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Biochemical Changes in Groundnut (*Arachis hypogaea L.*) Infected by Stem and Pod Rot Disease caused by *Sclerotium rolfsii* Sacc

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Peroxidases (POD), poly phenol oxidases (PPO) and phenyl alanine ammonia lyase (PAL) from healthy and *Sclerotium rolfsii* inoculated stems of 6 genotypes of groundnut were estimated after 3 days of inoculation. The activity of PO, PPO and PAL began from 3 rd DAI and gradually increased up to 6 th DAI and thereafter declined. PO, PPO, and PAL activity was more in infected stem sample compared to healthy stem. Among the genotypes resistant and moderately resistant genotypes recorded maximum PO, PPO and PAL activity whereas susceptible genotypes recorded less activity. Further, biochemical constituents of healthy and stem rot infected tissues of different genotypes of groundnut were evaluated. The results revealed that total sugar content was more in resistant genotypes (12009 and 17110), moderately resistant genotypes (17159 and 17169) when compared to highly susceptible genotypes (TMV-2 and KRG-1). Further, it is also found that healthy stems of groundnut recorded more sugar, reducing and non reducing sugar content compared to diseased stems. Phenol and protein contents in healthy and infected stems varied among genotypes. Phenol and protein contents were more in infected stem as compared to healthy stem. Further, resistant and moderately resistant genotypes showed more contents of phenol and protein than susceptible genotypes.

Keywords: Groundnut, *Sclerotium rolfsii*, Biochemical constituents, Defense enzymes.

Groundnut (*Arachis hypogaea* L.) is one of the important oil seed crop of India, growing in the semi-arid tropics. Diseases of groundnut reduce yield and quality and increase the cost of production wherever the crop is grown (Wynne *et al.*, 1991). Among the groundnut diseases, stem and pod rot caused by *Sclerotium rolfsii* Sacc. is emerging as a major problem and has become an economically important soil borne pathogen. This disease causes severe damage and yield losses over 25% have been reported (Mayee and Datar 1988). Its soil borne nature and scarcity of resistant germplasm make its management a challenge for pathologists across the globe. Like many plant species, groundnut employs a diverse array of defenses that minimizes losses during pathogen attack. Besides pre existing physical and chemical barriers, a variety of defense mechanisms are activated upon pathogen attack (Huang *et al.*, 2008). Biochemical changes in many plant-pathogen interactions are accompanied by the rapid increase in phenolic compounds and related enzymes, often termed the hypersensitive response (Mondal *et al.*, 2012). It is revealed from certain studies on biochemical changes during pathogenesis that certain defense biomolecules such as phenols, sugars as well as enzymes like peroxidase, polyphenols are formed to increase in levels so as to after resistance against the pathogen (Jiang *et al.*, 2009). Such changes can be attributed

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to a variety of mechanisms of defense as exhibited by the host during pathogenesis (Jayaraj *et al.*, 2010). Different types of chemical changes in infected host tissues have been reported in many host-pathogen systems but there is no report seems to be available for groundnut. However, changes in these biochemical parameters in the genotypes of groundnut and their associated stem and pod rot resistance are unknown. Thus, the objective of the present work was to study some biochemical parameters and defense enzyme activity in groundnut infected by *Sclerotium rolfsii.*

MATERIALS AND METHODS

Enzyme assay

Healthy and infected seedlings of groundnut were taken and different enzymes *viz*., peroxidase and polyphenol oxidase and phenylalanine ammonia lyase were assayed at 4 different stages (0, 3, 6 and 9 days) to know about their role in defense mechanism of groundnut plants against stem rot disease.

Assay of peroxidase

The peroxidase activity was assayed spectrophotometrically (Hartee, 1955).

Preparation of enzyme extract

One gram of plant sample was homogenized in 3 ml of 0.1 M phosphate buffer, pH 6.5 at 4 °C. This mixture was filtered through 4 layer muslin cloth. The filtrate was centrifuged at 12000 rpm at 4 °C for 20 minutes. The supernatant was collected and used for estimation of peroxidase activity.

Assay

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 °C). The change in absorbance was recorded at 470 nm at a time interval of 30 sec. upto 3 min in Hitachi U-2900 spectrophotometer. The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance at 420 nm min/g/ on fresh weight basis (Hammerschmidt *et al*., 1982).

Assay of polyphenol oxidase (PPO)

The polyphenol oxidaseactivity was determined as per the procedure given by Mayer *et al.* (1965).

Preparation of enzyme extract

One gram of plant sample was homogenized in 5 ml of 0.1M phosphate buffer, pH 7.0 at 4 °C. This mixture was filtered through 4 layer muslin cloth. The filtrate was centrifuged at 10000 rpm at 4 °C for 20 minutes. The supernatant was collected and used for estimation of polyphenol oxidase activity.

Assay

One gram of leaf and roots were used for phenol oxidase estimation: the reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 7.0) and 500ìl of the enzyme extracts. To start the reaction, 500ìl of 0.01 M catechol was added. The change in absorbance was recorded at 495 nm at a time interval of 30 sec. upto 3 min in Hitachi U-2900 spectrophotometer. The polyphenol oxidase activity was expressed as changes in absorbance at 495 nm/min/g fresh weight of tissue.

Assay of Phenylalanine Ammonia Lyase (PAL)

PAL activity was determined as the rate of conversion of L-phenyl alanine to trans-cinnamic acid at 290 nm as per the method described by Ross and Sederoff (1992).

Preparation of enzyme extract

one gram of sample was homogenised with 5 ml of 0.1 M ice cold sodium borate buffer (pH 8.8). The homogenate was filtered through 4 layer muslin cloth. The filtrate was centrifuged at 15000 rpm at 4 °C for 20 min. The supernatant was collected and used for estimation of PAL activity.

Assay

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 reaction was arrested by adding 0.5 ml of 1M TCA and incubated at 37 °C for 5 min. The blank contains 0.4 ml of crude enzyme extract and 2.7 ml of 0.1M borate buffer (pH 8.8) and absorbance was measured at 290 nm in Hitachi U-2900 spectrophotometer. Standard curve was drawn with graded amounts of cinnamic acid dissolved in acetone. The enzyme activity was expressed as ìM of trans-cinnamic acid/ min/g fresh weight of tissue.

Biochemical constituents

A pot experiment was conducted in glasshouse conditions. The experiment was carried out in a randomized complete block design (RCBD) with 3 replications. Seeds of genotypes were sown

in plastic pots of 5" diameter filled with sterilized soil. There were five seeds per pot for each genotype and were sown equi-distantly at a depth of 4 cm. The stem rot pathogen, *S. rolfsii* that was multiplied on sorghum grains was added to the pots at 30 DAS by spreading the inoculum on surface of soil. After 20 days inoculation stem rot infected plants were collected for biochemical studies.

For assaying biochemical constituents healthy and infected tissues of genotypes of groundnut were analyzed for contents of various biochemical constituents *viz.*, total sugars, reducing sugars, non-reducing sugars, phenols and protein to know their possible role in imparting disease resistance. For estimation of sugars and phenols both healthy and diseased samples were used. Ethanol extraction of plant material was made by following the method of Mahadevan *et al*. (1965). Ethanol extraction was used for estimating phenols and sugars. Reducing sugars were estimated by method of Nelson (1944), total sugars was calculated by the method of Inman (1962) and Nelson (1944). And non-reducing sugars were calculated by subtracting the reducing sugars from total sugar content. Total phenols were estimated as per Bray and Thorpe (1954). The estimation of proteins was done as per method developed by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Enzyme assay

Enzyme assay results revealed that increase in the activity of peroxidase (PO) began from 3rd DAI and gradually increased up to 6 th DAI and thereafter declined (Table 1). Our results suggest that suppression of peroxidase was found to be one of the important factor responsible for the successful pathogenesis in groundnut *S. rolfsii* system. Similarly, Reddy and Sireesha (2013) showed increased levels of peroxidase activity in groundnut infected with *S. rolfsii*. PPO catalyzes the last step in the biosynthesis of lignin and other oxidative phenols. PPO activity began from 3 DAI and reached its peak at 6 DAI and reduction of its activity was observed 9 DAI (Table 2). This finding has close similarity with rice-*Rhizoctonia* interacting system (Mondal *et al.*, 2012). Hence these results indicate that the oxidative enzyme converts phenolic compounds of plants to polyphenols and quinones which are toxic substances to the extracellular enzymes produced by the pathogens. PAL is an enzyme of the general phenyl propanoid metabolism and controls a key branch point in the biosynthetic pathways of flavonoid phytoalexins which are antimicrobial compounds. In this present study, the highest PAL activity was found at 6 DAI and thereafter decrease in PAL activity was observed at 9 DAI (Table 3). Reduction of phenyl propanoid levels by co-suppression of PAL increases disease susceptibility (Maher *et al.*, 1994).

Biochemical constituents

An insight into the data (Table 4) showed that infected samples of different genotypes showed less total, reducing and non reducing sugar content compared to healthy samples with respect to genotypes total sugar content was more in resistant genotypes (12009 and 17110), moderately resistant genotypes (17159 and 17169) when compared to highly susceptible genotypes (TMV-2 and KRG-1). Reducing and non reducing sugar content in healthy and stem rot infected plants followed the same trend of results as observed in total sugars. These results are in agreement with the findings of Verma and Singh (1994) and Sultana *et al*. (1998) who reported higher amount of sugars in healthy plant parts as compared to diseased ones. Reddy and Sireesha (2013) reported that healthy plants recorded higher amount of sugars than diseased plants. The reduction in sugar content after infection may be due to rapid hydrolysis of sugars during pathogenesis through enzymes (hydrolases) secreted by pathogens and subsequent utilization by pathogens for their development. In our studies phenol contents in healthy and infected stems varied among genotypes. The content ranged from 0.30 to 0.48 mg/g fresh wt. in healthy stems while it was 0.39 to 0.57 mg/g fresh wt. in infected stems of different genotypes. The highest phenol contents of 0.48 and 0.47 mg/g fresh wt. were recorded in the healthy groundnut stems of resistant genotypes, 12009 and 17110, respectively (Table 5). While, same genotypes showed less contents (0.57 and 0.69 mg/g fresh wt., respectively) in stem rot diseased plants. Resistant genotypes recorded slightly more phenol content compared to moderately resistant genotypes. The high phenol content in resistant

genotypes may be due to more sugar as it acts as precursor for synthesis of phenolics. This is in agreement with the findings of Ekbote and Mayee (1983). Similarly Ganguly (1995) and Singh (2000) who observed higher phenol content in diseased plant parts of resistant varieties. Bhagat and Chakraborty (2010) reported that the total phenol contents decreased in susceptible varieties and increased in resistant varieties following inoculation with S. *rolfsii*. The post-infection increase in phenolic content could be due to a number of factors including enhancement of

Table 1. Assay of peroxidase enzyme in resistant, moderately resistant and highly susceptible genotypes of groundnut

S. No.	Genotype	Disease reaction	Peroxidase (Changes in absorbance 470 nm /min/ mg protein)							
			0 DAI		3 DAI		6 DAI		9 DAI	
			Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1	12009	R	1.68	1.68	2.56	2.68	2.62	2.74	2.61	2.65
2	17110	R	1.90	1.91	2.59	2.95	2.87	3.04	2.87	2.98
3	17159	MR	1.57	1.57	2.20	2.44	2.26	2.46	2.27	2.39
4	17169	MR	1.59	1.59	2.37	2.50	2.53	2.80	2.50	2.51
5	KRG1	HS	0.96	0.96	1.98	2.27	2.18	2.47	2.18	2.44
6	TMV2	HS	1.08	1.08	1.95	2.24	2.19	2.44	2.12	2.40

R: Resistant;MR: Moderately resistant;HS: Highly susceptible

Table 2. Assay of polyphenol oxidase enzyme in resistant, moderately resistant and highly susceptible genotypes of groundnut

S. No.	Genotype	Disease reaction	Polyphenol oxidase (Changes in absorbance 420 nm /min/ mg protein)							
			0 DAI		3 DAI		6 DAI		9 DAI	
			Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1	12009	R	0.24	0.24	0.38	0.41	0.59	0.72	0.59	0.68
2	17110	R	0.25	0.25	0.32	0.44	0.62	0.71	0.61	0.59
3	17159	MR	0.13	0.13	0.29	0.31	0.44	0.75	0.42	0.70
4	17169	MR	0.19	0.19	0.30	0.48	0.59	0.76	0.57	0.62
5	KRG1	HS	0.13	0.12	0.31	0.41	0.36	0.57	0.31	0.44
6	TMV2	HS	0.15	0.15	0.35	0.50	0.41	0.54	0.42	0.52

R: Resistant;MR: Moderately resistant;HS: Highly susceptible

Table 3. Assay of phenylalanine ammonia lyase enzyme in resistant, moderately resistant and highly susceptible genotypes of groundnut

S. No.	Genotype	Disease reaction	Phenylalanine ammonia lyase (nmol trans-cinnamic acid/ hr/ mg protein)							
			0 DAI		3 DAI		6 DAI		9 DAI	
			Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1	12009	R	19.44	19.44	22.31	23.78	47.40	49.05	34.32	39.87
2	17110	R	19.14	19.14	22.59	23.15	26.08	33.05	25.14	28.1
3	17159	MR	18.87	18.87	26.86	33.54	45.36	52.34	45.10	49.76
4	17169	MR	18.50	18.50	21.58	22.42	23.85	25.04	21.57	23.89
5	KRG1	HS	16.19	16.19	19.93	20.88	21.98	33.08	24.16	27.97
6	TMV2	HS	18.65	18.65	21.91	22.69	24.16	32.20	22.59	24.91

R: Resistant;MR: Moderately resistant; HS: Highly susceptible

Table 4. Biochemical analysis for sugar content in resistant, moderately resistant and highly susceptible genotypes of groundnut

Sl. No.	Genotype	Disease reaction	Total Sugars (mg/g fresh wt)		Reducing sugars (mg/g fresh wt)		Non reducing sugars (mg/g fresh wt)	
			Healthy	Infected	Healthy	Infected	Healthy	Infected
1	12009	R	9.67	9.00	5.55	4.83	4.22	4.17
2	17110	R	11.35	10.44	7.05	6.69	4.30	3.75
3	17159	MR	8.70	7.91	4.58	4.28	4.12	3.63
4	17169	MR	7.68	7.25	4.08	3.95	3.60	3.30
5	KRG1	HS	6.87	6.37	4.24	3.78	2.63	2.59
6	TMV2	HS	6.07	5.80	3.73	3.62	2.34	2.18

R: Resistant; MR: Moderately Resistant; HS: Highly susceptible

Table 5. Phenol and protein analysis in resistant, moderately resistant and highly susceptible genotypes of groundnut

S. No.	Genotype	Disease reaction	Phenol (mg/g fresh wt)		Protein (mg/g fresh wt)	
			Healthy	Infected	Healthy	Infected
1	12009	R	0.48	0.57	5.12	7.02
2	17110	R	0.47	0.69	4.92	5.46
3	17159	MR	0.47	0.53	4.64	4.82
4	17169	MR	0.46	0.49	4.73	4.92
5	KRG1	HS	0.35	0.44	3.61	3.67
6	TMV2	HS	0.30	0.39	3.78	4.03

R: Resistant; MR: Moderately resistant; HS: Highly susceptible

synthesis, translocation of phenolics to the site of infection and hydrolysis of phenolic glycosides by fungal glycosidases to yield free phenols and the increase in level of phenolic compounds in infected leaves may be due to translocation of phenolics to the site of infection (Parashar *et al*., 1987 and Jabeen, *et al.*, 2009).

Whereas highest protein content of 7.02 and 5.46 mg/g fresh wt was recorded in diseased stems of resistant genotypes such as 12009 and 17110 (Table 2.) While, corresponding healthy stems of same genotypes showed lesser protein content (5.12 and 4.92 mg/g fresh wt, respectively). However, resistant and moderately resistant groundnut genotypes recorded more protein contents than highly susceptible genotypes. Similarly, Kaur and Dhillon (1990) observed an increased protein content of groundnut infected with *Cercospora personatum*. Higher total protein content in infected pods of T-9 variety of *Vigna mungo* susceptible to leaf crinkle virus was observed by Malik *et al*. (2002). It is a well-known fact that enzymes are proteins and the increased synthesis of proteins during the infection may be due to activation of enzymes which are essential for the synthesis of various defense chemicals (Vidyasekaran, 2001).

CONCLUSION

In conclusion biochemical defense is a powerful mechanism in plants which helps in resistance against disease. Accumulation of these substances provides workable protection against diseases. The concentration of total sugars, reducing sugars, non reducing sugars, phenols, poly phenol oxidases and peroxidises varies in healthy and diseased plant as well as in different

genotypes. This work it clear that biochemical defense is triggered in response to pathogen attack however, they were unable to totally inhibit the pathogen because of more virulence in pathogen. Further, individual components of the defense pathway should be enlightened for a broad spectrum resistance.

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