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BIOCHEMICAL ALTERATIONS IN LEAVES OF RESISTANT AND SUSCEPTIBLE PIGEONPEA GENOTYPES INFECTED SYSTEMICALLY BY STERILITY MOSAIC VIRUS

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ABSTRACT
The study on biochemical constituents were carried out in five pigeonpea genotypes due to infection of sterility mosaic virus in healthy and infected leaves at 30, 60 and 90 DAS. Overall results revealed that the chlorophyll content were decreased irrespective of genotypes. Phenol content and total sugars content was more in diseased sample when compared to healthy samples in both resistant and susceptible genotype but protein content was more in healthy leaves of resistant varieties but it was increased after viral infection which was more in susceptible varieties. PO, PPO and PAL content were more in resistant variety when compared to susceptible genotypes. Among the genotypes, the Bahar genotype at different days of interval resulted with the highest level of chlorophyll (2.66 mg/g), phenol (3.87 mg g^{-1}), protein (1.65 mg g^{-1}), total sugars (3.82 mg g^{-1}) peroxidase (3.92 changes in absorbance 470 nm/min/mg protein), polyphenol oxiase (0.68 changes in absorbance 420 nm/ min/mg protein) and phenylalanine ammonia lyase (18.92 nmol of trans cinnamic acid min^{-1} g^{-1}) at all days of interval when compared to the moderately resistant and susceptible genotypes. Hence, it is suggested that the genotype Bahar could be used as one of the resistant source against SMD.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is a drought-resistant pulse crop cultivated in semi-arid regions of the world (Saxena, 2008). In India, pigeonpea is cultivated in an area of about 36.3 lakh ha with an annual production of 27.6 lakh tonnes averaging a productivity of 760.33 kg ha^{-1} (Anon., 2014). It is known to be affected by many pests and diseases (Nene *et al.,* 1996) among these sterility mosaic disease (SMD) is the most economically important disease of pigeonpea in the Indian subcontinent (Singh *et al*., 1999).The SMD-affected plants show severe stunting and mosaic symptoms on leaves with complete or partial sterility (Nene, 1972). The SMD causal agent was recently identified as a novel virus provisionally named as the Pigeonpea Sterility Mosaic Virus (PPSMV) (Kumar *et al*., 2000). The virus is transmitted under natural conditions by the eriophyid mite *Aceria cajani* (Channabasavanna, 1966) and experimentally, by grafting (Ghanekar *et al.*, 1992). Certain resistant germplasm lines have been made available to the pulse breeders in the recent past, no information is available on the biochemistry of resistance mechanism in these germplasms (Kannaiyan *et al*., 1984) and it has been reported that resistance to any virus depends on plant metabolism (Ganapathy *et al*., 2011). Morphological traits have commonly been used for cultivar identification, the majority of which are controlled by several genes (Tsaftaris, 1987) and most are influenced in varying degrees by environmental conditions, however the biochemical markers, have no such disadvantage. Considering these observations, the present study was conducted to determine the effect of viral infection on the biochemical constituents in different genotypes of pigeonpea.

MATERIALS AND METHODS

Present study was carried out at department of Plant Pathology, College of Agriculture, Raichur during year 2014-16. Healthy and diseased pigeonpea leaf samples of the resistant (Bahar), moderately resistant (GRG-177, GRG-811) and susceptible (Maruti, TS-3R) were selected on the basis of their reaction to the sterility mosaic virus infection. Further, biochemical constituents *viz.,* chlorophyll (a, b and total chlorophyll), sugars (reducing, non reducing and total), total phenols, total proteins, peroxidase, polyphenol oxidase, phenyl alannine ammonia lyase were estimated from the leaves of pigeonpea cultivars applying standard and scientific protocols as mentioned bellow.

Chlorophyll estimation

Estimation of chlorophyll is carried out according to method given by Arnon (1949). Quantitative analysis of chlorophyll was done by using DMSO (Dimethyl sulphoxide) in the test tube. The optical density of coloured extracted solution was measured at 645 nm, 652 nm and 663 nm for chlorophyll a, chlorophyll b and total chlorophyll, respectively. The chlorophyll a, chlorophyll b and total chlorophyll were estimated using the formulae as given below

Chlorophyll a (mg g^{-1} tissue) = [12.7 (D663)-2.69 (D645)] x V /1000xW

Chlorophyll b (mg g^{-1} tissue) = [22.9 (D645) - 4.68 (D663)] x V/1000xW

Total Chlorophyll (mg g^{-1} tissue) = [20.2 (D645) + 8.02 (D663)] x V/1000 xW

Where:

D = Optical density at respective nm.

V = Final volume of chlorophyll extract in 80% acetone.

W = Fresh weight of the tissue extracted.

Total phenol

Folinciocalteau reagent method was used for estimating the total phenol (Bray and Thrope, 1954). One ml of ethanol leaf extract was taken in a boiling tube to which one ml of Folinciocalteau reagent and 2 ml of 20 per cent sodium carbonate were added. This mixture was heated exactly for one minute on a water bath. After cooling 2 ml distilled water was added and the blue colour development was read at 660 nm.

Total protein

Protein estimation was done by the following procedure of Lowry *et al*. (1951). Bovine serum albumin was used as the standard. The samples were diluted to 100 mg protein concentration per ml and known aliquots of the sample were made up to one ml with distilled water. To this five ml of alkaline copper solution was added and mixed well. After 10 min 0.5 ml of 1 N FCR was added and mixed well. The colour developed after 30 min was measured at 660 nm against a reagent blank.

Total sugar

The total sugar estimated after acid hydrolysis of non-reducing to reducing sugar by following Nelson's modification of Somogyi's method (Nelson, 1944). One ml of alcoholic extract was evaporated in hot water bath and made up to five ml using distilled water. One ml of each sample (alcoholic extract) was pipette to a test tube added with one ml of mixture of alkaline copper reagent. The tubes were then cooled under running tap water. After cooling one ml of arsenomolybdate reagent was added. The above solution was diluted to 15 ml after 15 min. The absorbance of the solution was measured in spectrophotometer at 510 nm. The amount of reducing sugars was determined by using standard curve prepared.

Peroxidase activity

Peroxidase activity was assayed, following the method described by Hartee (1955). Reaction mixture consisted of 1.5 ml of Guaicol solution, 100 ml of enzyme preparation and 100 ml of 1 percent H_2O_2. At the start of the enzyme reaction, the absorbance of the mixture was set to zero at 420 nm and change in the absorbance were recorded at 30 seconds intervals. Boiled enzyme preparation served as control. Peroxidase activity was expressed as changes in absorbance/ minute/ gram fresh weight.

Polyphenol oxidase activity

Polyphenol oxidase activity was assayed using the method described by Mayer *et al.* (1965). Standard reaction mixture contained 1.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.5 ml of enzyme preparation and 0.5 ml of 0.01 M catechol. At the start of the enzyme reaction the absorbance was set to zero at 495 nm. The changes in the absorbance were recorded at 30 seconds intervals and polphenol oxidase activity was expressed as changes in the OD of the reaction mixture per minute per 200 mg of fresh weight of tissue.

Phenylalanine ammonia lyase (PAL) activity

Plant samples (500 mg) were homogenized in 2 ml of ice cold 0.1 M sodium borate buffer at pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 g for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm (Dikerson *et al*., 1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The amount of transcinnamic acid synthesized was calculated (Dikerson *et al*., 1984) and enzyme activity expressed as n mol transcinnamic acid min^{-1} g^{-1} tissue.

RESULTS

Total chlorophyll estimation studies revealed that at 30 DAS the maximum total chlorophyll content in healthy leaves of resistant genotype 2.66 mg/g was observed in Bahar followed by 2.21 mg/g in GRG-177. The least total chlorophyll content was recored in susceptible genotype Maruti (1.30 mg/g). Similarly at 60 and 90 DAS, the maximum total chlorophyll content was observed in Bahar 2.32 mg/g and 1.98 mg/g in healthy leaves, respectively, whereas in infected leaves, total chlorophyll content was more in Bahar 1.86 mg/g and 1.32 mg/g, respectively as given in Table 1.

In the present investigation, resistant genotype of Bahar (0.91-3.87 mg/g) recorded comparatively higher amount of phenols than the moderately resistant genotypes GRG-177 (0.71-3.37 mg/g) and GRG-811 (0.53-2.17 mg/g)), but very less amount recorded in susceptible genotypes Maruti (0.25-2.50 mg/g) and TS-3R (0.28 -2.60 mg/g) at 30, 60 and 90 DAS in healthy and diseased sample respectively (Table 2).

With respect to the protein content, soluble protein content was comparatively more in resistant genotype Bahar with 1.42-1.65 mg/g, than the moderately resistant genotype GRG-177 (1.22-1.46 mg/g) and in GRG-811 (1.29-1.73 mg/g), but very high amount recorded in infected leaves of susceptible genotypes Maruti 1.82 mg/g and TS-3R 1.95 mg/g diseased samples, respectively (Table 3).

In the present investigation, the resistant genotype of pigeonpea Bahar exhibited more amount of toal sugar, (3.39-3.82 mg/g and 3.52 to 3.95 mg/g), reducing sugars (2.26-2.35 and 2.34-2.44 mg/g), and non reducing sugars (1.13-1.48 mg/g and 1.18-1.51 mg/g) as compared to the susceptible inbreed lines Maruti of total sugars, (1.89-2.20 mg/g and 2.17-2.42 mg/g), reducing sugars (1.45-1.61 and 1.61-1.63 mg/g), and non reducing sugars (0.44-0.58 mg/g and 0.61-0.79 mg/g) in healthy and infected leaves during the growth of 30 DAS and it was same trend in 60 and 90 DAS as given in Table 4.

An increase in PO activity began with the infection of sterility mosaic disease. More PO activity was recorded in diseased

Table 1: Effect of Sterility mosaic disease on chlorophyll content (mg/g) in different genotypes of pigeonpea

Genotypes	Chlorophyll Content (mg/g)															
	30 DAS						60 DAS						90 DAS			
	Healthy			Infected			Healthy			Infected			Healthy			Infected
	Chl. 'a'	Chl. 'b'	Total Chlro -phyll	Chl. 'a'	Chl. 'b'	Total Chlro -phyll	Chl. 'a'	Chl. 'b'	Total Chlro -phyll	Chl. 'a'	Chl. 'b'	Total Chlro -phyll	Chl. 'a'	Chl. 'b'	Total Chlro -phyll	Chl. 'a'	Chl. 'b'	Total Chlro -phyll
Bahar (R)	1.88	0.78	2.66	1.75	0.52	2.27	1.63	0.69	2.32	1.38	0.48	1.86	1.50	0.48	1.98	0.98	0.34	1.32
GRG – 177 (MR)	1.50	0.71	2.21	1.4	0.48	1.88	1.1	0.68	1.78	0.98	0.34	1.32	1.00	0.32	1.32	0.91	0.27	1.18
GRG – 811 (MR)	1.38	0.68	2.06	1.18	0.42	1.6	1.2	0.35	1.55	0.78	0.2	0.98	0.78	0.21	0.99	0.5	0.19	0.69
Maruti (S)	1.00	0.30	1.30	0.91	0.27	1.18	0.92	0.27	1.19	0.47	0.16	0.63	0.56	0.19	0.75	0.21	0.07	0.28
TS-3R (S)	1.04	0.32	1.36	0.81	0.22	1.03	0.83	0.24	1.07	0.75	0.22	0.97	0.73	0.08	0.81	0.39	0.05	0.44
S.Em ±	0.01	0.01	0.04	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.01	0.01
C.D. at (1%)	0.04	0.06	0.17	0.03	0.04	0.07	0.07	0.03	0.04	0.03	0.04	0.05	0.04	0.04	0.03	0.07	0.03	0.03

(R) - Resistant, (MR) - Moderately resistant, (S) - Susceptible, DAS: Days after sowing

plants compared to healthy pigeonpea leaves. The highest PO activity was noticed in resistant and moderately resistant genotypes than Maruti and TS- 3R. At 30 DAS, highest PO activity of 3.925 changes in absorbance 470 nm/min/mg protein was recorded in diseased samples of resistant variety Bahar as compared to healthy leaves (3.831 changes in absorbance 470 nm/min/mg protein) of same genotype. A slight increase in PO activity of diseased samples was noted at 30 and 60 DAS infection, later it was decreased in diseased leaves as given in Table 5.

In all genotypes initially PPO activity was increased but after 60 days there was slight decrease in resistant and moderately resistant genotypes but in susceptible genotypes it was decreased over the period. At 30 DAS healthy leaves of resistant and moderately resistant varieties Bahar, GRG-177, GRG- 811 recorded (0.38, 0.29 and 0.31 Changes in absorbance 420 nm/ min/mg protein) respectively exhibited slightly higher PPO activity than the corresponding leaves of susceptible varieties. At 60 and 90 DAS infected leaves of susceptible genotypes showed more PPO activity compared to healthy leaves of same genotypes (Table 6).

In healthy plants, of resistant variety PAL activity was 18.94 nmol trans cinnamic acid/ hr/ mg protein at initial days which increased to 23.45 nmol trans cinnamic acid/ hr/ mg protein at 90 DAS. In the case of diseased plants, PAL activity reached a maximum of 27.72 nmol trans cinnamic acid/ hr/ mg protein at 90 DAS (Table 7).

DISCUSSION

Chlorophyll is a vital pigment helps in photosynthesis and directly related to the productivity. Leaf chlorophyll content can be directly related to stress physiology affecting the growth and yield. Virus infection induces changes in leaf pigmentation, hence the symptoms are expressed. Stimulation of chlorophyllase which attack chlorophyll and inhibition of chloroplast development have been reported in virus infected plant it is mainly due to the disruption of chloroplast in chlorosis induced tissue as reported by Singh and Shukla (2009); Pineda *et al*. (2008). Chlorophyll content decreased after infection and maximum decrease was found in susceptible genotype compared to the resistant genotype. Similar results on reduction of chlorophyll content after pathogen infection was obtained by earlier workers Mali *et al.* (2000) and Arora *et al*. (2009).

Upon infection of the pathogen the host phenolic compounds may increase and contribute to enhance the mechanical strength of the host cell wall by the synthesis of lignin and suberin that are involved in the physical barrier that can block the spread of the pathogen and acceleration of phenols synthesizing pathway following pathogen infection. (Singh *et al*., 2014; Ngadze *et al*., 2012). However, phenols in quinone forms and in oxidized state are effective in checking the pathogen, by limiting the growth of the pathogen including the inactivation of enzymes produced by it. Our results are in close agreement to earlier studies by a number of workers, where it has been shown that a resistant variety had a higher level of phenolics than the susceptible one. This is in agreement with the findings of Rathi *et al*. (1986); Mali *et al.* (2000);

Table 2: Effect of sterility mosaic disease on phenol content (mg/g) in different genotypes of pigeonpea

Genotypes	Phenols (mg/g)					
	30 DAS		60 DAS		90 DAS	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Bahar (R)	2.75	3.87	1.71	1.75	0.91	1.01
GRG-177 (MR)	2.60	3.37	1.28	1.31	0.71	0.98
GRG-811 (MR)	2.09	2.17	1.06	1.12	0.53	0.75
Maruti (S)	2.05	2.50	0.28	0.62	0.25	0.35
TS- 3R (S)	2.12	2.60	0.90	1.15	0.28	0.46
S.Em ±	0.10	0.16	0.07	0.06	0.03	0.02
C.D. at (1%)	0.40	0.75	0.31	0.33	0.14	0.10

Table 3: Effect of sterility mosaic disease on soluble protein content (mg/g) in different genotypes of pigeonpea

Genotypes	Soluble protein (mg/g)					
	30 DAS		60 DAS		90 DAS	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Bahar (R)	1.42	1.45	1.59	1.63	1.61	1.65
GRG-177 (MR)	1.22	1.30	1.36	1.42	1.38	1.46
GRG-811 (MR)	1.29	1.36	1.43	1.50	1.41	1.60
Maruti (S)	1.11	1.73	1.29	1.79	1.30	1.82
TS- 3R (S)	1.25	1.79	1.42	1.93	1.43	1.95
S.Em ±	0.02	0.01	0.01	0.02	0.02	0.02
C.D. at (1%)	0.08	0.07	0.06	0.07	0.06	0.10

Table 4: Effect of sterility mosaic disease on sugar content (mg/g fresh weight) in different genotypes of pigeonpea

Genotypes	Sugars																	
	30 DAS						60 DAS						90 DAS					
	Healthy			Infected			Healthy			Infected			Healthy			Infected		
	TS*	RS	NRS	TS	RS	NRS	TS	RS	NRS	TS	RS	NRS	TS	RS	NRS	TS	RS	NRS
Bahar (R)	3.82	2.34	1.48	3.95	2.44	1.51	3.53	2.33	1.18	3.65	2.43	1.22	3.39	2.26	1.13	3.52	2.34	1.18
GRG-177 (MR)	3.75	2.29	1.46	3.86	2.37	1.49	3.37	2.25	1.12	3.54	2.36	1.18	3.19	2.22	0.97	3.36	2.35	1.01
GRG-811 (MR)	3.65	2.27	1.38	3.88	2.43	1.45	3.39	2.20	1.19	3.59	2.41	1.18	3.24	2.10	1.14	3.41	2.29	1.12
Maruti (S)	2.20	1.61	0.58	2.42	1.63	0.79	2.03	1.60	0.43	2.23	1.62	0.61	1.89	1.45	0.44	2.19	1.60	0.59
TS-3R (S)	2.24	1.74	0.50	2.50	1.89	0.61	2.12	1.72	0.40	2.33	1.81	0.52	1.91	1.52	0.39	2.20	1.50	0.70
S.Em ±	0.04	0.01	0.01	0.03	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.01	0.01	0.02	0.02	0.02	0.01	0.01
C.D. at (1%)	0.16	0.05	0.04	0.11	0.08	0.07	0.05	0.05	0.04	0.15	0.06	0.06	0.07	0.04	0.10	0.06	0.05	0.03

(R) - Resistant, (MR) - Moderately resistant, (S) - Susceptible, DAS: Days after sowing TS: Total sugars, RS: Reducing sugars, NRS: Non reducing sugars,

Table 5: Effect of sterility mosaic disease on peroxidase activity in different genotypes of pigeonpea

Genotypes	Peroxidase (PO)(Changes in absorbance 470 nm/min/mg protein)					
	30 DAS		60 DAS		90 DAS	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Bahar (R)	3.831	3.925	3.835	3.900	3.837	3.898
GRG-177 (MR)	3.621	3.821	3.692	3.842	3.697	3.840
GRG-811 (MR)	3.520	3.70	3.595	3.754	3.690	3.750
Maruti (S)	2.012	2.256	2.220	2.260	2.225	2.205
TS- 3R (S)	2.150	2.345	2.29	2.450	2.350	2.155

Table 6: Effect of sterility mosaic disease on polyphenoloxidase activity in different genotypes of pigeonpea

Genotypes	Polyphenoloxidase (PPO)(Changes in absorbance 420 nm/min/mg protein)					
	30 DAS		60 DAS		90 DAS	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Bahar (R)	0.38	0.39	0.62	0.72	0.59	0.65
GRG-177 (MR)	0.29	0.31	0.46	0.69	0.44	0.52
GRG-811 (MR)	0.31	0.33	0.58	0.70	0.56	0.64
Maruti (S)	0.27	0.41	0.37	0.54	0.32	0.44
TS- 3R (S)	0.28	0.50	0.42	0.57	0.43	0.52

(R) - Resistant, (MR) - Moderately resistant, (S) - Susceptible, DAS: Days after sowing

Shilpashree (2013) and Ranchana *et al.* (2015) .Increase in protein contents observed in infected pigeonpea leaves may also be correlated with respiration, rapid respiration probably helps in the synthesis of more amino acids. In the present study healthy leaves of resistant genotypes recorded more of soluble protein than the susceptible ones. Rao *et al.* (1989) stated that the increased protein content in virus infected plants due to increased activity of RNA synthetase or RNA polymerase. These results are in similar with the findings of Sinha and Shrivastava (2010); Sanjay and Alok (2010) and Ashfaq *et al*. (2010).

The sterility mosaic infected plant have got higher level of total

Table 7: Effect of sterility mosaic disease on phenylalanine ammonia lyase activity in different genotypes of pigeonpea

Genotypes	Phenylalanine ammonia lyase (PAL)(nmol trans- cinnamic acid/ hr/mg protein)					
	30 DAS		60 DAS		90 DAS	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
Bahar (R)	18.94	21.11	23.34	26.52	23.45	27.72
GRG-177 (MR)	16.78	18.05	20.92	24.23	23.10	23.39
GRG-811 (MR)	17.82	19.10	21.24	25.35	21.32	24.56
Maruti (S)	13.56	15.59	15.75	16.82	17.89	16.52
TS- 3R (S)	14.68	16.72	15.79	18.84	15.92	17.95

(R) - Resistant, (MR) - Moderately resistant, (S) - Susceptible, DAS: Days after sowing

sugars may be due to a stimulated respiration in sterility mosaic infected plant, this increased rate of respiration demanding large amount of substrate is responsible for breakdown of carbohydrate into simple sugars by increase activity which will convert starch into simple sugars resulting in increased level of sugars in sterility mosaic infected plant and also the accumulation of more reducing sugars in infected plants may be due to the response of pathogen stress as reported by Haq *et al.* (2011) and Chavan and Suryvanshi (2014).

Peroxidase is one of the first enzymes responding and providing fast defense against plant pathogens. PO participates in a variety of plant defense mechanisms in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses. The cell wall of plants appears to be a major site for defense related peroxidase polymerization reactions such as lignification, suberization and cross-linking of structural cell wall proteins (Singh *et al*., 2014). The increased PO activity observed in the present study may be triggered by cellular damage caused by virus replication. These results are in an agreement with Rathi *et al*. (1986); Clarke *et al*. (2002) and Karthikeyan *et al*. (2007) who also observed significant increase in peroxidase activity.

PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules, thus creating an unfavorable environment for pathogen development. Upon infection with SMD, the only appreciable change recorded was the activity of PPO in susceptible variety where it increased considerably after 30 days. In the resistant variety its activity was practically unaffected following inoculation. Total soluble phenols together with PPO play a role in resistance to viral pathogens. The results are in agreement with the few other investigations Anuradha *et al*. (2015) and Rathi *et al.* (1986).

PAL is an enzyme of the general phenyl propanoid metabolism and controls a key point in the biosynthetic pathways of flavanoid phytoalexins, which are antimicrobial compounds. Results of PAL activity in the sterility mosaic virus infected pigeonpea leaves in comparison with healthy leaves indicated higher activity in virus inoculated plants compared to the healthy plants. The activity was found to increase at all stages in case of both healthy and infected plants. These results are similar in findings of Patel *et al*. (2015) and Sindhu (2001) reported an enhanced PAL activity in black eye cowpea mosaic virus inoculated cowpea plants compared to healthy.

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