown spot which is caused by *Alternaria citri* fungus is an emerging threat to the successful production of citrus (Nawaz *et al.,* 2020; Gai *et al*., 2021). It causes a huge economic loss in different citrus growing areas of the world (Aiello *et al.,*2020). *Alternaria citri* attack induces different types of biochemical changes in citrus plant. It increases or decreases the concentrations of certain biochemical compounds but still there is little effort regarding the plant-pathogen interaction and biochemical substances (Yeats and Rose, 2013). These alterations in biochemical profiling are helpful to study the biochemical mechanism and plant-pathogen interaction. Virulence factors released by pathogens are recognized by the host plants through their receptor molecules. Moreover, these molecules are essential for the rapid activation of host plant defense like HR (Hypersensitive response) that inhibit the further spread of pathogens from point of infection to other parts of the plant. Activities of biochemical enzymes fluctuate after attack of pathogen on citrus and most probably increased (Abedi and Pakniyat, 2010). In diseased plant parts, consumption or translocation of these antioxidant enzymes is influenced by pathogens that play a key role in the severity of disease (Huber and Jones, 2013). Phenols as well as their oxidizing products affect host plant resistance after the establishment of infection while POD play a prime role in non-specific defenses of plants under stressed conditions as well as in presence of injury at sub cellular levels (Pandey *et al.,* 2017). Production of ROS (Reactive Oxygen Species) is an important event in interaction between host and pathogen (Kumar *et al*., 2011). Attack of pathogen results in high levels of ROS which is induced by signaling compounds like salicylic acid during HR and PCD (Programmed Cell Death). Superoxidase plays a vital role in defense mechanism of plants towards disease caused by virus, fungi or bacteria (Voloudakis *et al.,* 2006). The current study of biochemical alterations was designed to identify the source of resistance as well as to analyze alterations in the concentrations of catalases, peroxidases, total Soluble Sugars, superoxide dismutase, total soluble proteins, total phenolic contents, and hydrogen peroxide from inoculated

and un-inoculated leaves of citrus cultivars. Additionally, to explore the possibility of biochemical alterations and biochemical markers to identify resistance source and defense of citrus towards brown spot of citrus.

MATERIALS AND METHODS

Isolation and purification of pathogen from infected leaves

For isolation of pathogen, leaves sample showing characteristic symptoms of brown spot of citrus were cut into small pieces (2-3mm size). After this, these pieces were surface sterilized with 1% sodium hypochlorite (NaClO) followed by 2-3 consecutive washing with distilled water. These pieces were left for some time for surface drying and then placed on Petri Plates containing PDA media. These Petri Plates were incubated at at 25 °C for 24 hours. After incubation, black to grey colored fungal colonies were observed around experimental samples on PDA media plates. Some of these colonies were selected and Purification was done by picking relevant fungal colonies from previously grown fungal cultured plates with help of sterilized needle and was placed on fresh PDA (Potato Dextrose Agar) media plates. Long term preservation of fungal pathogen was done by transferring the inoculum from growing culture to test tube (16×100 mm) slants containing PDA media and air tight the lid of test tube to avoid contamination. The test tube was placed in a refrigerator (PEL: PRGD-145) at 4°C for future use (Humber, 1997).

Morphological and biochemical identification of pathogen

Pure colonies obtained from infected sample having same morphology were proceeded further for biochemical identification of associated fungal pathogen. The identification of pathogen was done according to Fawole and Oso, (1995). A drop of distilled water was placed on a clean slide and small portion of mycelium was picked from the fungal culture by using sterilized needle and was placed on clean slide. The mycelium was spread well on the slide with the aid of two sterilized needle and cover slip was placed gently on the slide. Then slide was examined under microscope (Model: OVE-MG 8751/1) at 10X and 40X magnification. Morphological characteristics of the pathogen such as spore shape, type of hyphae and asexual reproductive structure of fungi were observed (Muhammad *et al*., 2018).

Establishment of field experiment for pathogenicity test on different citrus cultivars

One-year old plants of three resistant (Mayer lemon, Eruka lemon and Citron) and three susceptible (Washington navel, Valentia late and Kinnow) varieties were collected from fruit nursery Institute of Horticultural Sciences (IHS), UAF. These plants were grown in pots (22×16 cm) filled with sterilized soil by arranging them under CRD (Completely Randomized Design) in the greenhouse of Department of Plant Pathology near CAS (Centre for Advanced Studies), UAF, Pakistan. Fungal isolates were revived from 4°C and concentration (10^4 spore/mL of H_2O) was maintained through hemocytometer (Model: T20B05) (Peever *et al*., 1999). Inoculation was done through hypodermic syringe method. Fungal suspension was taken in syringe (23Gx 1") and was injected into midrib and veins of lower surface of leaves (Kepczynska and Król, 2012) early in the morning (when max. no. of stomata were opened). Distilled water was used as a control to inoculate citrus leaves and plants were closely monitored for the appearance of brown spot symptoms.

Sample preparation for biochemical analysis from inoculated and un inoculated citrus leaves

Plant sample containing fresh leaves from both inoculated (Resistant and susceptible) and uninoculated (Resistant and susceptible) plants were collected in brown bags (13" ×9.5") and were cut into small sections). 0.5 g of leaf sample was taken and was grinded in pestle and mortar along with KH_2PO_4 buffer. These samples were centrifuged (Horizon 6 Flex) at 12000 rpm for 5 minutes and supernatant was collected for analysis of biochemical compounds

Estimation of total phenolic content (TPC) from inoculated and un-inoculated leaves of citrus plants

For determining the total phenolic contents (TPC), enzyme extract (100 µL) was prepared. Reaction mixture was consisting of 200 µL F-C reagent (10%), 700mM Na_2Co_3 800 µL and was left for 1 hour. Absorbance was estimated through spectrophotometer at 765nm (Barba *et al.,* 2013).

Estimation of total soluble sugars (TSS) from inoculated and uninoculated citrus leaves

For determining total soluble sugar (TSS), anthrone reagent ($C_{14}H_{10}O$) technique (Yemm and Wills, 1954) was used. 100 mg sample along with 5mL of hydrochloric acid (2.5N) was

hydrolyzed in a boiling water bath at 25ºC for three hours. Sodium carbonate (Na_2CO_3) was used in order to neutralize the solution until effervescence ceased. The specific standard was made by taking 0, 0.2, 0.4, 0.6, 0.8, 1 mL working standard and 0 was taken as blank. Distilled water was added in each tube to make the volume (up to 1mL) and then anthrone reagent (4 ml) was added, solution was heated in boiling water bath. Spectrophotometer (Hitachi U-2001: 121-003 was used to determine the absorbance at wavelength of 630 nm.

Estimation of superoxide dismutase (SOD) from inoculated and uninoculated citrus leaves

Estimation of superoxide dismutase (SOD) from citrus leaves was done by preparing the reaction mixture by mixing of 100 µL enzyme extract, methionine (200 µL), NBT (100 µL), triton X (200µL), potassium phosphate buffer (500 µL, pH 5) and distilled water (800 µL).Mixture was placed for fifteen minutes under UV(ultraviolet light) for 15 minutes. After this 100 µL riboflavin (Vitamin B2) was added and absorbance was estimated through spectrophotometer (Hitachi U-2001: 121-003) at 560nm (Giannopolitis and Ries, 1977).

3.6.4 Estimation of peroxidase (POD) from inoculated and uninoculated citrus leaves

For determining Peroxidase (POD) from the leaves of citrus, reaction mixture was made by mixing enzyme extract (100 µL), 18mM guaiacol (100 µL), KH_2PO_4 buffer (800 µL, pH 5) and 42mM hydrogen peroxide (100 µL). Absorbance was estimated at 470nm through Spectrophotometer (Liu *et al*., 2007).

Estimation of total soluble proteins (TSP) from inoculated and uninoculated citrus leaves

To estimate the total soluble proteins (TSP), enzyme extract of leaves was prepared according to Bradford (1976) method. Enzyme extract (40 µL) was made in potassium phosphate buffer (KH_2PO_4, pH 5), vertex (V-3: ELMI) it and then centrifugation was done.at 12000 rpm for 5 minutes. Bradford Reagent (160 µL) was added in extract and left it for 5 minutes. Absorbance was measured at a wavelength of 595nm by using spectrophotometer.

Estimation of catalase (CAT) from inoculated and uninoculated citrus citrus leaves

For estimation of catalase (CAT) from the leaves of citrus, reaction mixture was prepared by mixing enzyme extract (100 µL) and 100 µL of 5.95mM hydrogen peroxide. Absorbance was recorded at 240nm by using Spectrophotometer (Liu *et al*., 2009).

Estimation of H_2O_2 Concentration from inoculated and uninoculated citrus leaves

For determining H_2O_2 concentration, fresh leaves were collected from inoculated and un-inoculated cultivars. A sample of fresh leaf (50mg) was taken and was grinded within a ($C_2HCl_3O_2$) buffer and centrifuged at 12000 rpm for 15 minutes at 4 °C. After this, 1.3mL potassium phosphate buffer (pH 7), 1mL of potassium iodide was mixed with 0.3mL supernatant was incubated (incubator, RTI-250) for 5 minutes. Filtrate was treated with KH_2PO_4 (pH 7) buffer and after this with KI. Resultant mixture was placed in digital incubator (RTI-250) for 5 minutes and their absorbance was taken at 390nm through absorbance reader (BioTek: 800TS) and amount of hydrogen peroxide (H_2O_2) was taken as $\mu mol \cdot g^{-1}$ FW (Velikova *et al*., 2000).

RESULTS

Estimation of total phenolic compounds (TPC, TSS, SOD and POD from (µg/g) leaves of inoculated and un-inoculated citrus plants

Substantial difference was present among un-inoculated (4.30 µg/g) and inoculated (1.76µg/g) leaves during diseased condition. Resistant 3.42µg/g and susceptible 2.64µg/g plants also indicated significant variation at $P<0.05$ with total variance of 90.75 percent (Table 1). Varieties showed their natural affinities against concentration of total phenolic compounds by expressing 0.46% total variance. Maximum concentration was expressed by varieties exhibiting their natural tendencies against the concentrations of TPC expressing 0.46% of total variance, Maximum concentration were displayed by variety namely Eruka lemon (3.50µg/g) and minimum by Valentia late with amount of 2.55 (µg/g) (Table 2) While in case of TSS, significant difference was noticed among un-inoculated (4.17µg/g) and inoculated (2.86µg/g) leaves indicating that TSS contents affects metabolic activities under diseased conditions. Resistant (3.72µg/g) and susceptible 3.31µg/g also indicated significant variation $P<0.05$ with 9.17% total variance (Table 1). Varieties showed their natural affinities against the concentrations of TSS by expressing 0.53% of total variance. Higher concentration was displayed by variety namely Eruka lemon (3.73µg/g) while lowest by Washington navel to amount of (3.24µg/g) (Table 2).

Regarding SOD concentrations, significant differences were observed between un-inoculated (averaging to 2.16 µg/g across the un-inoculated type) and inoculated plant leaves (averaging to 0.39 µg/g across the inoculated type) that SOD amount effect the metabolic activities after establishment of brown spot disease in citrus plants. Resistant cultivars (1.53µg/g) and susceptible (1.02 µg/g) also showed significant variation among the both group with the total

variance of 94.22 % at P<0.05 (Table 1). Varieties attained 0.25 % of the total variance with respect to the nitrogen concentration. On individual basis, Eruka lemon showed maximum concentration of SOD with average amount of 1.81µg/g while susceptible cultivar Valentia late showed 1.06µg/g after the analysis (Table 2) but in case of POD, significant variations were observed across the inoculated and un-inoculated leaves of citrus plant (on an average 3.16 and 1.26µg/g respectively) during the disease development with total variance of 96.29 %. Resistant (2.04µg/g) and susceptible plants (2.38µg/g) also showed significant variation at P<0.05 (Table 1). 0.63% of total variance was expressed by varieties in their natural affinities with respect to POD contents. Mayer lemon and Valentia late expressed minimum and maximum concentrations of POD to the extent 1.96 of and 2.47 µg/g respectively (Table 2).

Estimation of total soluble proteins TSP, CAT and H_2O_2 from the leaves of inoculated and un-inoculated leaves (µg/g) of citrus plants

In the case of TSP, both uninoculated (3.30 µg/g) and inoculated (0.94µg/g) leaves of citrus plants showed significant difference during disease condition with 87.90 percent of the total variance. Significant difference was also expressed by the resistant (2.54 µg/g) as well as susceptible (1.74 µg/g) plants. Varieties indicated their natural tendencies with respect to TSP concentration expressing 0.67 % of the total variance (Table 1). Mayer lemon and Valentia late expressed highest and lowest concentration of TSP (2.62 and 1.63µg/g) respectively (Table 3) while in catalases, significant difference was observed in both un-inoculated (2.28µg/g) and inoculated (5.19µg/g) leaves of citrus plants during the disease stress with 89.22 percent of total variance. Plants which were marked as resistant and susceptible also expressed significant alteration with 3.27µg/g and 4.20µg/g respectively (Table 1). Varieties with 1.15 percent of total variance expressed their natural affinities regarding CAT contents. Mayer lemon (3.09µg/g) and Valentia late (4.46µg/g) displayed their lowest and highest concentrations of CAT respectively (Table 3). Significant difference was present across un-inoculated (0.27 µg/g) and inoculated (1.14 µg/g) leaves of citrus plants expressing that H_2O_2 contents which affects metabolic activities under diseased stress. Plants which were marked as resistant and susceptible also expressed significant difference with the 0.63 µg/g and 0.77 µg/g H_2O_2 respectively at P<0.05 with 6.48 % of total variance (Table 1). Varieties expressed their natural affinities with respect to H_2O_2 concentrations which indicates the 0.97 % of the total variance. Highest concentrations were shown by varieties namely "Washington navel" to the extent of 0.83 µg/g and lowest by Eruka lemon to amount of 0.58 µg/g (Table 3).

Table 1: Nested Structured ANOVA for TPC, TSS, SOD, POD, TSP, CAT and H_2O_2 contents of inoculated and un-inoculated citrus plant leaves

SOV	DF	SS	MS	F value	Pr>F	Variance components	% of total variance component
			TPC (µg/g)				
Group (A)	1	173.3560	173.3560	21.563	0.043*	3.061	90.75
Type (B)	2	16.0789	8.0395	55.575	0.000*	0.292	8.67
Variety (C)	8	1.1573	0.1447	34.380	0.000*	0.016	0.46
Error	96	0.4039	0.0042	-	-	0.004	0.12
Total	107	190.9962	-	-	-	3.374	-
			TSS (µg/g)				
Group (A)	1	46.7614	46.7614	20.059	0.046*	0.823	89.37
Type (B)	2	4.6625	2.3312	44.520	0.000*	0.084	9.17
Variety (C)	8	0.4189	0.0524	6.114	0.000*	0.005	0.53
Error	96	0.8221	0.0086	-	-	0.009	0.93
Total	107	52.6650	-	-	-	0.921	-
			SOD (µg/g)				
Group (A)	1	69.196	69.196	36.370	0.026*	1.246	94.22
Type (B)	2	3.805	1.902	56.239	0.000*	0.069	5.23
Variety (C)	8	0.270	0.033	8.562	0.000*	0.003	0.25
Error	96	0.379	0.004	-	-	0.004	0.30
Total	107	73.652	-	-	-	1.323	-
			POD (µg/g)				
Group (A)	1	96.8889	96.8889	60.763	0.016*	1.765	96.29
Type (B)	2	3.1891	1.5945	15.061	0.002*	0.055	3.01
Variety (C)	8	0.8470	0.1059	90.924	0.000*	0.012	0.63
Error	96	0.1118	0.0012	-	-	0.001	0.06
Total	107	101.0367	-	-	-	1.833	-
			TSP (µg/g)				
Group (A)	1	150.362	150.362	16.095	0.057*	2.611	87.90
Type (B)	2	18.684	9.342	52.098	0.000*	0.339	11.42
Variety (C)	8	1.434	0.179	4330.862	0.000*	0.020	0.67
Error	96	0.0040	0.0000	-	-	0.000	0.00
Total	107	170.485	-	-	-	-	-
			CAT(µg/g)				
Group (A)	1	228.3769	228.3769	19.251	0.048*	4.011	89.22
Type (B)	2	23.7263	11.8631	24.932	0.000*	0.421	9.39
Variety (C)	8	3.8066	0.4758	43.485	0.000*	0.05	1.15
Error	96	1.0505	0.0109	-	-	0.011	0.24
Total	107	256.9602	-	-	-	4.494	-
			H_2O_2(µg/g)				
Group (A)	1	22.436	22.436	28.198	0.034*	0.401	92.54
Type (B)	2	1.591	0.795	20.98	0.001*	0.028	6.48
Variety (C)	8	0.303	0.037	2634.89	0.000*	0.004	0.97
Error	96	0.001	0.000	-	-	0.000	0.00
Total	107	24.332	-	-	-	0.433	-

Table 2: Amount of TPC, TSS, SOD and POD in reaction groups (Inoculated and Uninoculated), types (Resistant and Susceptible) and in varieties/ cultivars of citrus plant leaves

					TPC (µg/g)							
Varieties (C)	Mayer lemon		Eruka lemon		Citron		Valentia late		Wa. Navel		Kinnow	
Type (B)	**Resistant**						**Susceptible**					
Group (A)	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.
Amount of TPC in (C)	4.62	2.08	4.77	2.23	4.68	2.14	3.81	1.30	4.10	1.56	3.82	1.29
Av. Amount of TPC in (C)	3.35		3.50		3.41		2.55		2.83		2.55	
Av. Amount of TPC in (B)	Resistant = **3.42**						Susceptible = **2.64**					

Av. Amount of TPC in (A)				Inoculated = **1.76**						Un-Inoculated = **4.30**		
TSS (µg/g)												
Amount of TSS in (C)	3.08	4.36	3.11	4.35	3.09	4.34	2.56	3.97	2.55	3.94	2.77	4.10
Av. Amount of TSS in (C)	3.72		3.73		3.71		3.26		3.24		3.43	
Av. Amount of TSS in (B)				Resistant = **3.72**						Susceptible = **3.31**		
Av. Amount of TSS in (A)				Inoculated = **2.86**						Un-Inoculated = **4.17**		
SOD (µg/g)												
Amount of SOD in (C)	0.52	2.34	0.44	3.18	0.52	2.23	0.29	1.7	0.30	1.83	0.32	1.72
Av. Amount of SOD in (C)	1.43		1.81		1.37		0.99		1.06		1.02	
Av. Amount of SOD in (B)				Resistant = **1.53**						Susceptible = **1.02**		
Av. Amount of SOD in (A)				Inoculated = **0.39**						Un-Inoculated = **0.39**		
POD (µg/g)												
Amount of POD in (C)	2.91	1.02	3.05	1.15	3.14	1.02	3.33	1.62	3.25	1.30	3.30	1.50
Av. Amount of POD in (C)	1.96		2.1		2.08		2.47		2.27		2.40	
Av. Amount of POD in (B)				Resistant = 2.04						Susceptible = 2.38		
Av. Amount of POD in (A)				Inoculated = 3.16						Un-Inoculated =1.26		

Table 3: Amount of TSP, CAT and H_2O_2 in reaction groups (Inoculated and Un-inoculated), types (Resistant and Susceptible) and in varieties/ cultivars of citrus plant leaves

TSP(µg/g)												
Varieties (C)	Mayer lemon		Eruka lemon		Citron		Valentia late		Wa. Navel		Kinnow	
Type (B)	**Resistant**								**Susceptible**			
Group (A)	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.	Inoc.	Unin.
Amount of TPC in (C)	1.20	4.04	3.63	1.18	3.14	3.88	0.63	2.63	0.84	2.93	0.70	2.73
Av. Amount of TPC in (C)	2.62		2.68		2.53		1.63		1.88		1.71	
Av. Amount of TPC in (B)				Resistant = 2.51						Susceptible = **1.74**		
Av. Amount of TPC in (A)				Inoculated = **0.94**						Un-Inoculated = 3.30		
CAT **(µg/g)**												
Amount of TSS in (C)	4.51	1.68	4.64	1.83	4.89	2.09	5.98	2.95	5.46	2.44	5.67	2.72
Av. Amount of TSS in (C)	3.09		3.23		3.49		4.46		3.95		4.19	
Av. Amount of TSS in (B)				Resistant = 3.27						Susceptible = 4.20		
Av. Amount of in (A)				Inoculated = 5.19						Un-Inoculated = 2.28		
H_2O_2 (µg/g)												
Amount of SOD in (C)	1.15	0.27	0.92	0.25	1.00	0.23	1.14	0.33	1.35	0.31	1.32	0.23
Av. Amount of SOD in (C)	0.71		0.58		0.61		0.73		0.83		0.77	
Av. Amount of SOD in (B)				Resistant = **0.63**						Susceptible = **0.77**		
Av. Amount of SOD in (A)				Inoculated = **1.14**						Un-Inoculated = **0.27**		

DISCUSSION

Brown spot disease of citrus is a devastating threat to citrus industry that affect the quality as well as quantity of the produce (Moosa *et al*., 2022; Grati *et al.*, 2022). When pathogen attack on its host plant, it causes alterations in its cell metabolism and biochemical compounds. Biochemical compounds like superoxide dismutase, Peroxidase, catalyase, hydrogen peroxide, total soluble proteins, total soluble phenols, total soluble sugars play an important role in inducing resistance in plants towards pathogens (Singh *et al*., 2019). Present study was designed to observe alterations in biochemical compounds infected with brown spot of citrus that can assist researchers and scientists in selection of resistant cultivar and better preventive measures against citrus brown spot disease, So, amounts of TSS, TPC, SOD, CAT, H2O2 and POD were estimated in resistant as well as susceptible cultivars of citrus.

After attack, pathogen produces toxic metabolites in the host plant that disturb its physiological function like respiration, photosynthesis, translocation, transpiration, development and growth and in biochemical compounds (Xie *et al*., 2016). In normal conditions, plants use available oxygen to proceed their growth while under stress like attack of pathogen, production of ROS cause photo-oxidative stress in biochemical compounds and internal structures of cell (Mittler, 2017).During plant-microbe interaction, plants respond by inducing defense mechanism such as hypersensitive response (HR), cell wall strengthening, antimicrobial action and antioxidant enzymes like peroxidases, superoxide dismutase, glutathione reductase and catalases (Valko *et al*., 2006). Antioxidant enzymes including POD, CAT and SOD are basic constituents in preparation of H_2O_2. During attack of pathogens, reactive oxygen species (ROS) produced which contained hydroxyl radicals, superoxide radicals (O^{2-}) and hydrogen peroxide (Chen *et al*., 2008). SODs are also associated with the production of H_2O_2 through alterations of superoxide radicals to H_2O_2 (Valko *et al*., 2006).

Phenolic compounds act as an antioxidant and protect cellular organelles, organic substances like protein, lipids, RNA and DNA from oxidative stress. Moreover, their rapid accumulation at infection site limit the multiplication of pathogens as indicated by Cherif *et al*. (1992). However, pathogen reduced the availability of these contents in host plants thereby affecting their defense mechanism. In present study, total phenolic contents were present in higher concentration in resistant and un-inoculated citrus cultivars than susceptible and inoculated citrus cultivars. These results are supported by the findings of Meena *et al*. (2017) and

Hameed *et al*. (2021) who observed higher amounts phenolic contents in resistant and uninoculated plants.

Peroxidase (PODs) performs numerous physiological functions of the plants including biosynthesis of lignin, auxin metabolism, cell wall stiffening and protect the plants from various pathogens (Bhardwaj *et al*., 2014). PODs Higher amount of POD was associated with resistance in plants against pathogens. In current work, it was observed that inoculated plants contained high amount of POD while un-inoculated and resistant plants have less amount of POD. These research findings are in line with outcomes of Ahmed *et al*. (2019) and Hameed *et al*. (2021). SOD is considered as first line of defense to produce reactive oxygen species (ROS) as a result of pathogen attack and its quick introduction helped to identify virulence factors of the pathogen. SODs level in resistant cultivar is involved in maximizing H_2O_2 contents while in current study enhanced level of H_2O_2 was suppressed by the action of pathogen due to the higher level of CAT whereas decreased amounts of SODs are responsible for production of CAT that are involved in the removal of H_2O_2. In present study, SOD concentration was found less in susceptible and inoculated plants which is also witnessed by Ahmed *et al*. (2019) and Hameed *et al*. (2021).

Catalase (CAT) is an important enzyme that is involved in scavenging H_2O_2 from plant materials as indicated by Nafie and Mazen. (2008). Higher SOD and lower CAT contents in resistant plants were associated in mediating hydrogen peroxide level thereby inducing resistance in citrus plants while metabolic activities of hydrogen peroxide are regulated by production or destruction of these enzymes (Imlay, 2008). Outcomes of current findings are supported by the results of Kumar *et al*. (2011) and Ahmed *et al*. (2019) who also noticed higher amounts of CAT in inoculated and susceptible cultivars than un-inoculated and healthy varieties.

Total Soluble sugars (TSS) are considered as a metabolic substrate that have vital role in the structural development and metabolism of the plants. Soluble Sugars contribute in the defense mechanism of plants by activating the defense related genes and through formation of cellulose, lignin and phytoalexins. Moreover, fructose and glucose are the primary source of energy for plant cells (Salerno and Curatti, 2003). Several researches showed that carbohydrate metabolism is influenced by the attack of pathogens thereby resulting in the reduction of sugar contents in infected leaves (Sain and Gour, 2008). In current study, reduction in total soluble sugar was observed in infected and susceptible cultivars which is witnessed by Milena *et al*. (2019) and Hameed *et al*. (2021).

Total soluble proteins (TSP) performs various enzymatic, structural as well as functional roles in plant growth and development. Moreover, proteins play an imperative role in managing plant diseases as they limit the multiplication of bacteria, fungi and viruses by retarding the pathogen spread from infected to healthy tissues. (Nafees *et al*., 2019b). Protein contents are reduced in host plants after attack of pathogens (Martin, 2003). In current study, proteins contents were less in susceptible and inoculated citrus cultivars as compared to resistance and un-inoculated varieties which is also witnessed by the work of Milena *et al*. (2019) and Hameed *et al*. (2021).

CONCLUSION

Concentrations of Total Phenolic Contents, Total Soluble Sugars, SOD, Total Soluble Proteins were reduced while POD, CAT and H_2O_2 were increased after attack of *Alternaria citri.* Such changes in biochemical compounds can be further used in breeding program for the identification and development of resistance source against brown spot of citrus. These outcomes can also be used as effective disease controlling mechanism.

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