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Temporal and Spatial Changes in Host Defense Enzymes in Response to *Fusarium* Wilt in Pigeonpea (*Cajanus cajan* (L.) Millspaugh)

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Authors' contributions

This work was carried out in collaboration between both authors. Author JD collected and maintained the pathogen and host cultivars, performed pot experiment for disease development, performed enzyme analyses of collected host tissue samples and recorded the data. Author NL proposed conception of the study concerning defense enzyme analysis, prepared experimental plan, discussed the results and prepared the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Fusarium wilt caused by *Fusarium udum* Butler (*Fud*) is a constant threat to pigeonpea productivity in several parts of the world. Understanding the molecular basis of pigeonpea-*Fud* interaction is necessary to improve resistance to *Fud* and thereby the productivity of pigeonpea. Temporal and spatial changes were studied in defense enzymes namely catalase, peroxidase (PO), polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL), β-1,3-glucanase and chitinase at pre-initiation (S1), disease initiation (S2) and severe diseased (S3) stages in root, stem and leaf tissues of 2 susceptible and 2 resistant cultivars of pigeonpea inoculated with *Fud*. Comparison of various treatment combinations revealed that all the defence enzymes showed temporal and spatial variation in activity/expression pattern. PO increased in all the tissues of all the varieties from S1 to S2 and declined thereafter in S3 stage, and induction was higher in resistant cultivars. Catalase and PPO were induced highly in resistant cultivars inoculated with *Fud,* and the pattern was stage-specific and tissue-specific in nature. Catalase induction occurred at S2 in uninoculated plants

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whereas it started at S1 in *Fud* inoculated plants. PPO level diminished from S2 to S3 stage, and in susceptible varieties, it reached even below the control. PAL, β-1,3-glucanase and chitinase activity were induced at S1, and S2 and values at S3 decreased progressively. Induction of chitinase was significantly high in leaves whereas β-1,3-glucanase activity was high in stem tissues. Both control and challenged plants had a higher level of β-1,3-glucanase activity at later stages of disease development, but the proportionate increase was much higher in resistant cultivars. The activity/expression pattern of these defence enzymes reveals their use as biochemical markers for resistance and provides scope for manipulating their expression and development of wilt-resistant transgenic pigeonpea.

Keywords: Pigeonpea; India; Fusarium udum; Pathogenesis; Defence enzymes; antioxidant enzymes; PR proteins.

ABBREVIATIONS

gfw^{-1} : per gram fresh weight
gdw^{-1} : per gram dry weight
PAL : Phenylalanine Ammonia Lyase
PO : Peroxidase
PPO : Polyphenol Oxidase
PR : Pathogenesis-Related
ROS : Reactive Oxygen Species

1. INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is among the world's most important pulse crops. Around 76% of the total global area and around 73% of total global production of pigeonpea is in India. Globally it is cultivated on 4.75 million hectares producing 3.68 million tonnes with the productivity of 774.30 kg/ha [1]. Wilt caused by *Fusarium udum* Butler (*Fud*) is an important disease of pigeonpea in north, central and south India inflicting heavy losses in yield estimated to be 470 thousand tones in India and 531 thousand tones in the world [2]. Plants activate a large array of defense mechanisms in response to pathogen attack and a crucial factor determining the success of these mechanisms is the speed of their activation. There is considerable interest in understanding how plants recognise pathogen attack and control the expression of defence mechanisms. After pathogen recognition, highly localised biochemical events are rapidly induced to inhibit the further development of the attacking pathogen. The rapid production and accumulation of reactive oxygen species (ROS), particularly the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), culminates with a hypersensitive response (HR) and localized programmed cell death (PCD) to impair pathogen establishment and development inside the host tissues and thus deprive the pathogen of further access to nutrients [3]. Concomitant with or following HR, several pathogen defence-related genes are translated into antioxidant enzymes and "pathogenesis Related" (PR)-proteins [4] that confer resistance to the plant in defense against pathogen attack. Enzymes of the phenyl-propanoid pathway e.g. phenyl alanine ammonia lyase (PAL) are involved in the synthesis of phenolic compounds like flavonoids, salicylic acid, coumarins etc. which are known as phytoalexins. Differential expression of PAL genes was investigated using northern blot analysis in chickpea seedlings challenged with *Fusarium oxysporum* f. sp*. ciceri* [5]. Datta and Lal [6] observed temporal and spatial changes in phenolic compounds in response to infection by *Fusarium* wilt pathogen in chickpea and pigeonpea and suggested that interaction between *Fusarium* and host plants was found to enhance defence responses against wilt disease in resistant cultivars. Broetto et al. [7] also reported similar results in bean, *Phaseolus vulgaris* L. infected by *Fusarium oxysporum* f. sp. *phaseoli*. The host proteins important for defence against pathogens also include enzymes like polyphenol oxidase, peroxidase, superoxide dismutase and PR-proteins *viz*. chitinase and β-1,3-glucanase [3,4,8].

Higher activity of β-1,3-glucanase enzyme in highly resistant cultivars and lower in susceptible ones suggested the possible role of β-1,3-glucanase as a biochemical marker for screening of pearl millet cultivars for downy mildew [9]. Giri et al. [10] reported differential induction of chitinase in susceptible and resistant cultivars of chickpea and decrease in β-1,3-glucanase in the resistant cultivars in response to the pathogen infection. The observed decrease in the resistant cultivars may be associated with the reduction in pathogen invasion as a result of the defence reaction. Stevenson et al. [11] reported that root exudates of chickpea plants containing phytoalexins contribute at least partly to

resistance against wilt as they show anti- *F. oxysporum* activity *in vitro*. In a recent study, Oliveira et al. [12] studied time course activities of antioxidant enzymes and PR proteins in cowpea plants infected with *Colletotrichum gloeosporioides* and observed differential induction among resistant and susceptible genotypes and confirmed participation of peroxidase, chitinase and β-1,3-glucanase as important component of host defense. Similarly, Pareek et al. [13] recorded higher induction/activation of chitinase and β-1,3-glucanase in resistant genotypes of *Vigna acontifolia* inoculated with *Macrophomina phaseolina* indicating these enzymes as a part of general defense response against fungal pathogens. Gharbi et al. [14] compared biochemical defense responses of susceptible and resistant cultivars of *Olea europaea* to *Verticillium* wilt and observed an early activation of PO and PPO, early and simultaneous upregulation of chitinase and β-1,3-glucanase, correlated with reduced susceptibility in resistant cultivars.

Biochemical and molecular mechanisms of resistance provide information for better understanding of the pathogens and their reaction to crop and therefore may help in management of disease. Keeping this in view, the present investigations were undertaken to correlate and analyse the biochemical basis of disease resistance, by measuring levels of PO, catalase, PPO (as antioxidant enzymes), PAL (enzyme involved in phenyl-propanoid pathway), β-1,3-glucanase and chitinase, spatially and temporally in wilt resistant and wilt susceptible cultivars of pigeonpea.

2. MATERIALS AND METHODS

2.1 Plant Material

Two wilt resistant and wilt susceptible cultivars of pigeonpea (*C. cajan* (L.) Millspaugh) were used for biochemical and molecular study. The test cultivars comprised of Asha (ICPL 87119), Maruthi (ICP 8863) in resistant group and Bahar and Type 7 as susceptible.

2.2 Inoculation of Plants with the Fungus and Sample Collection for Biochemical Analyses

The isolates of *Fud* were collected from wilt affected plants of pigeonpea. The pathogenic fungal isolates were collected from fourth-node stem sections of wilted pigeonpea plants according to Tullu et al. [15] and were colonized on filter paper, dried in the transfer hood, and aseptically cut into small pieces. These fungi were colonized on filter paper and dried in the transfer hood. The colonized filter paper was aseptically cut into small pieces and placed in potato-dextrose broth and incubated to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheese cloth to remove mycelia. The spore (conidia) suspension was pelleted by centrifugation. After discarding the supernatant, the conidia were washed with sterile water to adjust the spore suspension to 1 x 10^6 spores ml^{-1} with a haemocytometer. These isolates were further characterized at the laboratories of Department of Life Sciences, and Department of Biochemistry, I.B.S.B.T, C.S.J.M. University, Kanpur. Single spore culture of fungus was obtained by serial dilution method. Isolated fungus was identified as *F. udum* and its pathogenicity was tested on pigeonpea cultivar Bahar in pot experiments. Plastic pots of 30 cm diameter were surface-sterilized with 0.1% (w/V) mercuric chloride ($HgCl_2$). Pots were filled with 2 kg sterilized soil (sterilized thrice at 1.1 kg/cm^2 for 1 h for 3 days). Seven days before sowing, pots were inoculated with the 14-day-old culture of the pathogen multiplied on sand maize meal water medium (90 g sand, 10 g maize meal and 20 ml distilled, sterilized water) @ 50 g kg^{-1} soil. Seeds were surface-sterilized using 2% sodium hypochlorite for 3 min, and rinsed in sterile water. Ten seeds of each cultivar were sown in each pot for disease scoring. The root, shoot and leaf tissues were collected separately from 3 randomly chosen plants/treatment at 7, 15 and 30 days after sowing (DAS) and were frozen immediately in liquid nitrogen to store at -20°C. These tissue samples of root, shoot and leaf were subjected to isolation of major biomolecules and assessing their activity. Biochemical basis of disease resistance was studied by changes in major biomolecules in *Fusarium* wilt resistant and susceptible genotypes.

2.3 Extraction of Antioxidant Enzymes

The root, shoot and leaf tissue samples (500 mg fresh weight) of 3 chosen plants were separately ground in 0.1 M phosphate buffer (pH 7.0) using a pre-chilled mortar and pestle, and homogenate was centrifuged at 15000 X g at 4°C for 30 min and aliquots were used as a source of enzyme.

2.4 Isolation and Activity Assay of Peroxidase (EC 1.11.1.7)

Peroxidase activity was determined according to Kar and Mishra [16] with the following modifications. Assay mixture for the peroxidase comprised: 2 ml 0.1 M of phosphate buffer, pH 7.0, 1 ml 0.01 M of pyrogallol ($C_6H_6O_3$), 1 ml of 0.005 M hydrogen peroxide (H_2O_2), and 1 ml of the 2 times diluted enzyme extract (1:2 ratio dilution). The reference cuvette contained equal volume of the inactivated enzyme (by boiling) and pyrogallol. This was incubated for 5 min. at 25°C after which the reaction was stopped by adding 0.5 ml of 5% (v/V) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm. One unit of peroxidase activity is defined as the amount of the enzyme that caused a change of 0.01 absorbance unit per minute under assay condition and it was expressed as U per gram fresh weight (U gfw^{-1}).

2.5 Activity Assay of Polyphenol Oxidase (EC 1.14.18.1)

Assay mixture of polyphenol oxidase comprised of same ingredients as in case of peroxidase [16] except H_2O_2.

2.6 Activity Assay of Catalase (EC 1.11.1.6)

The activity of catalase was assayed using method of Braber [17] with the following modification. Five ml of the assay mixture for the catalase activity comprised: 300 µmoles of phosphate buffer, pH 6.8 100 µmoles of H_2O_2, and 1 ml of diluted enzyme extraction (1:2 dilution). After incubation at 25°C for 1 min, the reaction was stopped by adding 10 ml of 2% (v/V) H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple colour persisted for at least 15 sec. A control was run at the same time in which the enzyme activity was stopped at "zero" time. Catalase activity was expressed as µmole of H_2O_2 used per minute under the assay condition described, considering the concentration of H_2O_2 using the extinction coefficient 0.036/µmol/ml.

2.7 Isolation and Activity Assay of Phenylalanine Ammonia Lyase (EC 4.3.1.5)

Tissues were homogenized in 0.1 M Borate buffer (pH 8.8) at 4°C. Homogenate was centrifuged at 4°C and 20800 rpm and the supernatant used as enzyme source. The activity of L-phenylalanine ammonia lyase was determined by the method of Zucker [18] with certain modifications. Assay mixture consisted of 1.5 ml Borate buffer, 1.0 ml H_2O, 1.0 ml phenylalanine solution (10 µM) and 0.5 ml enzyme extract. After incubation of this assay mixture for 2 h in a 38°C water bath, the change of absorbance at 290 nm compared to a reference sample without added substrate was measured with a spectrophotometer. PAL activity was expressed as ΔA_{290nm} ml^{-1} h^{-1}.

2.8 Isolation and Activity Assay of Chitinase (EC 3.2.1.14)

Defatted and depigmented tissue powder was stirred with extraction buffer (1:6 w/V in 0.1 M phosphate buffer, pH 6.9 containing 0.05 M NaCl) at 4°C for 12 h. The mixture was centrifuged at 10,000 rpm for 20 min and the proteins from the supernatants were precipitated by adding ammonium sulphate [$(NH_4)_2SO_4$] to 90% saturation (60 g $(NH_4)_2SO_4$/100 ml extract). The precipitated proteins were collected by centrifugation, resuspended and dialysed against the extraction buffer [10]. Chitinase activity was determined according to Chen et al. [19] and Tsukomoto et al. [20]. The reaction mixture contained 1.0 ml of colloidal chitin solution (7 mg), 1.0 ml of sodium acetate buffer (50 mM, pH 5.2) and 1.0 ml of 1:1 diluted enzyme. After incubation at 50°C for 1 h the released reducing sugar was measured as N-acetyl glucosamine (NAG) equivalents by the method of Reissig et al. [21].

2.9 Isolation and Activity Assay of β-1,3-Glucanase (EC 3.2.1.39)

β-1,3-glucanase activity was estimated using the procedure of Koga et al. [22]. The assay mixture contained 1.0 ml of suitably diluted enzyme and 1.0 ml of 1% laminarin solution in sodium acetate buffer (50 mM, pH 5.2). The mixture was incubated at 40^0C for 30 min and the product released was reducing sugar which was measured as glucose equivalents [23].

2.10 Statistical Analyses

Data from the enzyme assays were subjected to analysis of variance (ANOVA) followed by Tukey's test, and values were expressed as Mean±SE.

3. RESULTS

3.1 Spatial and Temporal Activity of Peroxidase

The activities of peroxidase increased in all the tissues of all the varieties from pre-initiation (S1) to disease initiation stage (S2) and declined thereafter in severe diseased stage (S3) as shown in Table 1. This change was prominent in case of all challenged as well as un-inoculated plants. After 15 DAS at S2, irrespective of whether the plants were challenged or un-inoculated, there was a significant increase in peroxidase activity in all the tissues compared to the S1 stage. At this stage, conspicuous changes occurred at the rate of increase in activity of the enzyme between resistant and susceptible cultivar upon inoculation with respect to their corresponding control. Upon *Fud* infection, peroxidase activity increased by 6.31% and 25.7% in the roots of resistant varieties, Asha and Maruthi (Table 1) whereas, in the susceptible cultivars Type 7 and Bahar, rates of increase were 1.6 and 3%, respectively, with respect to their controls. The rate of increase was more in the stem and leaf tissues of resistant varieties. Activity increased by 12% in the stem of resistant cv. Maruthi and 2% in susceptible cv. Type 7 upon inoculation. The highest level of increase (13.5%) in peroxidase activity was observed in leaves of Maruthi and the lowest increase (5%) was observed in Bahar leaves. Irrespective of inoculated or un-inoculated plants, there was a significant decrease in enzyme activity in S3. But the comparative level of induction of peroxidase was higher in resistant cultivars than in susceptible cultivars.

3.2 Spatial and Temporal Activity of Polyphenol Oxidase

In pigeonpea roots in control condition, prior to disease initiation, the highest activity (53.42 U gfw^{-1}) was noticed in susceptible variety Bahar and lowest (46.40 U gfw^{-1}) in resistant cultivar, Maruthi and the same was true for stem tissue (Table 2). At S2 stage, enzyme activity was elicited to 21.19 % and 52.0% in the stem of the resistant cultivars, Asha and Maruthi respectively compared to their un-inoculated control plants. Upon *Fud* inoculation, the activity increased by 9.80% and 7.56% in the stem tissue of susceptible cultivars Type 7 and Bahar respectively. Similarly, in root tissues at S2 stage, the increase in percentage was 16.80, 23.36, 13.44 and 0.77 in Asha, Maruthi, Type 7 and Bahar, respectively, as compared to control. In pigeonpea, it was interesting to see that induction of the activity was up to S3 stage in resistant cultivars. In resistant varieties, the enzyme accumulated in a significantly higher rate as compared to the untreated plants. The level of the enzyme activity was remarkably decreased from S2 to S3 stage and the level reached even below the control level in susceptible cultivars.

3.3 Spatial and Temporal Activity of Catalase

Type 7 (control) showed highest (212 µmol H_2O_2 used min^{-1} gfw-1) and Maruthi (control) exhibited the minimum (179 µmol H_2O_2 used min^{-1} gfw-1) activity in stem tissue at S1 stage. The activity of catalase in leaf tissues was significantly higher compared to root and stem (Table 3). Catalase activity in leaves at S1 stage was as high as 222.39 µmol H_2O_2 used min^{-1} gfw-1 (WR 315, control). Activity of catalase at S2 stage in susceptible cultivar Bahar increased by 13%, whereas, in resistant cv. Asha and Maruthi, the increase was 12.7% and 25%, respectively, compared to their corresponding controls in root tissues (Table 3). Highest induction was attained at S3 stage in the same tissues. For example, in resistant cv. Maruthi upon infection the increase was 1.65 times with respect to its control. In case of stem tissue, the same pattern of enzyme activity was followed and increase in induction was highest in cv. Asha at S3 (16.47%) over control. The same pigeonpea cultivar showed highest increase in catalase activity in leaf tissue as well. Another significant pattern observed in all the cultivars was the increase in enzyme activity of challenged tissues at S2 stage compared to S1 stage.

3.4 Spatial and Temporal Activity of Phenylalanine Ammonia Lyase

It is evident that irrespective of whether the plants were inoculated or un-inoculated, there was a significant increase in PAL activity from S1 to S3 stage (Table 4). The activity either slightly decreased or remained unchanged at S3 stage. At S1 stage, highest PAL activity was observed in susceptible cultivar Bahar under un-inoculated condition. At S2 stage, the increase in activity was mainly in control samples of the root tissues of all varieties (Table 4). In resistant cultivars, Asha and Maruthi, PAL activity was increased by

Table 1. Peroxidase activity (U g^{-1} fresh weight) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	229.4±11.03	725.0±16.69	192.4±6.22	246.2±6.51	699.6±0.57	183.6±9.05	246.6±16.12	628.4±6.22	147.2±2.26
	Fud	293.2±16.97	770.8±24.89	229.0±0.28	215.2±0.57	710.2±1.98	211.4±1.41	264.8±1.70	692.6±25.17	192.8±1.13
Maruthi	Control	257.8±7.64	637.2±2.83	179.4±7.64	265.8±14.99	618..4±3.96	206.2±10.47	238.4±13.58	659.8±0.28	207.0±3.68
	Fud	357.2±0.57	801.0±8.20	179.0±0.28	181.0±5.37	694.8±7.35	229.8±4.81	281.0±24.61	748.8±7.92	242.0±8.49
Type 7	Control	208.4±9.62	624.6±0.28	315.6±6.22	231.0±1.98	665.0±3.11	207.2±3.39	143.4±4.81	538.4±1.70	153.4±1.41
	Fud	245.0±7.07	634.2±2.55	240.8±11.88	152.2±13.86	679.4±0.85	127.4±7.64	184.4±19.23	585.8±5.37	114.0±7.35
Bahar	Control	279.6±9.62	570.0±30.83	249.8±4.81	142.0±0.57	637.4±6.51	142.0±0.57	176.2±0.28	613.0±13.86	200.6±3.11
	Fud	272.0±1.70	587.4±7.07	185.8±1.41	239.8±11.03	664.2±0.28	182.0±1.13	252.8±7.35	642.8±13.58	150.2±13.86

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

Table 2. Polyphenol oxidase activity (U g^{-1} fresh weight) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	49.88±3.90	60.22±0.51	47.74±0.03	46.74±0.03	48.12±0.40	45.60±0.00	51.60±2.43	50.46±0.03	44.96±0.06
	Fud	51.72±0.00	53.22±0.37	54.82±0.65	52.20±0.45	58.32±1.36	54.10±0.37	53.04±0.34	53.24±0.45	50.78±1.16
Maruthi	Control	46.40±0.57	57.00±0.68	44.00±0.17	46.38±0.20	49.60±0.62	60.74±0.37	48.80±0.00	52.12±0.34	48.10±0.20
	Fud	65.30±0.48	70.32±0.28	52.46±0.31	51.32±0.06	75.64±0.74	67.32±4.02	52.36±1.07	54.86±0.20	52.02±0.14
Type 7	Control	47.20±1.39	48.48±0.11	46.50±0.03	48.46±0.14	51.00±0.06	48.86±0.37	48.30±0.48	47.98±0.20	45.24±1.36
	Fud	48.82±0.14	55.00±0.51	45.34±0.20	50.78±0.14	55.92±0.91	46.24±0.06	42.30±0.20	49.08±0.23	49.30±3.48
Bahar	Control	53.42±1.39	56.94±0.31	53.82±0.14	50.80±0.00	52.90±0.71	47.80±0.11	47.04±0.00	48.00±3.73	50.80±0.23
	Fud	53.68±0.68	57.38±0.03	48.88±0.06	59.58±0.88	56.90±1.56	47.36±1.24	54.22±0.03	52.12±0.45	49.32±0.23

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

Table 3. Catalase activity (µmol H_2O_2 used min^{-1} g^{-1} fresh weight) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	272.9±1.41	241.7±4.40	243.5±5.97	195.6±2.28	170.2±3.45	171.4±0.71	214.5±0.31	217.5±2.67	221.2±4.63
	Fud	227.9±10.99	272.3±1.65	275.0±6.83	168.6±2.67	196.9±1.88	199.7±1.88	244.6±2.12	247.1±0.08	248.3±2.43
Maruthi	Control	242.2±1.41	194.6±5.18	209.7±2.20	179.5±4.87	201.7±1.81	182.9±6.51	234.4±1.02	234.9±1.81	237.7±2.35
	Fud	194.8±1.41	243.4±0.55	347.3±10..92	193.4±1.49	215.6±2.04	202.4±9.65	235.7±4.55	240.2±7.93	244.8±1.57
Type 7	Control	204.1±2.75	210.6±3.38	246.0±1.96	212.7±1.41	182.8±4.87	218.5±2.35	191.1±2.04	182.2±1.41	192.3±3.06
	Fud	209.6±1.26	211.8±3.22	199.7±3.61	192.8±5.73	194.4±3.06	204.7±0.31	180.5±0.94	190.1±0.55	184.4±4.63
Bahar	Control	214.1±1.10	215.5±2.90	219.5±3.22	201.5±1.49	216.0±5.42	222.2±9.42	252.0±0.31	252.3±1.02	248.3±0.31
	Fud	240.0±2.28	243.7±6.83	248.8±0.24	228.2±2.20	231.9±3.61	237.0±1.81	273.0±5.49	272.1±0.47	272.4±0.78

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

Table 4. Phenylalanine ammonia lyase activity (ΔA_{290nm}ml h^{-1}) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	0.42±0.01	1.11±0.00	1.11±0.01	0.21±0.01	0.89±0.00	0.95±0.00	0.43±0.04	1.09±0.00	1.06±0.01
	Fud	0.72±0.01	1.51±0.02	1.44±0.01	0.31±0.04	1.00±0.02	1.06±0.00	0.63±0.01	1.32±0.04	1.33±0.02
Maruthi	Control	0.08±0.01	0.81±0.01	0.76±0.01	0.34±0.01	1.03±0.02	1.08±0.02	0.49±0.00	1.18±0.01	1.19±0.01
	Fud	0.50±0.02	1.39±0.15	1.30±0.12	0.36±0.01	1.12±0.01	1.06±0.02	0.54±0.01	1.23±0.00	1.26±0.03
Type 7	Control	0.37±0.01	1.00±0.10	1.56±0.01	0.39±0.01	1.07±0.01	1.20±0.01	0.27±0.01	0.97±0.00	0.97±0.01
	Fud	0.79±0.00	1.06±0.03	1.09±0.06	0.22±0.00	0.96±0.01	0.93±0.01	0.30±0.01	0.97±0.00	0.97±0.00
Bahar	Control	0.51±0.00	1.17±0.01	1.26±0.08	0.75±0.01	0.96±1.01	1.52±1.01	0.52±0.05	1.17±0.00	1.30±0.02

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

36% and 72%. In the susceptible varieties, activity was increased by 6% and 9% in root tissues as compared to their respective controls. The significant increase in activity by 12% and 9% was observed in stem tissues of Asha and Maruthi, respectively, at S2 stage. PAL activity in susceptible cv. Bahar increased by only 3% in stem tissue and by 15% in leaf tissue at S2 stage as compared to its control. At S3 stage, in case of *Fud* treated pigeonpea, highest activity of PAL was observed in root tissue of Asha and lowest in Type 7. At this stage, stem and leaf tissue of resistant cultivars showed only a marginal increase in PAL activity. In case of susceptible varieties, the activity either remained unchanged or fell below the constitutive level of PAL activity.

3.5 Spatial and Temporal Activity of Chitinase

Induction of chitinase in pigeonpea was significantly high in leaves. The enzyme activity patterns of chitinase were, similar to those observed for peroxidase and polyphenol oxidase, with a marked increase in different tissues upon inoculation of the plants with *Fud.* Maximum enzymatic activity was observed in resistant cultivars at S2 stage. It was observed that the enzyme activity increased progressively from S1 to S2 stage, and there after showed decline with progression of the disease and at S3 stage enzyme activity was very low (Table 5). In contrast, these changes were much less conspicuous in case of un-inoculated plants, where the increase or decrease was only marginal suggesting the role of chitinase in inducing resistance to wilt disease in plants. In the leaf tissues, the increase was very clear in all the varieties and at S1 stage, the enzyme activity was up to 4.37 U gfw^{-1} in control samples of Bahar (Table 5). At S2 stage, an increase in activity by 3.10 and 3.58-fold in the resistant variety Asha and Maruthi and by 2.29 fold in leaf tissues of susceptible variety compared to control. Similarly, in the resistant varieties, enzyme activity increased by 3.0-fold in root and stem tissues, whereas in susceptible variety, it increased by 2.0-fold compared to their respective individual controls at S2. At S3 stage, the activity was slightly decreasing or it was almost unchanged in all the cases (Table 5).

3.6 Spatial and Temporal Activity of β-1,3-Glucanase

Glucanase activity was high in stem and leaf tissues, and upon inoculation of the plants with *Fud*, a marked increase in glucanase activity in different tissues was observed. In general, maximum enzymatic activity was observed in resistant cultivars at S2 stage and was more or less maintained up to S3 stage. This is in contrast to the activity pattern of other enzymes, where the levels showed significant decrease at S3 stage. Both control and challenged plants had higher levels of enzymatic activity at later stages of disease development, but the proportionate increase was much higher in resistant cultivars (Table 6). At S1 stage, maximum enzymatic activity was up to 0.76 mM glucose $h^{-1}gfw^{-1}$ in control root samples of Asha, while in cv. Bahar, the activity was lowest (0.47 mM). Similarly, there was marked increase in glucanase activity at S2 stage. The resistant cultivar Maruthi showed maximum increase (17 fold) in glucanase activity in leaves. This increase in activity was lower in susceptible cultivars (1.3-fold) in root tissues (Table 6).

4. DISCUSSION

A variety of complex mechanisms that involve the biosynthesis and accumulation of metabolites, novel and constitutive proteins were revealed that directly or indirectly function in the plant's defense response to pathogens. In the present study, interaction between *Fud* and the host plants, pigeonpea cultivars was observed to enhance the defense responses against wilt disease in resistant cultivars of pigeonpea. The induction of plant's own defense system started only after the infection by *Fud*, and subsequently resulted in hypersensitive reaction conferring resistance. Also, pigeonpea plants exposed to *Fud* showed enhanced synthesis of pathogenesis related proteins, activities of PAL and other antioxidant enzymes relative to their controls.

4.1 Spatial and Temporal Activity of Anti-Oxidant Enzymes (PO, PPO and Catalase)

The presented results revealed increased PO, PPO and catalase activities in pigeonpea tissues after inoculation with *Fud* and the activities were higher in the resistant cultivars at disease initiation stage in comparison to susceptible cultivars. These findings are in conformity with Rathi et al. [24] who reported higher PO activity in leaves of resistant variety. Inoculation with *Fusarium* caused an increase in enzyme activity in leaves of both susceptible and resistant varieties, but at S3 stage, activities recorded significant decline in the susceptible varieties falling even below the control levels in few cases.

Table 5. Chitinase activity (U g^{-1} fresh weight) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	3.04±0.00	3.42±0.01	3.38±0.00	3.24±0.02	3.56±0.00	3.52±0.02	3.15±0.01	3.44±0.08	3.51±0.05
	Fud	3.36±0.02	10.19±0.13	10.10±0.02	3.50±0.07	10.43±0.12	10.18±0.06	3.30±0.07	10.68±0.07	10.71±0.00
Maruthi	Control	2.83±0.01	3.66±0.06	3.57±0.03	2.91±0.00	3.50±0.00	3.53±0.00	2.95±0.00	3.17±0.04	3.14±0.00
	Fud	3.30±0.00	10.03±0.22	9.47±0.04	3.07±0.36	11.07±0.01	10.91±0.38	3.39±0.01	11.36±0.13	11.44±0.21
Type 7	Control	3.59±0.05	4.77±0.04	4.78±0.02	4.38±0.00	5.34±0.00	5.25±0.01	3.98±0.04	4.22±0.00	4.31±0.02
	Fud	4.22±0.01	9.47±0.12	8.62±0.02	5.11±0.04	9.43±0.00	8.31±0.05	4.55±0.01	9.69±0.05	8.86±0.08
Bahar	Control	4.37±0.10	4.81±0.00	4.54±0.00	3.83±0.01	4.64±0.03	4.57±0.03	3.88±0.01	4.18±0.04	4.12±0.04
	Fud	5.93±0.00	9.39±0.04	8.32±0.01	5.71±0.00	9.11±0.04	8.63±0.04	4.09±0.01	9.58±0.08	5.91±0.02

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

Table 6. Glucanase activity (mM h^{-1} g^{-1} fresh weight) in different tissues of pigeonpea cultivars differing in susceptibility to *Fusarium* wilt

Genotypes	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
Asha	Control	0.76±0.08	0.79±0.02	0.75±0.05	1.15±0.05	1.22±0.02	0.78±0.04	0.42±0.00	0.67±0.11	0.76±0.00
	Fud	0.92±0.47	1.48±0.09	1.35±0.04	1.36±0.01	1.56±0.03	1.73±0.03	0.72±0.04	2.43±0.02	1.85±0.02
Maruthi	Control	0.54±0.01	0.79±0.24	0.79±0.76	.63±0.00	0.58±0.54	0.69±0.02	0.31±0.01	0.56±0.02	0.52±0.04
	Fud	0.86±0.11	1.98±0.00	1.23±0.00	0.80±0.02	1.78±0.50	1.45±0.00	2.47±0.05	9.41±0.05	1.31±0.37
Type 7	Control	0.52±0.51	0.79±0.01	1.08±0.01	0.44±0.02	0.71±0.00	0.74±0.12	0.78±0.07	0.50±0.01	0.54±0.01
	Fud	0.95±1.02	1.06±0.01	0.82±0.01	1.16±0.03	1.14±0.78	1.17±0.01	1.08±0.01	0.71±0.00	0.62±0.24
Bahar	Control	0.47±0.01	0.59±0.03	0.55±0.00	0.43±0.00	0.56±0.08	0.81±0.01	0.45±0.00	0.51±0.00	0.62±0.05
	Fud	0.73±0.01	0.67±0.01	1.11±0.00	0.73±0.01	0.83±0.17	0.45±0.01	0.71±0.03	0.82±0.01	0.98±0.00

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3: Severe disease stage (30 DAS)
All values are mean of three replications ± SE

However, the rate of increase in activity continued up to 30 DAS, upon induction, though the total enzyme concentration was declining at this stage. Xue et al. [25] reported 2-fold increase in total peroxidase in bean tissues after inoculation with binucleate *Rhizoctonia* species in comparison to control.

The PO activity also showed a marked increase in stems and leaves, but not in roots. This spatial regulation of enzyme activity in distant tissues has been reported by previous researchers [26]. Tissues of plants attacked by necrotizing pathogens including *Rhizoctonia* and *Colletotrichum* species have been shown to accumulate a substantial amount of the enzymes not only at the site of infection but also in distant tissues. The presence of enzymes in infected plants has been associated with lesion development and limiting the spread of the disease [27-29]. The PO action could still happen in an indirect way by the activity of byproducts with antimicrobial activity or by inducing the formation of structural barriers. Higher activity of PPO was observed in pigeonpea resistant cultivars than susceptible cultivars, upon inoculation with *Fud* and the difference was more prominent in root and stem tissues than in the leaf tissues. In pigeonpea, increased PPO activity strongly indicates Asha and Maruthi's resistance was partially conferred by higher induction of polyphenol oxidase up to 52% over the susceptible varieties upon *Fusarium* infection. Similar results in chickpea were recorded by Raju et al. [30] who reported higher PPO activities in resistant cultivar ICCV-10 upon infection. The induced PPO-2 isoform in roots and shoots treated with salicylic acid, spermidine and the pathogen imparted defence response against the pathogen invasion. However, no induction of PPO was observed in susceptible cultivars. The present results confirm the reports of Raju et al. [30] where no induction was noticed in susceptible cultivars in either root, shoot or leaves at S3 upon inoculation. Studies conducted by Constabel et al. [31] and Stewart et al. [32] showed that plant PPOs are induced in response to mechanical wounding, fungal and bacterial infection, and by treatment with signalling molecules such as jasmonic acid/methyl jasmonate (MeJA), systemin and salicylic acid. The transgenic tomato plants with PPO over-expression gene exhibited high resistance to *Pseudomonas syringae,* the causative agent of speck disease compared to control plants [33]. Localized inoculation of tomato leaflets with *Pseudomonas syringae* induces a significant increase in PPO activity and leads to systemic resistance to the subsequent infection by *P. syringae* [34]. Thipyapong and Stiffens [35] also reported the PPO catalyzed phenolic oxidation in limiting disease development. Therefore, induction of PPO and PO is quite likely to govern mechanism of biochemical resistance in resistant cultivars that arose due to host-pathogen interaction. However, in un-inoculated condition, no significant changes were observed in PPO level between resistant and susceptible cultivars but significant changes in the induction of enzyme from S1 through S3 level were observed in all varieties. In the case of cv. Maruthi, upon inoculation, PPO level increased 40.7% in root tissue and up to 52.5% in stem. This establishes the fact that enzymes are induced at high amount only after pathogen infection and disease initiation. Another important observation was that PPO induction was more in stem tissues, whereas peroxidase induction was higher in leaf tissue. Raju et al. [30] also reported similar spatial distribution of PO and PPO activity in chickpea in response to *F. oxysporum* f. sp. *ciceri* infection.

Catalase induction started at S2 in un-inoculated control plants. Increase in induction was significant in leaf tissues upon infection compared to controls and it continued up to S3 stage. These results match with Subramanian et al. [36] and El-Khallal [37] who reported that level of H_2O_2 and other enzyme activities increased to the level of tolerance or susceptibility to *Fusarium* wilt. They also reported that susceptible cv. BG 256 showed increase in enzymatic activities by 30% over the resistant varieties at S3 stage in leaves after infection. This might be due to the invasion of the pathogen; the host cell wall degrades with the release of particular enzyme at the vicinity of the infection site.

4.2 Spatial and Temporal Activity of Phenyl Alanine Ammonia Lyase

PAL activity increased in response to pathogen treatments and resembles observations on accumulation of phenolics and PAL by Arfaoui et al. [5] in chickpea seedlings treated with *Rhizobium* Pch43 followed by *F. oxysporum* f. sp. *ciceri* race 0. The chickpea seedlings exposed to cell wall protein of *F. oxysporum* f. sp. *ciceri* showed enhanced synthesis of phenols, pathogenesis-related proteins and activities of PAL and PO relative to water treated controls [38]. Stadnik and Buchenauer [39] also

reported the enhancement of PAL activity and accumulation of cell wall-bound phenolic compounds in wheat plants treated with benzothiadiazole (BTH), a novel systemic acquired resistance (SAR) inducer in response to powdery mildew infection. Our study also indicates a rapid increase of PAL activity in pathogen treated pigeonpea seedlings, except for the control treatment. Dixon et al. [40] observed an elicitor dose-response effect (isolated from *Colletotrichum lindemuthianum*) in the activity of PAL in bean cells suspension cultures and two maxima of the enzyme activity in response to the application of smaller doses of elicitors.

Invasion of root tissues by the pathogen resulted in decreased activity of PAL in susceptible cultivar, whereas, its increased activity in resistant cultivar might have prevented the fungal invasion and thus the PAL activity maintained at higher levels during the experimental period. Increased accumulation of PAL with pathogen could re-establish the notion that PAL is synthesized more rapidly in resistant cultivars than that in susceptible cultivars in response to invasion of pathogen.

4.3 Spatial and Temporal Activity of Chitinase and β-1,3-Glucanase

Depolymerization of cell wall by the combined activities of chitinase and glucanase is reported to kill fungi *in vitro* [28,29] and cell wall fragments are shown to induce defense reactions in plants by switching on genes responsible for the synthesis of pathogenesis-defence related proteins [41]. Inhibition of growth of several fungi requires the presence of chitinase and β-1,3-glucanase activities [38]. Ferraris et al. [42] found that infection with *F. oxysporum* f. sp. *lycopersici* caused several fold increases in chitinase, β-1,3-glucanase, glucosidase and N-acetyl-glucosaminidase activities in susceptible varieties and resistant tomato cultivars from 5 to 90 days after inoculation. The present study also proved that chitinase activity was increasing 3.08 and 2.33 fold in root tissue, 2.45 fold and 3.43 fold in stem tissue, 3.15 fold and 3.5 fold in leaf tissues of pigeonpea resistant cultivars.

β-1,3-glucanase activity increased from 1.27 to 17-fold in the infected resistant cultivars as compared to 1.0-1.5 fold in infected susceptible cultivars at S2 stage which conforms the findings of Singh et al. [43]. Xue et al. [25] observed relatively higher activity of chitinase in the cotyledons of bean plants than hypocotyl and a significant increase in all cellular fractions of PO, β-1,3-glucanases and chitinase compared with diseased and control plants. β-1,3-glucanase activity was also reported to increase up to 18 fold in induced bean hypocotyl tissues. Similar results were found in the present study where the induction of chitinase activity was lower in root tissues. Similar increases in β-1,3-glucanase, but not as pronounced were reported from other studies using incompatibility interactions between soybean and *Phytophthora megasperma* f. sp. *glyciensi* [44], between bean cultivars and *Colletotrichum lindemuthianum* [45] and between *Vigna unguiculata* (L. Walp.) and *Fusarium oxysporum* [46]. Present study showed that chitinase significantly accumulated in different tissues after the onset of disease infestation. Igratius et al. [47] observed that seed associated chitinases of barley were different from those identified in leaves infected with powdery mildew. Anuratha et al. [27] isolated an infection related chitinase transcript that was only induced upon infection of rice with the sheath blight pathogen *Rizoctonia solani*. Both antifungal hydrolases are induced in coordination with other PR proteins in typical SAR responses [48].

Increased activity of defense enzymes in pigeonpea tissues after inoculation with *Fud* was in conformity with Cachinero et al. [49] where defense responses were induced in both pre-induced and non-induced plants infected by pathogens. They concluded that the suppression of *Fusarium* wilt possibly involved in an inhibitory effect of pre-induced as well as induced plant defenses against the pathogen. Present work also reveals that in pigeonpea resistant cultivars Asha and Maruthi, the increase in β-1,3-glucanase activity is comparatively lower in root tissue than in leaf tissue (Table 6). Giri et al. [10] also recorded similar lower activity of β-1,3-glucanase in roots of resistant cultivars of chickpea. Benhamou et al. [50] observed accumulation of these enzymes to be faster in incompatible interactions of tomato and *F. oxysporum* f. sp. *lycopersici* or *F. radicis-lycopersici* than in a compatible interaction. In the present study, lower activity of β-1,3-glucanase was observed in resistant variety of pigeonpea in the stem tissue as compared to the susceptible cultivar at S2 stage. Beckman and Roberts [51] in their model for host pathogen interaction suggested callose deposition and lignification as one of the plant's defense response against wilt disease and rate of these processes determines the degree of reduction of pathogen invasion.

Beffa et al. [52] demonstrated in tobacco that, when expression of β-1,3-glucanase is specifically blocked by the antisense mRNA technique, callose deposits are protected from degradation, resulting in resistance to viral infection. Levels of chitinase and β-1,3-glucanases were also reduced when the infection was confined at S3 stage. The observed decrease in β-1,3-glucanase activity may be associated with the reduction in the pathogen invasion as a result of defense reaction. In the current study, leaf tissue of pigeonpea resistant cv. Maruthi showed 17-fold increase in comparison to control at S2 stage and lower proportionate enhancement was found in root and stem tissue. Moreover, these two enzymes act synergistically in the partial degradation of fungal cell walls. The combination of these two enzymes could strongly inhibit growth of many fungi, including those that could not be inhibited by chitinase or β-1,3-glucanase alone [53]. Chitinases in combination with β-1,3-glucanase protect the plants from fungal infection by their lytic actions on fungal cell walls or by releasing oligosaccharide signal molecules that can activate an array of plant defences.

The resistant cultivars showed higher PO activities (2-5 fold more) than the susceptible cultivars. In root tissue of Maruthi, 25% increase in PO activity over the control suggests that probably this enzyme strengthens the defence response by enhancing lignification of cell walls. The induction of PPO activities increased only after challenging with pathogen, suggesting that PPO acts by phenol oxidation. Raju et al. [30] screened different genotypes of chickpea for the induction of PPO isoforms and found that the resistant genotypes against wilt disease expressed multiple isoforms of PPO, while the susceptible genotypes did not. Resistant cv. Maruthi showed 1.5-fold increase in activity at S2 stage in the stem tissue, whereas susceptible cv. Type 7 and Bahar showed 8-10% increase upon infection. One interesting finding was that there was almost no change in activity of PPO in Bahar roots at S2 after infection started, supporting the view that susceptible cultivars could not withstand the challenge. On the other hand, catalases implicated in several plant defence mechanisms, mainly in the generation of ROS have also been associated with induced resistance response. The results presented here indicate that all the three enzymes activities increased in different tissues of host plants after inoculation with *Fud* but catalase activity was pronounced up to S3 stage in contrary to other enzymes. In the leaf tissue of Asha (resistant) and Bahar (susceptible), the same level of activity enhancement was observed. Surprisingly, there was no significant change in Maruthi leaf tissue at S3 stage compared to other varieties, though in the S2 stage, the induction was more in this cultivar compared to the susceptible ones. These effects can be associated with the reason that the susceptible varieties express multiple isoforms of catalase, whereas the resistant genotypes did not.

Priming resistance by inducing these genes could be an efficient and inexpensive way of achieving the control of *Fusarium* wilt in pigeonpea. In case of pigeonpea resistant cultivar Maruthi, the increase was 71.6% in root tissue at S2 stage. In contrast, susceptible cultivar Type 7 did not show significant changes in the stem and leaf tissues at S2 stage. Several hypotheses can be formulated to explain the level of phenolics [6] and PAL observed in the plants following *Fud* infection. It may result from the *de novo* mRNA synthesis or accumulation of defense related genes such as PAL. Accumulation of phenolics may also occur, upon challenge by *Fusarium,* from the release of phytoalexins from their preformed conjugated forms. Furthermore, it is likely that these two mechanisms generating phytoalexins can act synergistically. The presented results are very important for future studies focusing on the transgenic pigeonpea development by isolation and transfer of disease resistance genes from resistant cultivar to susceptible cultivar during the stage of their potential expression in the tissues, upon challenging with pathogen. This will be an integrated management strategy to protect the crop plants from further pathogen attack.

5. CONCLUSION

To develop an effective strategy for management of wilt diseases, understanding of the molecular basis of pathogenesis and resistance mechanism is very important. Plants activate a large array of defence mechanisms in response to pathogen attack. A crucial factor determining the success of these mechanisms is the speed of their activation. Consequently, there is a considerable interest in understanding how plants recognize pathogen attack and control expression of defence mechanisms The present results demonstrate that resistant cultivars resulted in a significant increase in PO, PPO, PAL, chitinase

and β-1,3-glucanase and the expression of defense-related genes encoding PR proteins can be used as markers for the establishment of resistance. The chitinase and β-1,3-glucanase enzymes act synergistically on the fungal cell wall resulting in degradation and loss of inner content of a cell and indicate the possibilities of their involvement in defence response in pigeonpea against *Fusarium* wilt. A direct role for β-1,3-glucanase in defense of plants against pathogens may be proposed because the substrates for these enzymes are major components of cell walls of many fungi. Induced resistance is multi-component and it is necessary to investigate further other mechanisms involved in host-pathogen interaction, either alone or collectively.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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