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Studies on Biochemical Changes in Fusarium Wilt Resistant and Susceptible Genotypes during *Fusarium udum* and *Heterodera cajani* interaction in Pigeonpea

Rajalaxmi S. Keshgond[1] and S. Lingaraju[2]

[1]Department of Plant Pathology, UAS, Dharwad - 5800 05, India.
[2]Professor of Plant Pathology & Head, Institute of Organic Farming, UAS, Dharwad - 5800 05, India.

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Biochemical changes were studied in Fusarium wilt resistant and susceptible genotypes during *Fusarium udum* and *Heterodera cajani* interaction. Fusarium wilt resistant genotypes, *viz.* WRP-1 and ICP-8863 recorded increased activities of peroxidase, polyphenol oxidase, phenylalanine lyase and total phenols than Fusarium wilt susceptible genotype, GS-1. A decrease in total sugars was observed in all the three genotypes. Maximum decrease in total sugars was recorded in the Fusarium wilt susceptible genotype, GS-1 followed by ICP-8863 and WRP-1.

Keywords: Pigeonpea, Fusarium wilt, Pigeonpea cyst nematode.

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is an important food legume predominantly cultivated in about 50 countries in tropical and subtropical regions of Asia and Africa. The Indian subcontinent alone contributes nearly 92 per cent of the total pigeonpea in the world. Although India leads the world both in area and production of pigeonpea, its productivity is lower (671 kg/ha) than the world average (742 kg/ha) [FAOSTAT 2010] as we are not able to realize its yield potential due to several biotic and abiotic factors. Pigeonpea wilt complex incited by *Fusarium udum* (fungus) and *Heterodera cajani* (Pigeonpea cyst nematode; PCN) is a major one. Although *H. cajani* and *F. udum*, account for significant yield losses caused singly, crop suffers heavily when it is attacked by both the pathogens in interaction that lead to disease complex situation; the per cent of wilt incidence in such cases may go as high as 93.60 (Hassan, 1984) to 100.00 (Sharma and Nene, 1990). Synergistic interaction of these two pathogens has resulted into accentuation of wilt disease and wilt resistance break down in Fusarium wilt resistant genotypes. It is now known that the wilt resistance character of some pigeonpea cultivars may be broken due to the interaction of wilt fungus and a nematode, while in some other cultivars it may not be broken suggesting that loosing or holding of the wilt resistance is genotype- specific (Sharma and Nene, 1990; Lingaraju and Benagi, 2004).

Plants employ a vast array of defense mechanisms in response to pathogen attack of which biochemical factors form a major group. It would be helpful in managing pigeonpea wilt complex disease through developing Fusarium resistant cultivars that hold resistance in the presence of the pigeonpea cyst nematode. The aim of present study was to know the changes in different biochemical factors, *viz*. Peroxidase (PO), Polyphenol Oxidase (PPO), Phenylalanine Lyase (PAL), Total Phenols and Total Sugars in Fusarium wilt resistant pigeonpea genotypes that hold and lose the resistance in fungus-nematode interaction conditions.

* To whom all correspondence should be addressed.

MATERIALS AND METHODS

Isolation and maintenance of the fungus

Fungus was isolated by standard tissue isolation method from the pigeonpea plants showing wilting symptoms collected from the farmers' fields. The pure culture of this fungus was obtained by single spore isolation. The pure culture was maintained by subculturing once in a month and preserved in refrigerator. The identity of the fungal species associated was done by comparative morphological studies and pathogenicity test.

Collection and maintenance of Pigeonpea cyst nematode cultures

Cyst nematode infested pigeonpea cultivated soils were obtained from the field and the soil was processed for cysts as follows: 200 cc of soil was washed with clean tap water and sieved using a coarse mesh sieve (20 size – 840 µm) to remove stones and other materials. The filtrate was sieved with 72 mesh (212 µm) sieve recommended for extraction of cysts of *Heterodera cajani.* Vulval cone top structures of the cysts were studied to confirm their identity.

Sterile earthen pots of size (30 cm diameter) were filled with sterile 1:1 riverbed sand and soil mixture and watered. Healthy seeds of pigeonpea cultivar GS-1, WRP-1 and ICP-8863 were soaked in clean tap water overnight. Next day, six seeds were sown per pot and watered sparsely. Watering subsequently was done as per the requirement. Fifteen days after sowing (DAS), the seedlings were thinned to retain three seedlings per pot. Plumpy cysts were selected and inoculated to pigeonpea seedlings near root zone in the pots in greenhouse. Sufficient time (60 days) was given for *H. cajani* to complete three generations and then cysts were retrieved from the pots through processing (as already mentioned) as and when required for the experiment.

Pigeonpea genotypes used for the present study

From the earlier studies by Lingaraju and Benagi (2004), it has been established that some Fusarium wilt resistant pigeonpea genotypes hold resistance under wilt complex (fungus-nematode interaction) conditions. Hence three pigeonpea genotypes were selected to compare the changes in biochemical factors mentioned in different conditions of presence of *Fusarium udum* or *Heterodera cajani* and/or both. The pigeonpea genotypes used were;

1. GS-1: Fusarium wilt susceptible genotype
2. WRP-1: Fusarium wilt resistant genotype that holds resistance in presence of PCN
3. ICP-8863: Fusarium wilt resistant genotype that looses resistance in presence of PCN

Treatments imposed:

T1: Healthy - Neither *Fusarium udum* nor *Heterodera cajani*
T2: *Fusarium udum* (15 DAS)
T3: *Heterodera cajani* (15 DAS)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS

Inoculations for interaction studies

Fungal inoculum consisting of 5 per cent of giant culture of *Fusarium udum* prepared on maize meal sand medium (90:10) with 40 x 10^8 cfu/ g of giant culture and *H. cajani* @ 500 juveniles per pot (30 cm diameter) were inoculated 15 DAS as per the treatments mentioned.

Sampling

The sampling was done at 60 DAS. The root and stem samples collected were placed in the ice box containing ice cubes and brought to the laboratory. Further these were used for biochemical studies.

Enzyme extraction

The stem and root samples collected from different treatments were immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1 M (pH 7.0) at 4^0C. The homogenate was centrifuged for 20 min at 10000 rpm. Protein extracts prepared were used for estimation of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase.

Assay of Peroxidase (PO)

Peroxidase activity was assayed using a spectrophotometer (Hartee, 1955). The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of the enzyme extract and 0.5 ml of one per cent H_2O_2. The reaction mixture was incubated at room temperature (28±10^0C). The change in absorbance was recorded at 30 sec. interval for 3 min. The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance at 420 nm min^{-1} g^{-1} on fresh weight basis (Hammerschmidt *et al.,* 1982).

Assay of Polyphenol oxidase (PPO)

PPO activity was determined as per the procedure given by Mayer *et al.* (1965). One gram of roots was used for phenol oxidase estimation: the reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extracts. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm/ min/g fresh weight of plant tissue.

Assay of Phenylalanine Ammonia Lyase (PAL)

The PAL assay was conducted as per the method described by Ross and Sederoff (1992). The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCL (pH 8.8) and 600 µl of 1 mM PAL was incubated for 60 min. The reaction was arrested by adding 2N HCl. Later, 1.5 ml of toluene was added, vortexed for 30 sec, centrifuged (1000 rpm, 5 min.) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 295 nm against the blank of the toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. The enzyme activity was expressed as µM of trans-cinnamic acid/ min/g fresh weight of plant tissue.

Assay of Total phenols

The total phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of tissue was homogenized in 10 ml of 80 per cent methanol and agitated for 15 min at 70^0C, 1 ml of methanol extract was added to 250 µl of Folin Ciocalteau reagent (1N) and the solution was kept at 25^0C. After 3 min, 1 ml saturated solution of Na_2CO_3 and 1 ml of distilled water was added and the reaction mixture was incubated for 1 hr. at 25^0C. The blue colour developed was measured using a spectrophotometer at 725 nm. The content of the total soluble phenol was calculated according to a standard curve obtained from a Folin Ciocalteau reagent with a phenol solution (C_6H_5OH) and expressed as phenol equivalents in mg/g fresh weight of plant tissue.

Assay of Total sugars

The total sugar content was estimated as per the procedure given by Hegde and Hofreiter (1962). One gram of infected root sample was taken and powdered. The fresh material was extracted using aqueous ethyl alcohol using pestle and mortar. The solvent was evaporated by keeping 0.2 ml of extract in water bath to the sugar in test tube and one ml water was added to dissolve the sugars and 4 ml of Anthrone's reagent was added. The content was heated for eight min in boiling water bath and cooled. The green colour developed was measured using a spectrophotometer at 630 nm with suitable blank. The OD value was referred to a glucose standard and was expressed in mg/g of plant tissue.

RESULTS AND DISCUSSION

Changes in Peroxidase activity in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

The data on estimation of changes in peroxidase activity in pigeonpea genotypes with

Table 1. Changes in Peroxidase activity in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

Treatment	PO activity (changes in absorbance $min^{-1}g^{-1}$) Genotype		
	GS-1 (wilt susceptible)	WRP-1 (wilt resistance holding)	ICP-8863 (wilt resistance breaking)
T1: Healthy- Neither *Fusarium udum* nor *Heterodera cajani*	1.431 (-) (-)	2.433 (-)	2.000
T2: *Fusarium udum* (15 DAS)	1.589 (11.04)	3.742 (53.80)	2.018 (0.90)
T3: *Heterodera cajani* (15 DAS)	1.498 (4.68)	3.784 (55.53)	2.094 (4.70)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS	1.989 (38.99)	3.989 (63.95)	3.082 (54.10)
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS	1.842 (28.72)	3.842 (57.83)	3.042 (52.10)

interactions of *F. udum* and *H. cajani* has been presented in table 1. There was an increase in the activity of peroxidase in all the genotypes when inoculated with *Fusarium udum* and *Heterodera cajani*. The highest peroxidase activity was recorded in the wilt resistance holding genotype WRP-1 that accounted for 63.95 per cent increase over healthy (63.95 units change in absorbance/ min/g) followed by the wilt resistance breaking genotype ICP 8863 which recorded for 54.10 per cent increase over healthy (54.10 units change in absorbance/ min/g) and least was recorded in the wilt susceptible genotype GS-1 with 38.99 per cent increase over healthy (1.989 units change in absorbance/ min/g) in the treatment involving simultaneous inoculation of *F. udum* and *H. cajani* @ 15 DAS compared to the other treatments.

Rai *et al*. (2011) observed maximum peroxidase activity in tomato Fusarium wilt resistant cultivar Flora Dade (2.073 units/g) and minimum in the susceptible cultivar Sel-18(0.241 units/g). Higher levels of PO were observed in Fusarium resistant variety compared to partially resistant variety (Parmar and Subramanian, 2012). Pigeonpea Fusarium wilt resistant variety ICPL 87119 had higher levels of peroxidase with 3.1755 unit/g than the susceptible variety T 1515 with 2.1096 unit/g (Mary and Subramanian, 2014). Increased PO levels were observed in Fusarium head blight resistant wheat cultivar Sumai 3 compared to the susceptible cultivar Falat when inoculated with *Fusarium graminearum* (Sorahinobar, 2015).

Peroxidase plays an integral part in the biosynthesis of plant cell wall components, *viz*. lignin, suberin and cross-linked extension (Lamport *et al*., 1986). The lignifications and wall thickenings are part of defense response to pathogens particularly fungi. Besides these, PO also plays an important role in one of the earliest observable event of plant's defense response, *i.e*. oxidative burst (Wojtaszek, 1997). PO oxidizes phenolics to highly toxic quinines in the presence of hydrogen peroxide and hence has been assigned a role in disease resistance (Loebenstein and Linsey, 1961; Vidhyasekaran, 1988).

Changes in Polyphenol oxidase activity in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

An increase in the activity of polyphenol oxidase was observed in the wilt resistance holding genotype WRP-1 that recorded 75.00 per cent increase over healthy (0.1190 units change in absorbance/ min/g) followed by the wilt resistance breaking genotype ICP 8863 with 66.10 per cent increase over healthy (0.0980 units change in absorbance/ min/g) and least increase was observed in the wilt susceptible genotype GS-1 with 14.52 per cent increase over healthy (0.071 units change in absorbance/min/g)in the treatment involving simultaneous inoculation of *F. udum* and *H. cajani* @ 15 DAS compared to the other treatments (table 2).

Higher PPO activity was recorded in Fusarium wilt resistant cultivars of tomato, *viz*. FEB-2, FEB-4, Flora Dade and NF-31 as compared

Table 2. Changes in Polyphenol oxidase activity in pigeonpea genotypes with interactions of *F. udum and H. cajani*

Treatment	PPO activity (changes in absorbance $min^{-1}g^{-1}$) Genotype		
	GS-1 (wilt susceptible)	WRP-1 (wilt resistance holding)	ICP-8863 (wilt resistance breaking)
T1: Healthy- Neither *Fusarium udum* nor *Heterodera cajani*	0.0620 (-)(-)	0.0680 (-)	0.0590
T2: *Fusarium udum* (15 DAS)	0.0680 (9.68)	0.0720 (5.88)	0.0620 (0.30)
T3: *Heterodera cajani* (15 DAS)	0.0580 (-6.45)	0.0690 (1.47)	0.0640 (8.47)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS	0.071 (14.52)	0.1190 (75.00)	0.0980 (66.10)
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS	0.0750 (20.97)	0.1099 (61.62)	0.0880 (49.15)

to the susceptible cultivars (Sel-7, Sel-18 and Punjab Chhuhara) (Rai *et al*., 2011). Srivastava *et al*. (2014) observed that there was increased accumulation of PAL in the roots of pigeonpea Fusarium wilt resistant varieities (ICP 8863, ICP 9174, JA 4 and Banda Palera) than the susceptible ones (Type 7, Bahar, ICP 2376 and UPAS 120) when inoculated with *Fusarium udum*. Patel *et al*. (2015) observed that Fusarium wilt resistant genotypes of pigeonpea (ICPL 87119 and BDN 2) recorded higher levels of PPO in infected and non-infected conditions compared to the susceptible ones (T1515 and ICP 2376). Sorahinobar (2015) observed increased PPO activity in Fusarium head blight resistant wheat cultivar Sumai 3 compared to the susceptible cultivar Falat when inoculated with *Fusarium graminearum.*

PPO is important in the initial stage of plant defense where membrane damage causes release of phenols such as chlorogenic acid. PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules, thus creating an unfavorable environment for pathogen development (Jockusch 1966; Mohamed *et al*. 2012).

Changes in Phenylalanine Lyase activity in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

An increase in the activity of phenylalanine ammonia lyase was observed in wilt resistant genotypes, *viz*. WRP-1 with 56.09 per cent increase over healthy and ICP-8863 with 46.34 per

Table 3. Changes in Phenylalanine Ammonia Lyase in pigeonpea genotypes with interactions of *F. udum and H. cajani*

Treatment	PAL (µM of transcinnamic acid $min^{-1}g^{-1}$) Genotype		
	GS-1 (wilt susceptible)	WRP-1 (wilt resistance holding)	ICP-8863 (wilt resistance breaking)
T1: Healthy- Neither *Fusarium udum* nor *Heterodera cajani*	0.85 (-)	0.88 (-)	0.82 (-)
T2: *Fusarium udum* (15 DAS)	0.90 (5.88)	1.18 (43.90)	1.10 (34.14)
T3: *Heterodera cajani* (15 DAS)	0.88 (3.52)	1.12 (36.58)	1.15 (40.24)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS	0.78 (-8.23)	1.28 (56.09)	1.20 (46.34)
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS	0.68 (-20.00)	1.24 (51.21)	1.18 (43.90)

Table 4. Changes in total phenols in pigeonpea genotypes with interactions of *F. udum and H. cajani*

Treatment	Total phenols (mg g^{-1} of sample) Genotype		
	GS-1 (wilt susceptible)	WRP-1 (wilt resistance holding)	ICP-8863 (wilt resistance breaking)
T1: Healthy- Neither *Fusarium udum* nor *Heterodera cajani*	0.3220 (-)	0.2840 (-)	0.2360 (-)
T2: *Fusarium udum* (15 DAS)	0.2980 (-7.45)	0.2300 (-19.01)	0.3220 (36.44)
T3: *Heterodera cajani* (15 DAS)	0.2700 (-16.14)	0.2980 (4.92)	0.3280 (38.98)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS	0.2440 (-24.22)	0.4120 (45.07)	0.4220 (78.81)
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS	0.2500 (-22.36)	0.3920 (38.02)	0.3820 (61.86)

cent increase over healthy when inoculated with both pathogens, whereas there was decrease in the PAL activity in the wilt susceptible genotype GS-1 with 8.23 per cent decrease over healthy in the treatment involving simultaneous inoculation of *F. udum* and *H. cajani* @ 15 DAS compared to the other treatments. Least PAL activity was observed in the wilt susceptible genotype GS-1 with 20.00 per cent decrease over healthy in the treatment involving inoculation of *H. cajani* @ 15 DAS followed by *F. udum* inoculation @ 30 DAS (table 3).

Our results are in accordance with results obtained by Sarwar *et al*. (2001) who observed increased PAL activity in both Ascochyta blight resistant and susceptible genotypes when inoculated with *Ascochyta rabiei* but the increase was more pronounced in resistant varieties compared to the susceptible ones. Bhagat and Chakraborty (2010) recorded increased levels of PAL in tea cultivars resistant to *Sclerotium rolfsii* compared to the susceptible ones when inoculated with *S. rolfsii*. Mary and Subramanian (2014) observed 1.4 fold times increased activity of PAL in pigeonpea Fusarium wilt resistant variety (ICPL 87119) compared to the susceptible variety (T 1515).

PAL is the first enzyme of phenylpropanoid metabolism in higher plants and it plays a significant role in regulating the accumulation of phenolics, phytoalexins and lignins, the three key factors responsible for disease resistance (Vidhyasekaran, 1988).

Changes in total phenols in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

The data on changes in total phenols in pigeonpea genotypes with interactions of *F. udum* and *H. cajani* is presented in table 4. An increase in the total phenols was recorded in the wilt resistance breaking genotype ICP-8863 with 78.81 per cent increase over healthy followed by the wilt resistance holding genotype WRP-1 with 45.07 per cent increase over healthy and least was recorded in the wilt susceptible genotype GS-1 with 24.22 per cent decrease over healthy in the treatment involving simultaneous inoculation of *F. udum* and *H. cajani* @ 15 DAS compared to the other treatments.

The present results are in close agreement to earlier studies by other researchers, where it has been shown that a resistant variety had a higher level of phenolics than the susceptible one: in tomato against Fusarium wilt and leaf curl virus (Banerjee & Kalloo, 1989; Singh *et al*., 2002). Parmar and Subramanian (2012) found an increase in phenol content in resistant variety (76.30 µg/g) when compared to partially resistant variety (63.80 µg/g). An increase of 1.25 fold times was recorded in pigeonpea Fusarium wilt resistant variety (ICPL 87119) compared to the susceptible variety (T 1515) when inoculated with *Fusarium udum* (Mary and Subramanian, 2014).

The exact mode of action of phenolics compounds in reducing the incidence of Fusarium wilt is not yet very well known. However, phenols

Table 5. Changes in total phenols in pigeonpea genotypes with interactions of *F. udum and H. cajani*

Treatment	Total sugars (mg g^{-1} of sample) Genotype		
	GS-1 (wilt susceptible)	WRP-1 (wilt resistance holding)	ICP-8863 (wilt resistance breaking)
T1: Healthy- Neither *Fusarium udum* nor *Heterodera cajani*	3.65 (-)	2.94 (-)	2.90 (-)
T2: *Fusarium udum* (15 DAS)	3.42 (-6.30)	2.90 (-1.36)	2.84 (-2.06)
T3: *Heterodera cajani* (15 DAS)	3.50 (-4.10)	3.14 (6.80)	3.10 (6.89)
T4: *Heterodera cajani* + *Fusarium udum* @ 15 DAS	3.00 (-17.80)	2.72 (-7.48)	2.60 (-10.34)
T5: *Heterodera cajani* @ 15 DAS + *Fusarium udum* @ 30 DAS	3.25 (-10.96)	2.91 (-1.02)	2.80 (-3.44)

in quinone forms and in oxidized state are effective in checking the pathogen, including the inactivation of enzymes produced by it (Sequeria, 1983).

Changes in total sugars in pigeonpea genotypes with interactions of *F. udum* and *H. cajani*

The disease reaction has been correlated with the sugar level in different crop plants. Generally high levels of total sugars in the host plant are stated to be responsible for disease resistance. A decrease in total sugars was observed when both *F. udum* and *H. cajani* were inoculated simultaneously @ 15 DAS in all the three genotypes. Maximum decrease in total sugars was recorded in the wilt susceptible genotype GS-1 with 17.80 per cent decrease over healthy followed by wilt resistance breaking genotype ICP-8863 with 10.34 per cent decrease over healthy and then by the wilt resistance holding genotype WRP-1 with 7.48 per cent decrease over healthy. The least decrease in total sugars in the wilt resistance holding genotype WRP-1 with 1.02 per cent decrease over healthy in the treatment involving inoculation of *H. cajani* @ 15 DAS followed by *F. udum* inoculation @ 30 DAS (table 5). These results are in conformity with the reports of Patil *et al*. (2011) in wheat against spot blotch in tetraploid wheat; Ramdayal and Joshi (1968) in barley against leaf spot pathogen, Mandokhot *et al*. (1979) and Levy and Cohen (1984) in case of maize against Turcicum blight and Subramanyam *et al*. (1990) in wheat against *Exerohilum hawaiiensis.* Sunkad and Kulkarni (2006) reported that groundnut varieties resistant and moderately resistant to rust recorded more sugars than susceptible ones after infection.

Infection by some pathogens bring changes in respiratory pathway and photosynthesis which are the vital processes taking place inside the plant leading to wide fluctuations in sugars (Klement and Goodman, 1967).

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