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Involvement of Some of Defense Responsive Enzymes against *Fusarium* Wilt in Chickpea Cultivars

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A pot culture experiment was conducted involving five each of resistant and susceptible chickpea cultivars grown in normal, sterilized and wilt sick soils in order to determine the activities of six defense responsive enzymes in their root portions at 10, 20 and 30 DAS. The study indicated that β, 1-3-glucanase and chitinase activity in root portions was significantly higher in resistant than susceptible cultivars from 10 to 30 DAS. However, pronounced increase was observed in resistant cultivars under wilt sick soil condition. The higher peroxidase activity was observed in resistant than susceptible cultivars particularly those grown under wilt sick soil conditions as compared to normal and sterilized soil condition. During 10 to 30 DAS under wilt sick soil condition, the increase in the activity of polyphenol oxidase was relatively higher in resistant cultivars than the susceptible ones. The increase in phenylalanine ammonia-lyase activity was comparatively higher from 10 to 30 DAS under wilt sick soil condition in resistant cultivars than under normal and sterilized soil conditions. The trypsin inhibitor activity was found to be significantly higher in resistant cultivars than the susceptible ones from 10 to 30 DAS. However, increase in activity was more pronounced under wilt sick soil condition than under sterilized soil condition. Based on the results of this investigation, the cultivar ICCV-96030 appeared to be highly sensitive to wilt sick condition than the sensitive national check JG-62, where as the cultivar Vishal had comparatively higher level of defense responsive enzymes under wilt sick soil condition and appeared to be more tolerant.

Key words Chickpea, *Fusarium oxysporum* f.sp. *ciceri,* β, 1-3-glucanase, Chitinase, Peroxidase, Polyphenol oxidase, Phenylalanine ammonia lyase, Trypsin inhibitor.

Chickpea (*Cicer arietinum* L.) is the largest produced food legume in South Asia and the third largest produced food legume globally, after common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.). Chickpea is grown in more than 50 countries, but developing countries account for over 95% of its production. Over 75% of the chickpea production comes from South Asia, where India is the largest chickpea producing country accounting for 67% of the global chickpea production[1]. Several diseases are known to limit worldwide production of chickpeas, of which *Fusarium oxysporum* f.sp. *ciceri* is one of the most important. An annual loss in chickpea grain yield of about 10 to 15% has been reported for this disease[2]. It is difficult to manage the disease either through crop rotation or application of chemicals because of its soil borne nature. An outbreak of *Fusarium* wilt of chickpea would result in a dramatic reduction in area of production, due to increased cost of production making chickpea less competitive compared to other crops. Persistence of the pathogen in soil and its capacity to survive there for years even in the absence of host makes it's control very difficult. Instead, the use of wilt resistance chickpea cultivars, when they are available is the most effective and eco-friendly

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method of managing the disease[3]. It is therefore prime importance to know about biochemical parameters impart the resistance. Plant resistance induces the accumulation of hydrolytic enzymes such as chitinase and β-1,3 glucanase [4]. The defence gene products peroxidase (PO) and polyphenol oxidase (PPO) catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) is involved in the synthesis of phytoalexins and phenolics[5]. Pathogenesis-related proteins (PRs) such as and β-1,3 glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8 and PR-11) degrade the fungal cell wall and cause lysis of fungal cell walls. Furthermore, chitin and glucan oligomers released during degradation of the fungal cell wall by the action of lytic enzymes act as elicitor that elicit various defense mechanism in plant[4]. PAL catalyzes the deamination of phenylalanine to produce trans-cinamic acid the first step controlling the rate of phenylpropanoid metabolism[5]. The production of phenylpropanoid compound is important in plant development, plant microbe signalling and plant defense[4]. Proteinase inhibitor are highly stable defensive protein of plant tissues that are both developmentally regulated and induced in response to insect and pathogen attacks[6]. Proteinase inhibitors (PR-6) in plant are also able to suppress enzymatic activities of phytopathogenic micro organisms [7] In the present study, we reported some defense responsive enzyme such as β, 1-3-glucanase, chitinase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and trypsin inhibitor against *Fusarium* wilt of chickpea.

MATERIALS AND METHODS

Plant material

Five tolerant cultivars viz., Vijay, Vishal, Virat, Vihar and Phule –G-94-55 and five susceptible cultivars JG-62, ICCV-96030, Chafa, Vikas and Phule – G-5 were obtained from chickpea breeder, All India Co-ordinated Pulses Improvement Project, MPKV, Rahuri for analysis in the present investigation.

Isolation, purification and mass multiplication of Fusarium oxysporum f.sp.ciceri

The seeds of susceptible cultivar JG-62 were sown in earthen pots containing wilt sick soil obtained from wilt sick plot of the experimental farm of the All India Co-ordinated Pulses Improvement Project, MPKV, Rahuri. The roots of infected JG-62 cultivar were used for the isolation of *Fusarium oxysporum* f.sp.*ciceri* fungi. The culture was purified, maintained and multiplied on crushed cotton seeds. The fungus grown on crushed cotton seeds was mixed in sterilized soil and incubated for 30 days then this soil was used as wilt sick soil in the experiment Seeds of resistant and susceptible cultivars of chickpea were sown in earthen pots containing three different soil *viz*., normal, autoclaved (sterilized) and wilt sick soil in the duplicate set. The fresh root samples of the above cultivars were collected at an interval of 10, 20 and 30 DAS, and analyzed for different defense responsive enzyme.

Pot Experiment

The pot cultured experiment was carried out in three set using Factorial Completely Randomized Block Design (FCRBD) to evaluate the performance of resistance and susceptible cultivar on wilt disease of chickpea. In the first duplicate set the seeds of resistant and susceptible cultivars were sown in earthen pots containing 10 kg sterilized (control) soil, in second duplicate set the seeds of the resistant and susceptible cultivars sown in earthen pots containing 10 kg wilt sick (*F.oxysporum* f. sp. *Ciceri*) soil and in the third duplicate set, the seeds of resistant and susceptible cultivars were sown in earthen pots containing 10 kg normal soil.

Assay of β-1, 3-glucanase

The 0.5 g fresh root sample was macerated with chilled 6 ml of 0.2M Tris-HCl buffer of pH 7.5. The homogenate was centrifuged at 11,000 g at 4^0C for 30 min and the supernatant was used as the source of crude enzyme. One ml reaction mixture contained 950 µl of laminarin and 50 µl of crude enzyme extract and was incubated at 37^0C for 30 min[8]. The reducing sugar released into the solution at the end of reaction was estimated by Nelson-Somogyi method[9]. The protein content in the crude enzyme extract was estimated according to the method of Lowry[10].The activity was expressed as mg of glucose released g^{-1} of soluble protein hr^{-1}.

Assay of Chitinase

Fresh root samples of 0.5g was macerated with 6ml of 0.1M sodium citrate buffer in pre cooled

mortar and pestle. The homogenate was centrifuged at 10000g for 10 min at 10^0C and the supernatant was used as crude source of chitinase. For the assay, 1ml of supernatant ,4ml of chitin suspension containing 15 mg BSA were incubate in water bath at 37^0C for 3hr. One ml water and 1ml of reaction mixture was boiled in centrifuge tube covered with glass marble for 10 min and was centrifuged. An aliquot of 0.5 ml was taken for the estimation of N-acetylglucosamine as per the method of Nelsom-Somogyi[9]. The soluble protein content in the crude enzyme extracted was estimated according to method of Lowry *et al* (1951)[10]. The activity was expressed as mg of N-acetylglucosamine released g^{-1} of soluble protein hr^{-1} [11,12].

Assay of Peroxidase

Root sample of 0.5g was macerated separately with 6ml of 0.1M phosphate buffer in prechilled mortar and pestle. The homogenate was centrifuged at 15000g at 4^0C for 30 min. One ml supernatant was diluted to 10 ml with distilled water and was used as the enzyme source. The assay mixture of peroxidase contained 3.6 ml of 0.1 M phosphate buffer (p^H 7.0), 1ml of 0.005 M hydrogen peroxidase, 1ml of 0.01M pyrogallol and 1ml of well diluted enzyme extract. The absorbance was read at 420 nm on a spectronic-20 spectrophotometer for every 30 sec interval upto 3min and the reaction was stopped by adding 2.5 N H_2So_4 exactly after 3min. One unit of peroxidase activity was determined as an increase in O.D by 0.001. The enzyme activity was calculated for one gram of sample and expressed as unit min^{-1} mg^{-1} soluble protein[13].

Assay of Polyphenol oxidase activity

The enzyme extract was prepared as described under the assay of peroxidase and was used as the enzyme source. The assay mixture of polyphenol oxidase contained 2ml of 0.01M phosphate buffer (p^H 7.0), 1ml of well-diluted enzyme extract. The absorbance was read at 420 nm on spectronic-20 spectrophotometer after every 30 sec interval and the reaction was stopped exactly after 3 min by addition of 2.5 N H_2So_4. One unit of enzyme activity was calculated as the change in absorbance by 0.001 min^{-1} mg^{-1} soluble protein[13].

Assay of Phenylalanine ammonia-lyase activity

Fresh root sample of 0.5g was macerated separated with 6ml of 0.05 M borate buffer (p^H 8.5) containing 2-mercaptoethanol and insoluble polyvinyl pyrrolidone in pre-chilled mortar and pestle. Homogenate was centrifuged at 20,000g at 4^0C for 20 min. The supernatant obtain was used as enzyme source. The assay mixture containing 1ml aliquots of supernatant and 110ml of 100mM L-phenylalanine were incubated at 40^oCfor 30 min. Then 1ml of 4% TCA was added in it to terminate the reaction. Similarly the TCA was added in one of the test tube at 0 min to serve as a blank. The assay mixture was incubated with TCA for 5 min at room temperature and centrifuged at 10,000g for 5 min and the absorbance of the supernatant was read at 290 nm. PAL activity was calculated as m moles of cinnamic acid produced g^{-1} soluble protein hr^{-1} under the specific condition[4].

Estimation of Trypsin Inhibitor activity

The modified method of Kakade[14] was used for assaying the trypsin inhibitor activity (TIA) from the root portions of resistant and susceptible cultivars of chickpea. Root sample 0.5g was cut into small pieces and macerated in pre-chilled mortar and pestle with chilled 6 ml 0.05 M Tris HCl buffer (P^H8.2). The homogenate was filtered throw four layered of muslin cloth and centrifuged at 10,000g for 10 min at 4^0C the supernatant was used as a source of trypsin inhibitor (TI). Reaction mixture contains 0.2 to 1.0 ml of TI extracted and 0.5ml of the stock trypsin solution was brought to the volume of 2ml with Tris-HCl buffer. The test tubes were placed in a water bath at 37°C. One ml of 30 per cent acetic acid was added to one of the triplicate tubes which served as a blank. Then previously warmed at 37°C 7 ml of BAPA solution was added in each test tube. After 10 min, the reaction was terminated by adding 1 ml of 30 per cent acetic acid. After through mixing, the absorbance of each solution was measured at 410 nm against the blank. One trypsin unit was taken as an increase of 0.01 absorbance at 410 nm per 10 ml of the reaction mixture under the conditions defined. Trypsin inhibitor activity is defined as the number of trypsin units inhibited mg^{-1} soluble protein min^{-1}.

Statistical analysis

The pot cultured experiment was carried out in three set using Factorial Completely Randomized Block Design (FCRBD).

RESULTS

Glucanase activity

It was observed that all resistant cultivars had higher activity of β-1,3-glucanase at 10, 20 and 30 DAS in the roots of chickpea plants grown in normal, sterilized and wilt sick soils than the susceptible ones(Table 1). The mean β-1,3-glucanase activity in resistant cultivars increased from 1.96 to 8.17, 1.64 to 7.83 and 3.00 to 10.24 mg of glucose released g^{-1} soluble protein hr^{-1} in normal, sterilized and wilt sick soils, respectively. However in susceptible cultivars, the activity increased from 1.05 to 5.03, 0.91 to 4.84 and 2.02 to 4.62 mg of glucose released g^{-1} soluble protein hr^{-1} in normal, sterilized and wilt sick soils, respectively. Thus, it appeared that the increase of β-1,3-glucanase activity was lower in susceptible cultivars than the resistant ones during 10 to 30 DAS. Further, it was revealed that the induction of β-1,3-glucanase was higher in wilt sick soil condition followed by normal and sterilized soil conditions. Among the resistant cultivars, Vishal had comparatively higher glucanase activity at 10 (3.17), 20 (8.30) and 30 (10.59) DAS under wilt sick soil condition than the rest of the resistant cultivars.

Among the susceptible cultivars, the cultivar ICCV-96030 had very low activity than the rest of susceptible cultivars which indicated ICCV-96030 to be more susceptible to *Fusarium* wilt than the national check, JG-62.

Chitinase activity

It was observed that all resistant cultivars had higher activity at 10, 20 and 30 DAS of the plants grown in normal, sterilized and wilt sick soils than the susceptible ones. The mean chitinase activity increased from 1.58 to 4.80, 1.40 to 4.65

Table 1. β-1,3-glucanase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	β -1,3-glucanase activity(mg of glucose released g^{-1} soluble protein hr^{-1})								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant cultivars									
Vijay	1.49	6.10	8.06	1.38	6.09	7.56	2.79	8.03	10.03
Vishal	1.89	7.30	8.27	1.72	7.11	8.23	3.17	8.30	10.59
Virat	2.15	6.76	8.13	1.91	6.40	7.95	2.96	8.13	9.98
Vihar	2.25	6.87	8.31	1.57	6.67	7.63	3.00	8.18	10.36
Phule-G-9425-5	2.03	7.69	8.10	1.63	7.60	7.81	3.06	8.28	10.23
Mean	1.96	6.94	8.17	1.64	6.77	7.83	3.00	8.18	10.24
Susceptible cultivars									
JG-62	1.37	5.69	5.81	1.09	5.63	5.21	1.89	6.34	2.59
ICCV-96030	0.93	0.96	1.03	0.84	0.89	0.95	1.70	1.12	0.97
Chafa	1.02	5.76	6.15	0.91	5.70	5.98	2.31	5.98	6.37
Vikas	0.95	5.87	6.12	0.76	5.82	6.09	2.18	6.09	6.77
Phule G-5	0.98	5.89	6.07	0.95	5.47	5.99	2.03	6.81	6.41
Mean	1.05	4.83	5.03	0.91	4.70	4.84	2.02	5.27	4.62
			SE±				CD at 5 %		
Cultivar (A)			0.018				0.0504		
Soil (B)			0.0098				0.0276		
DAS(C)			0.0098				0.0276		
Interaction									
AXB			0.0311				0.0873		
AXC			0.0311				0.0873		
BXC			0.017				0.0478		
AXBXC			0.054				0.1512		

and 2.77 to 7.32 mg N-acetylglucosamine released g^{-1} soluble protein hr^{-1} in normal, sterilized and wilt soils, respectively in resistant cultivars. However in susceptible cultivars, the activity increased from 1.28 to 1.94, 0.79 to 1.64 and 1.37 to 3.18 mg N-acetylglucosamine released g^{-1} soluble protein hr^{-1} in normal, sterilized and wilt sick soils, respectively. Further, it was found that among the resistant cultivars, Vishal had comparatively higher chitinase activity at 10 DAS in normal (1.88), sterilized (1.69) and wilt sick soils (3.09 N-acetylglucosamine released g^{-1} soluble protein hr^{-1}).

Among the susceptible cultivars, ICCV-96030 had very low activity than the rest of susceptible cultivars which indicated that ICCV-96030 to be more susceptible to *Fusarium* wilt than the national check, JG-62.

Peroxidase activity

The peroxidase activity from five *Fusarium* wilt resistant and five susceptible cultivars was estimated from the root portions of the plants grown in normal, sterilized and wilt sick soils at 10, 20 and 30 DAS and is presented in Table 3. It was observed that all the resistant cultivars had comparatively higher activity at 10, 20 and 30 DAS of the plants grown in normal, sterilized and wilt sick soils than the susceptible ones. The mean peroxidase activity increased in resistant cultivars from 1.77 to 3.51, 1.48 to 3.38 and 3.64 to 9.21 units in normal, sterilized and wilt sick soils, respectively. However in susceptible cultivars, the activity increased from 1.09 to 1.86, 0.98 to1.67 and 1.93 to 2.28 units in normal, sterilized and wilt sick soils, respectively. Further, it was found that among the resistant cultivars, Vishal

Table 2. Chitinase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Chitinase activity (mg N-acetylglucosamine released g^{-1} soluble protein hr-1)								
	Normal soil			Sterilized soil			Wilt sick soil		
	DAS			DAS			DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	1.29	4.09	4.31	1.02	3.81	4.09	2.20	5.31	6.78
Vishal	1.88	4.30	5.12	1.69	4.10	5.09	3.09	5.67	7.69
Virat	1.42	4.20	4.79	1.29	3.96	4.69	2.88	6.02	7.98
Vihar	1.56	4.78	4.61	1.48	4.06	4.37	2.73	5.81	6.94
Phule-G-9425-5	1.76	4.52	5.20	1.54	4.19	5.02	2.97	6.16	7.21
Mean	1.58	4.38	4.80	1.40	4.02	4.65	2.77	5.79	7.32
Susceptible Cultivars									
JG-62	0.89	1.93	2.09	0.74	1.63	1.70	1.29	3.69	1.10
ICCV-96030	0.78	0.81	0.90	.56	0.58	0.60	1.11	1.02	0.92
Chafa	1.39	2.12	2.29	0.89	1.92	2.15	1.36	4.09	4.97
Vikas	1.56	1.93	2.08	0.91	1.53	1.79	1.62	3.89	4.69
Phule G-5	1.79	2.13	2.33	0.83	1.67	1.94	1.49	3.68	4.24
Mean	1.28	1.78	1.94	0.79	1.46	1.64	1.37	3.27	3.18
			SE±			CD at 5 %			
Cultivar (A)			0.014			0.040			
Soil (B)			0.0078			0.022			
DAS (C)			0.0078			0.022			
Interaction									
AXB			0.0248			0.069			
AXC			0.0248			0.069			
BXC			0.0136			0.038			
AXBXC			0.0431			0.012			

had comparatively higher peroxidase activity at 10 (4.28), 20 (8.08) and 30 DAS (10.10) in wilt sick soil, followed by Phule G-9425-5. Thus, higher peroxidase activity in resistant cultivars and more so in Vishal indicates that these cultivars are tolerant to *Fusarium* wilt and the cultivar Vishal seems to be more tolerant amongst them.

Among the susceptible cultivars, the ICCV-96030 had very low activity than the rest of susceptible cultivars, which indicated the ICCV-96030 to be more susceptible to *Fusarium* wilt than the national check, JG-62.

Polyphenol oxidase activity

The polyphenol oxidase activity from five *Fusarium* wilt resistant and five susceptible cultivars was estimated from the root portions of the plants grown in normal, sterilized and wilt sick soils at 10, 20 and 30 DAS and is presented Table 4. It was observed that resistant cultivars had the higher activity at 10, 20 and 30 DAS of the plants grown in normal, sterilized and wilt-sick soils than the susceptible cultivars. The mean polyphenol oxidase activity increased from 0.37 to 1.33, 0.30 to 1.17 and 0.98 to 5.94 units in normal, sterilized and wilt sick soils, respectively in resistant cultivars. However in susceptible cultivars, the activity increased from 0.25 to 0.50, 0.21 to 0.47 and 0.49 to 1.33 units in normal, sterilized and wilt sick soils, respectively from 10 to 30 DAS.

Thus, pronounced increase in polyphenol oxidase activity was observed in the resistant cultivars than the susceptible ones and the cultivars Vishal had comparatively higher activity than the rest of the resistant cultivars.

Phenylalanine ammonia-lyase activity

The changes in phenylalanine ammonia-lyase (PAL) activity in root portions of resistant and susceptible cultivars of chickpea is presented in Table 5. It was noticed that the activity increased in both the groups of the cultivars. However, the

Table 3. Peroxidase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Peroxidase activity (Units)								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	1.59	2.09	2.98	1.07	1.94	2.76	2.36	5.83	8.67
Vishal	1.88	2.53	3.46	1.75	2.38	3.34	4.28	8.08	10.10
Virat	1.84	2.75	3.38	1.57	2.35	3.25	3.62	6.93	9.17
Vihar	1.65	2.73	3.62	1.45	2.25	3.48	3.83	7.74	9.31
Phule-G-9425-5	1.90	3.01	4.12	1.55	2.81	4.04	4.12	6.73	8.79
Mean	1.77	2.62	3.51	1.48	2.34	3.38	3.64	7.06	9.21
Susceptible Cultivars									
JG-62	0.95	1.23	1.37	0.86	1.13	1.27	1.66	1.85	1.30
ICCV-96030	0.94	1.17	1.38	0.82	0.96	1.15	1.58	1.47	1.21
Chafa	1.13	1.55	1.94	0.99	1.35	1.71	2.10	2.73	2.86
Vikas	1.23	1.88	2.39	1.10	1.73	2.11	2.11	2.64	2.94
Phule G-5	1.19	1.94	2.22	1.12	0.89	2.09	2.21	2.86	3.07
Mean	1.09	1.56	1.86	0.98	1.21	1.67	1.93	2.31	2.28
			SE±			CD at 5 %			
Cultivar (A)			0.013			0.0036			
Soil (B)			0.0007			0.0020			
DAS(C)			0.0007			0.0020			
Interaction									
AXB			0.00228			0.00638			
AXC			0.00228			0.00638			
BXC			0.00124			0.00349			
AXBXC			0.00394			0.0011			

increase was more pronounced in the resistant that the susceptible cultivars. Further, under the pathogenic condition, i.e. wilt sick soil, the increase was higher in resistant cultivars than the plants grown under the normal and sterilized soils. During 10 - 30 DAS in normal soil, the mean increase was 1.06 to 2.00 µmoles of cinnamic acid g^{-1} soluble protein hr^{-1}, however in wilt sick soil, it was 1.49 to 4.58 µmole of cinnamic acid g^{-1} soluble protein hr^{1} in resistant cultivars. In susceptible cultivars, about 2.5 times lower activity was observed. Among the resistant cultivars during pathogenesis, higher activity was observed in Vishal at 10 (1.70), 20 (3.90) and 30 DAS (5.70).

Trypsin inhibitor activity

The trypsin inhibitor activity from the root portions of resistant and susceptible cultivars in relation to *Fusarium* wilt is shown in Table 6. It was observed that the resistant cultivars had the higher trypsin inhibitor activity when grown in normal, sterilized and wilt sick soils than the susceptible cultivars at 10, 20 and 30 DAS. The trypsin inhibitor activity increased from 7.98-10.87, 7.37 to 10.64 and 10.94 to 16.20 µg trypsin inhibited mg^{-1} soluble protein min^{-1} in normal, sterilized and wilt sick soils, respectively in resistant cultivars. However in susceptible cultivars, the activity increased from 4.62 to 7.58, 4.20 to 7.01 and 8.06 to 10.29 µg trypsin inhibited mg^{-1} soluble protein min^{1} in normal, sterilized and wilt sick soils, respectively. Among the resistant cultivars, Vishal had comparatively higher trypsin inhibitor activity at 10, 20 and 30 DAS in normal, sterilized and wilt sick soils.

DISCUSSION

In the present study the differential induction of defense responsive enzymes was observed in both the susceptible and resistance

Table 3. Peroxidase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Peroxidase activity (Units)								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	1.59	2.09	2.98	1.07	1.94	2.76	2.36	5.83	8.67
Vishal	1.88	2.53	3.46	1.75	2.38	3.34	4.28	8.08	10.10
Virat	1.84	2.75	3.38	1.57	2.35	3.25	3.62	6.93	9.17
Vihar	1.65	2.73	3.62	1.45	2.25	3.48	3.83	7.74	9.31
Phule-G-9425-5	1.90	3.01	4.12	1.55	2.81	4.04	4.12	6.73	8.79
Mean	1.77	2.62	3.51	1.48	2.34	3.38	3.64	7.06	9.21
Susceptible Cultivars									
JG-62	0.95	1.23	1.37	0.86	1.13	1.27	1.66	1.85	1.30
ICCV-96030	0.94	1.17	1.38	0.82	0.96	1.15	1.58	1.47	1.21
Chafa	1.13	1.55	1.94	0.99	1.35	1.71	2.10	2.73	2.86
Vikas	1.23	1.88	2.39	1.10	1.73	2.11	2.11	2.64	2.94
Phule G-5	1.19	1.94	2.22	1.12	0.89	2.09	2.21	2.86	3.07
Mean	1.09	1.56	1.86	0.98	1.21	1.67	1.93	2.31	2.28
			SE±			CD at 5 %			
Cultivar (A)			0.013			0.0036			
Soil (B)			0.0007			0.0020			
DAS(C)			0.0007			0.0020			
Interaction									
AXB			0.00228			0.00638			
AXC			0.00228			0.00638			
BXC			0.00124			0.00349			
AXBXC			0.00394			0.0011			

cultivars upon the normal wilt sick and sterilized soil condition. This could be a partially responsible for the resistance or susceptibility to various pathogens. The preferred site for infection by *Fusarium oxysporum* f. sp. *Ciceri* is the root tissues close to the point of seed attachment. Fungal hyphae colonize first the root xylem and then the xylem vessels of the stem. Therefore, the root plant tissues sampled in our study represent the plant fungus interaction leading to a compatible reaction is produced[16]

β-1,3-glucanase

The results on *F. oxysprorum* infection leading to induced synthesis of β-1,3-glucanse in both resistant and susceptible cultivars of chickpea in the present investigation confirms with earlier report of Rakshit[9]. Saikia[17] reported that β-1,3-glucanase activity significantly increased in chickpea after infection of *Fusarium* wilt

Enzymatic system involved in cell wall degradation by an antagonistic organism is β-glucan degrading enzymes because β-1, 3-glucan is a structural component of fungal pathogen cell walls. Gajera[18] reported that chitinase and β-1, 3-glucanase were positively correlated with each other to inhibit the growth of fungal pathogen in culture medium at 16 DAI.

Pathogenesis related protein such as chitinase and β-1, 3-glucanase are induced to enhance the resistance against the pathogen during the progression of infection in plant tissues[19,20]. The cowpea pathogen *F. solani* f.sp. *phaseoli* was inhibited by the exudates defense related proteins (chitinase and β-1, 3-glucanase) from cowpea seed[21]. Wu[22] demonstrated that β-1, 3-glucanase and chitunase activities in the leaves of watermelons treated with Fusaric acid were swiftly increased and the highest activities were obtained after 3 and 12hr. Raju[23] reported that 43 kDa β-1, 3-glucanase has been induced by

Table 4. Polyphenol oxidase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Polyphenol oxidase activity(units)								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	0.34	0.68	1.13	0.27	0.58	0.95	0.90	2.36	5.78
Vishal	0.42	0.90	1.61	0.38	0.85	1.42	1.09	3.24	6.34
Virat	0.37	0.80	1.53	0.33	0.71	1.38	1.10	3.07	5.45
Vihar	0.33	0.83	1.29	0.21	0.79	1.16	0.94	2.88	6.10
Phule-G-9425-5	0.41	0.77	1.08	0.35	0.64	0.93	0.89	2.90	6.00
Mean	0.37	0.79	1.33	0.30	0.72	1.17	0.98	2.89	5.94
Susceptible Cultivars									
JG-62	0.16	0.29	0.34	0.13	0.23	0.26	0.35	0.48	0.41
ICCV-96030	0.20	0.24	0.31	0.14	0.18	0.28	0.28	0.35	0.30
Chafa	0.30	0.39	0.60	0.25	0.35	0.58	0.59	0.93	1.88
Vikas	0.23	0.36	0.61	0.18	0.32	0.56	0.63	1.12	2.21
Phule G-5	0.37	0.45	0.64	0.35	0.41	0.60	0.60	1.05	1.83
Mean	0.25	0.35	0.50	0.21	0.30	0.47	0.49	0.79	1.33
		SE±				CD at 5 %			
Cultivar (A)		0.0013				0.0039			
Soil (B)		0.0007				0.0021			
DAS(C)		0.0007				0.0021			
Interaction									
AXB		0.002				0.0067			
AXC		0.002				0.0067			
BXC		0.0013				0.0037			
AXBXC		0.0041				0.0117			

Fusarium oxysporum f. sp. *ciceri*, only in roots and shoots of resistant cultivars but not in susceptible cultivar.

Chitinase

Deborah[24] showed that increased activities in combination of chitinase and glucanase affect the fungal pathogens. Thus, in the present investigation, higher activities of both the enzymes in combination in the tolerant cultivars might lead to overcoming the *Fusarium* wilt. Earlier researchers reported that chitinase activity was markedly increased in resistant cultivars of crop plants due to a disease such as *Alternaria triticina* in wheat[25], *Fusarium* wilt in chickpea[13].

Nagy[26] reported as increase in chitinase isoform in *Rhizoctonia* infected roots. Saikia[17] reported that chitinase activity significantly increased in chickpea when inoculated with *Fusarium* wilt. Chitinase alone with β-1, 3-glucanase imparts resistance against a wide array of plant pathogen[27].A direct role for chitinase in defense of plant against pathogens has been proposed because the substrate for those enzymes is a major components of the cell wall of many fungi[28]

Peroxidase

The activity of peroxidase was generally higher in the infected tissue of resistant varieties than in the infected susceptible ones[29]. Highest activity of peroxidase was found against *Fusarium* wilt in cumin [30]and in banana [31]. The results obtained in the present investigation are in agreement with these results. The cell wall building process includes peroxidase-mediated oxidation of hydroxy-cinnamyl alcohols into free radical intermediates, polysaccharide cross-linking, lignification and suberization. Thus in resistant cultivars, studied in the present investigation may have any one type of process or in combination of cited above**.** The enzyme peroxidase along with

Table 5. Phenylalanine ammonia-lyase activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Phenylalanine ammonia-lyase activity (µmoles of cinnamic acid g^{-1} soluble protein hr^{-1})								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	0.90	1.16	1.80	0.85	1.04	1.67	1.50	2.90	4.80
Vishal	1.31	1.74	2.34	1.26	1.69	2.21	1.70	3.90	5.70
Virat	0.97	1.39	1.81	0.89	1.33	1.62	1.30	3.50	4.70
Vihar	1.11	1.56	1.94	1.05	1.46	1.87	1.50	2.70	3.90
Phule-G-9425-5	1.03	1.47	2.11	0.96	1.42	2.09	1.40	1.90	3.80
Mean	1.06	1.46	2.00	1.00	1.39	1.89	1.49	2.98	4.58
Susceptible Cultivars									
JG-62	0.32	0.51	0.62	0.31	0.51	0.61	0.81	1.40	1.20
ICCV-96030	0.32	0.41	0.59	0.31	0.41	0.58	0.79	0.90	0.79
Chafa	0.47	0.58	0.66	0.45	0.57	0.64	0.80	1.60	2.00
Vikas	0.49	0.58	0.70	0.48	0.57	0.71	0.89	1.40	1.89
Phule G-5	0.49	0.59	0.68	0.45	0.56	0.68	0.99	1.80	2.30
Mean	0.41	0.53	0.65	0.40	0.52	0.64	0.86	1.42	1.64
			SE±				CD at 5 %		
Cultivar (A)			0.0249				0.06973		
Soil (B)			0.013				0.03819		
DAS(C)			0.013				0.03819		
Interaction									
A X B			0.0413				0.1207		
A X C			0.0413				0.1207		
B X C			0.0236				0.06615		
A X B X C			0.0747				0.2092		

polyphenol oxidase and other enzymes in tolerant cultivars in the present study might have restricted the fungal growth through either oxidative product of phenols, synthesis of phytoalexins, lignins or by less degradation of cell wall of the plant tissue[30].

Polyphenol oxidase activity

The results obtained and trends obtained in the present study are in accordance with the earlier findings[32,30]. The activities of polyphenol oxidizing enzymes such as peroxidase and polyphenol oxidase have been correlated in a plant defense system against the invasion of the pathogen. The enhanced activity of PPO in resistant cultivars might be a result of augmented oxidation of phenolic compounds resulting in the formation of toxic quinones (antimicrobial compounds) and their participation in defense mechanism of the host plant and hypersensitivity in resistant plants to viruses, bacteria and fungi[33]. The increased in PPO activity may be the result of protein synthesis or release of bound proteins during degenerative processes caused by the fungal infection[34]. It has also been suggested that PPO might function as an alternate electron transport chain and thus, serves as a terminal oxidase in infected plant tissue, where obstruction in the normal metabolic pathways leads to products interferring with the normal electron transport chain[35]. The precursors of phenolic compounds *via* shikmic acid pathway might be available for induction of PPO synthesis in infected tissue[36].

The PPO over expressing transgenic tomato plant exhibited high resistance to *Psedomonase syringae,* the causative agent of speack disease compared with control plant[37]. The PPO-2 isoform in roots and shoots treated with *T. harzianum* L1 with pathogen might have also been implicated in induced defence response against the pathogen invasion. Raju [23] have screened different genotypes for wilt disease expressed

Table 6. Trypsin inhibitor activity at 10, 20 and 30 DAS in root portions of wilt susceptible and resistant cultivars grown in normal, sterilized and *Fusarium* wilt sick soils

Cultivars	Trypsin inhibitor activity (µg Trypsin inhibited g^{-1} soluble protein min^{-1})								
	Normal soil DAS			Sterilized soil DAS			Wilt sick soil DAS		
	10	20	30	10	20	30	10	20	30
Resistant Cultivars									
Vijay	6.69	8.15	9.12	6.19	7.03	8.95	9.74	13.43	14.19
Vishal	10.13	12.13	13.13	9.81	11.82	12.81	12.50	17.68	19.22
Virat	8.97	9.70	12.92	8.06	8.21	12.67	10.86	16.65	15.66
Vihar	6.97	7.68	9.52	5.95	7.35	9.42	10.14	14.31	16.17
Phule-G-9425-5	7.16	8.44	9.68	6.86	8.07	9.38	11.49	14.29	15.75
Mean	7. 98	9.22	10.87	7.37	8.50	10.64	10.94	15.27	16.20
Susceptible Cultivars									
JG-62	3.05	4.85	7.46	2.79	4.26	6.77	9.15	10.56	12.2
ICCV-96030	3.09	5.43	6.59	2.50	5.09	5.90	5.86	7.62	6.14
Chafa	6.26	7.21	9.64	5.83	7.01	8.61	6.73	8.17	10.82
Vikas	4.80	4.89	6.38	4.08	4.40	6.24	9.36	11.63	12.13
Phule G-5	5.89	7.36	7.82	5.83	7.09	7.53	9.21	9.69	10.19
Mean	4.62	5.95	7.58	4.20	5.57	7.01	8.06	9.53	10.29
		SE±			CD at 5 %				
Cultivar (A)		0.02168			0.06071				
Soil (B)		0.01187			0.03325				
DAS(C)		0.01187			0.03325				
Interaction									
AXB		0.0375			0.1051				
AXC		0.0375			0.1051				
BXC		0.02057			0.057				
AXBXC		0.0650			0.1821				

multiple isoform of PPO, while the susceptible genotypes did not.

Phenylalanine ammonia-lyase

Lois[38] reported the *do novo* synthesis of PAL isozymes in response to fungal challenges and other biotic and abiotic stresses. Chakarbarty[39] revealed that the magnitude of induction was invariably higher in resistant lines than the susceptible plants in cotton due to infection of grey mildew. The higher PAL activity in the present investigation during 10 -30 DAS in wilt sick soil in resistant cultivars may be the response of *Fusarium* fungi. Thus, the results obtained are in agreement with these earlier reports. The enzyme PAL catalyzes the deamination of phenylalanine to produce *trans*-cinnamic acid and the production of phenylpropanoid compound is important in plant-microbe signalling and plant defense mechanism[5]. Dixon and Pavia[40] reported that the defense response of plants are induced by fungal elicitor that lead to biosynthesis of an array of stress-induced phenylpropanoid which are accumulated in plant during stress or disease conditions. Logemann[41] reported that fungal invasion triggers the transcription of mRNA that codes for PAL, thus increasing the amount of PAL in the plants, which then stimulates the synthesis of phenolic compounds. Thus in the present investigation, higher activity in resistant cultivars and particularly in Vishal under pathogenesis (grown under wilt sick soil) may be the result of expression of PAL gene due to fungal elicitor molecules.

Similar results were observed by many researchers. Jebakumar[42] reported that PAL activity increased in the leaves of black pepper when inoculated with *Phytophthora capsici*. Kale and Choudhary[43] observed that PAL activity reached to maximum level in resistant cultivars of groundnut after infection with *Cercosporidium personatum*.

Trypsin inhibitor activity

Mariana[44] reported that in the resistant cultivars, the induction of trypsin inhibitor activity was more pronounced than in the susceptible cultivars. The results obtained in the present investigation are in agreement with these earlier reports. This study was similar to Nair and Sandu[45] showed that the resistance to certain corn genotypes to fungal infection is related to the action of trypsin inhibitor, which is due to the lowering in the production and activity of fungal alpha-amylase which in turn reduce the availability of simple sugars for fungal growth. Ham[46] reported that in plants genes encoding inhibitors that inactivate proteinases of pathogenic organisms may reduce the ability of aggressor to digest host proteins and therefore limit the availability of amino acid source for the invader. However, early reports describe inhibitors that specifically inhibit proteolytic enzymes from micro-organisms and not digestive proteases of animals[7].

Protease inhibitors (PR-6) in plant are able to suppress enzymatic activities of phytopathogenic microorganisms[8]. Earlies, Jayalakshmi[47] have reported that seeds of cultivar JG-62 treated with *T. harzianum* L1 shows to produce trypsin and chymotripsin inhibitors and inhibited protease -2 of *Fusarium oxysporum* f.sp. *cicer.* Raju [23] showed that new trypsin and chymotrypsin were synthesized to inhibit the growth of *Fusarium oxysporum* f.sp. *cicer*. Protease inhibitor of potato were shown to inhibit the pathogenicity and growth of *Botrytis cineaara* [48]

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