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Biochemical traits in pearl millet (*Pennisetum glaucum*) against downy mildew disease

SARANG S SAPRE[1], VINAY PATEL[2], RAJIVKUMAR[3], Y M ROJASARA[4], K S RAO[5], J S PATEL[6] and J G TALATI[7]

B A College of Agriculture, Anand Agriculture University, Anand, Gujarat 388 110

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Pearl millet (*Pennisetum glaucum* L.) is the fifth important cereal crop in the world and the fourth important food crop in India after rice, wheat and sorghum. The crop is grown on the poorest soil and under harsh climatic conditions where no other crop can grow (Singh 1995). The major constraints to the productivity of crop include lesser area under hybrids and prevalence of different biotic and abiotic stresses. Downy mildew disease caused by *Sclerospora graminicola* (Sacc.) Schroet is the most serious constraint in realizing higher production of pearl millet causing upto 80% loss in grain yield (Arun *et al*. 2010). In India, the monetary loss due to a single epidemic of downy mildew is calculated to be £7.8 million (Deepak *et al*. 2007). Plant defence mechanisms operate through the activation of multiple defence proteins. In fungal plant pathogenesis, enzymes play a crucial role through external and internal interactions to resist the development of fungal pathogens. Enhanced biosynthesis and activity of some enzymes is one of the most important processes in plant defence and in some specific plant-fungal pathogen interactions. The presence or activities of enzymes can be used as biochemical markers for the degree of resistance or susceptibility (Raj *et al*. 2006).

Peroxidases participate in a variety of plant defence mechanisms in which H_2O_2 is often supplied by an oxidative burst, a common event in defence responses. They belong to a large multigene family, and participate in a broad range of physiological processes, such as lignin and suberin formation, cross-linking of cell wall components, and synthesis of phytoalexins, or participate in the metabolism of ROS (reactive oxygen species) and RNS (reactive nitrogen species), both switching on the hypersensitive response (HR), a form of programmed host cell death at the infection site associated with limited pathogen development (Almagro *et al*. 2009). A wide range of phenolic compounds are synthesized in plant tissues during normal growth and development via the phenyl propanoid pathway. These compounds are building blocks for cell wall structure and plant pigment production and serve as protection from ultra-violet light and as a defence against pathogens (Singh and Singh 1995). An important first line in plant defence against infection is provided by the very rapid synthesis of phenolics and their polymerization in the cell wall (Matern and Kneusel 1988). Silicon enhances resistance to diseases by acting as a physical barrier. It is deposited beneath the cuticle to form a cuticle-Si double layer. This layer can mechanically impede penetration by fungi and thereby disrupt the infection process (Ma and Yamaji 2006). Lignin is a major component of cell walls of vascular plants, was shown to accumulate in cell wall appositions and surrounding areas and is thus considered a first line defense against successful penetration of invasive pathogens. Lignification renders the cell wall more resistant to mechanical pressure applied during penetration by fungal appressoria as well as more water resistant and thus less accessible to cell wall-degrading enzymes (Vance *et al*. 1980). So keeping in view the above points this study was conducted to screen the pearl millet entries against downy mildew disease, using biochemical and histochemical parameters.

Eighteen entries were selected from PMDMVN (Pearl millet downy mildew virulence nursery) programme, grown in a downy mildew sick plot under epiphytotic conditions during *kharif* 2009 at RRS (Regional research station).

All the entries were grown in downy mildew sick plot, under epiphytotic conditions during *kharif* 2009, at Regional research station, Anand. Field screening was done using the infector row technique (Singh *et al.* 1993), which involved

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[1] PhD Scholar (e mail: srngsapre27@gmail.com), [2] PhD Scholar (e mail: vinayphd@gmail.com), [3] Assistant Professor (e mail: rajivnrc@yahoo.co.in), [4] Assistant Research Scientist (e mail: yogeshrojasara@gmail.com), [5] Professor (e mail: kayesrao@yahoo.com), [6] Professor and Head (e mail: jspatel@aau.in), [7] Professor (e mail: jgtalati@yahoo.co.in). [2,5] B R Doshi School of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat 388 120

sowing of infector row with a susceptible cultivar 2-3 weeks before sowing of the test entry. Conditions of humidity were maintained with frequent irrigation at early stages. The test entries were sown in 2 replications between the infector lines. The pearl millet entries were scored for disease incidence on 30 and 60 DAE (days after emergence). The disease incidence was based on observations taken at 60 DAE and was calculated as:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

Seedlings (150 mg) were homogenized in a pre-chilled mortar and pestle with 2 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.2) with the addition of 1% polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 15minutes at 4°C and the supernatant was used for the assay of peroxidase. The reaction mixture contained 2.99ml of 0.003% H_2O_2 (substrate) in 0.1M phosphate buffer (pH 6.0), 0.01% orthodianisidine dye (freshly prepared, dissolved in methanol). The reaction was initiated by the addition of 10µl of enzyme extract. The change in colour of oxidized dye was read at 460nm up to 1 minute at the interval of 15 seconds. The enzyme activity was expressed as change in OD/min/g fresh weight (Bisswanger 2011).

Phenol was estimated using Folin ciocalteau reagent method described by Malik and Singh, (1980).

Thirty days old pearl millet leaves were collected from each entry, dried at 60°C for 48 hr, ground and passed through 60-mesh sieve. Then sample pretreatment was done by Autoclave induced digestion method (AID) (Elliot and Snyder, 1991). Samples of plant tissue weighing 100mg were wetted with 2ml of 30% H_2O_2 in 100ml polyethylene tubes previously rinsed with 0.1M NaOH and double distilled water. 3ml of 50% NaOH was added to each tube with gentle vortexing. The tubes were individually covered with loose-fitting plastic caps. The rack of tubes was placed in an autoclave at 138 kPa for 1 hr. After atmospheric pressure was reached, the tubes were removed and the contents brought to 50ml with double distilled water.

For spectrophotometric analysis 5ml of sample solution was transferred to 50ml tube, then 30ml 20% acetic acid and 10ml ammonium molybdate solution (54g/l, pH 7.0) were added. It was kept for 5minutes and then 5ml 20% tartaric acid and 1ml reducing solution were immediately added. [The reducing solution was made by mixing solution A (2g of Na_2SO_3 and 0.4g 1-amino -2- naphthol 4-sulfonic acid in 25ml of distilled water) and solution B (25g of $NaHSO_3$ dissolved in 200ml of distilled water) and diluting to 250ml. It was stored in a tightly stoppered plastic bottle in the dark.] The total volume was adjusted to 50ml with 20% acetic acid. Following the addition of all the reagents, the samples were mixed well and allowed to stand for 30 minutes. The colour was read at 650nm (Wei-Min *et al*. 2005).

Silicon standard was prepared with sodium silicate, which contained 1 mg/ml silicon. Further steps were followed as for sample determination. [Note: All the plasticwares were rinsed with 0.1 M NaOH.]

The amount of phenol and silicon present in the sample was calculated as:

Phenol/Silicon (mg/g) = Sample OD × Standard OD × Dilution factor

Fifty days old leaves were taken from both resistant and susceptible entries grown in sick plot. Immediately after collection samples were fixed in formaldehyde acetic acid. Transverse hand sections were taken with sharp blade. Sections were stained with phloroglucinol/HCl reagent (Johansen 1940) for localization of lignin. Photomicrographs were taken with the help of Carl Zeiss digital camera attached with the microscope.

All the biochemical parameters were analyzed in three replications. The data obtained were analysed using a completely randomized design (CRD). Analysis of Variance (ANOVA), appropriate for the design, was carried out to determine the significance of differences among the entries for each of the characters under study. Correlation was also performed between disease incidence and biochemical traits. t-test was performed for testing the significance of the difference between means of resistant and susceptible groups.

Table 1 Downy mildew disease incidence in the field grown pearl millet entries

Entries	Total plants	DM infected plants (no.) at		DM incidence (%)*	Rating
		30 DAE	60 DAE		
700651	45	6	8	17.78	S
852B	49	23	45	91.84	HS
IP-18293	30	0	3	10.00	R
IP-18294	54	3	5	9.26	R
7042-S	64	35	40	62.50	HS
843-22B	57	0	0	0.00	HR
ICML-22	24	4	18	75.00	HS
HHB 67-2 improved	41	0	0	0.00	HR
JMSB-101	62	14	22	35.48	S
RHRB-58	67	0	0	0.00	HR
ICMR-312	32	1	1	3.13	R
PPMI-301	23	1	4	17.39	S
HTP 94/54	62	0	0	0.00	HR
81B	53	22	31	58.49	HS
88004B	56	0	0	0.00	HR
IP5272-1	61	7	23	37.70	S
RB-13	53	5	13	24.53	S
MRB-8	55	11	15	27.27	S

DAE, Days after seedling emergence, *Based on 60 DAE observation; HR, highly resistant, R, resistant, S, susceptible and HS, highly susceptible

The computer package SPSS (version 10.0) was used for correlation and t test.

The list of 18 entries and their disease incidence data recorded at 30 and 60 DAE stage is given in Table 1. Based on the percentage of disease incidence, the entries were grouped into highly resistant (843-22B, HHB 67-2 improved, RHRB-58, HTP94/54, 88004B) having no disease incidence, moderately resistant (IP 18293, IP 18294, ICMR-312) having disease incidence 1-10%, susceptible (700651, JMSB-101, PPMI-301, IP 18272-1, RB-13, MRB-8) having disease incidence 11-40% and highly susceptible (852B, 7042S, ICML-22, 81B) with disease incidence greater than 40%.

Peroxidase activity in plants of 30 and 50 DAE are shown in (Fig 1). It was found that characterization of genotypes based on peroxidase differed at two stages (Table 2). Concentration of peroxidase was less in resistant entries as compared to susceptible entries at 30 DAE, but the concentration of peroxidase increased with growth of plants being higher in resistant entries. At 30 DAE stage, peroxidase activity was found lower in higher resistant and resistant entries (37.66 to 157.33) exception HHB 67-2 improved and IP 18294 which showed slightly higher activity. Whereas in highly susceptible and susceptible entries the activity was higher (168.66 to 603.00) exception JMSB-101 showing slightly lower activity. The above results were in accordance to Arora *et al*. (1986) but contradicted with Thukral *et al*. (1986). At 50 DAE stage, peroxidase activity was found higher in highly resistant and resistant entries (386.50 to 685.50) except RHRB-58 which showed slightly lower activity. In comparison to resistant entries highly susceptible and susceptible entries showed lower activity (189.33 to 685.50) except JMSB-101 which showed slightly higher activity. The results at this stage matched with those obtained by Thukral *et al*. (1986). This shows that unlike the resistant entries, susceptible entries failed to show early reaction against

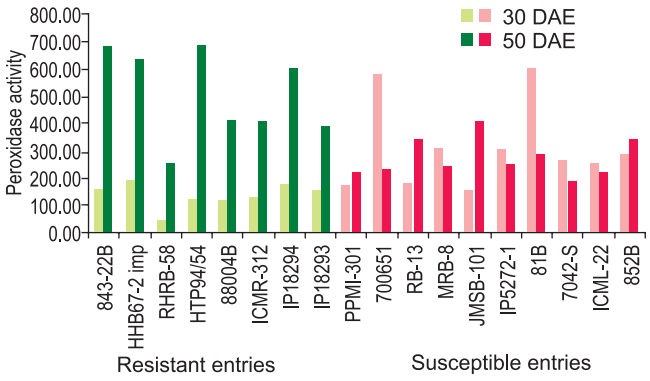

Fig 1 Peroxidase activity (Δ OD at 460 nm min/g/fw) in pearl millet entries

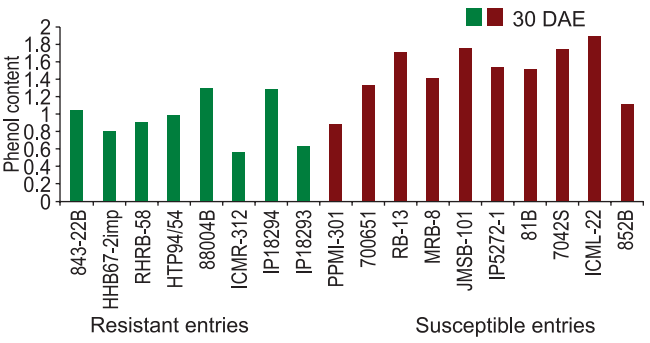

Fig 2 Total phenol content (mg/g) in pearl millet entries

Table 2 Peroxidase, total phenol and silicon content in pearl millet entries

Entries	Peroxidase activity (Δ/min/g fw)		Total Phenol (mg/g)	Silicon content (mg/g)
	30 DAE	50 DAE	30 DAE	30 DAE
700651***	579.00	230.33	1.33	64.78
852B****	283.66	341.66	1.12	57.19
IP-18293**	152.66	386.50	0.63	68.68
IP-18294**	172.33	600.66	1.27	72.88
7042-S****	261.66	189.33	1.74	29.61
843-22B*	157.33	685.50	1.04	80.99
ICML-22****	254.33	218.33	1.90	35.68
HHB 67-2 improved*	188.33	635.50	0.79	69.45
JMSB-101***	153.66	403.00	1.77	52.27
RHRB-58*	37.66	254.00	0.90	64.47
ICMR-312**	129.33	410.40	0.56	63.23
PPMI-301***	168.66	215.50	0.89	67.04
HTP 94/54*	114.00	682.50	0.99	75.40
81B****	603.00	286.33	1.51	44.33
88004B*	114.00	409.00	1.31	70.17
IP5272-1***	305.33	249.33	1.54	63.38
RB-13***	178.33	340.00	1.71	54.62
MRB-8***	307.67	238.00	1.40	57.75
SEm	16.36	17.00	0.02	1.78
CD	33.19	34.48	0.04	3.62
CV%	8.67	5.53	2.13	3.60

*Highly resistant, **resistant, ***susceptible, ****highly susceptible

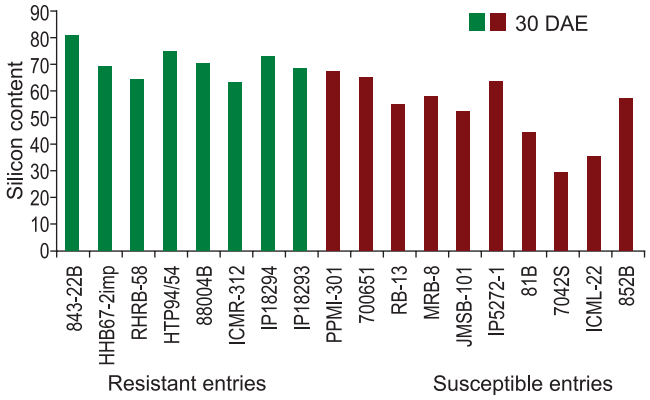

Fig 3 Silicon content (mg/g dry weight) in pearl millet entries

the pathogen attack and this may be the reason for establishment of pathogen in host and cause of disease.

Total phenol contents was higher in leaves of highly susceptible and susceptible pearl millet entries as compared to highly resistant and resistant pearl millet entries at 30 DAE stage as shown in (Fig 2). The present results for total phenols also agree with those in downy mildew infected plants (Kumhar *et al.* 1990, Shekhawat and Arya 1979 and Alagianaglingam *et al.* 1978). In highly susceptible and susceptible entries it ranged from 1.33-1.90 mg/g except 852B which had 1.12mg/g. In highly resistant and resistant entries it ranged from 0.56-1.31 mg/g. Conversion of phenols to quinines produced defence mechanism in host (Alagianaglingam *et al.* 1978). Higher phenol levels were registered in highly susceptible and susceptible entries. Malformaton and malfunction is result of higher phenolic content (Shekhawat and Arya, 1979). Phenol content showed positive correlation with disease incidence (r=0.59) which was significant at 0.01 level (Table 3).

Table 3 Correlation between biochemical traits and disease incidence

Biochemical parameter	Disease incidence
Peroxidase (30DAE)	0.47*
Peroxidase (50DAE)	–0.56*
Phenol (30DAE)	0.59**
Silicon (30DAE)	–0.78**

*Correlation is significant at 0.05 level (2-tailed), **correlation is significant at 0.01 level (2-tailed)

Silicon accumulated differentially in leaves of entries with varied level of resistance against downy mildew disease (Fig 3). Majority of highly susceptible and susceptible entries had silicon accumulation in the range of 29-44 mg/g and 52-67 mg/g dry weight respectively. Most of the resistant and highly resistant entries showed silicon accumulation ranging from 63-72 mg/g and 69-80 mg/g of dry weight respectively. The level of silicon accumulation indicated the degree of resistance against downy mildew disease. Silicon plays a role in resistance mechanisms through the formation of a physical barrier deposited beneath the cuticle. Silicon deposition mechanically impedes penetration by pathogenic structures and it also acts as modulator of host resistance by activating defence mechanisms. Silicon content showed negative correlation (r=-0.78) with disease incidence at 0.01 level (Table 3). Deepak *et al*. (2008) had presented conclusive evidence for the implication of silicon in the enhanced resistance to *Sclerospora graminicola*.

For peroxidase, total phenol content and silicon content test of significance was performed (Table 4) and it was found that peroxidase at both stages; silicon content showed highly significant differences at 0.01 level amongst the resistant and susceptible entries. But phenol content did not show significant difference amongst these entries.

Table 4 Test of significance between resistant and susceptible groups

Biochemical aspect	Type	Mean	Standard Deviation	SEd	Calculated t
POX (30DAE)	Susceptible	309.53	158.51	50.12	3.03**
	Resistant	133.21	46.92	16.59	
POX (50DAE)	Susceptible	271.18	69.24	21.89	3.71**
	Resistant	508.01	162.74	57.54	
Phenol (30DAE)	Susceptible	1.49	0.31	9.95×10^{-2}	0.96^{NS}
	Resistant	0.94	0.27	9.66×10^{-2}	
Silicon (30DAE)	Susceptible	52.96	12.51	3.96	3.74**
	Resistant	70.66	5.77	2.04	

NS, Non-significant; **significant at 0.01 level

The comparison of lignin deposition in leaf rachis of susceptible and resistant entry is shown in. Leaf rachis of resistant entry showed well developed vascular bundles with thick lignified walls. Pink color showed the presence of lignin in the cell walls of both parenchyma cells and vessel walls. Intensity of color showed the concentration of deposition of lignin in particular tissues. Vessel associated parenchyma cells showed faint pink color compared to vessel walls indicating less lignin. Leaf rachis from the susceptible leaf showed less lignin deposition compared to the resistant entry. Cell walls of parenchyma cells were thinner and not properly lignified. Vessel associated parenchyma cells were found to have equal deposition of lignin as seen in resistant entry. Lower epidermis had one layered thick cuticle. Yellowish orange color indicated the initiation of lignin deposition in the cell wall and middle lamella. Fibres also showed the secondary wall deposition with yellowish orange colour. Leaf rachis from susceptible entry showed very thin cuticle layer with no traces of lignin deposition. Fibres contained very thin secondary wall compared to resistant entry. These results suggest that resistant entry had higher lignin deposition as compared to susceptible ones. The epidermal thickness ranged from 37.5 to 12.5 µm, whereas the cuticular thickness ranged from 2.5 to 7.5 µm. The results were in accordance to those obtained by Yadav and Thakur (2001). Kumudini and Shetty (2002) suggested that lignin deposition was host structural response for cultivar resistance. Raj *et al*. (2012) also reported that induced resistance associated with PGPR against downy mildew disease in pearl millet led to enhanced lignification.

The study of peroxidase activity, phenolic content and silicon content in relation to the downy mildew incidence of 18 pearl millet entries revealed that peroxidase activity at 50 days stage and silicon content at 30 days stage were linearly related to degree of resistance, whereas phenol content and peroxidase activity at 30 days were linearly related to the degree of disease incidence. Test of significance results also showed that peroxidase activity and silicon content were appropriate biochemical traits to differentiate the resistant

and susceptible entries. Higher peroxidase activity in resistant entries can be correlated with higher lignin deposition at 50 days stage. Thus, peroxidases play a key role in scavenging reactive oxygen species as well as participate in physiological process such as lignin formation. Also, silicon plays an important role in plant defence by formation of a physical barrier preventing pathogen invasion.

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SUMMARY

Eighteen pearl millet (*Pennisetum glaucum* L.) entries were screened to find out biochemical traits in relation to downy mildew. Peroxidase activity was determined after 30 and 50 days, whereas phenol, silicon content were estimated at 30 days stage. Results showed that, at 30 days the peroxidase activity was lower in resistant entries and higher in susceptible entries, whereas this trend was reversed at 50 days. Negative correlation was obtained at 50 days between disease incidence and peroxidase activity. Phenol content showed positive correlation with disease incidence but the difference was non-significant amongst resistant and susceptible entries. There was a positive relationship between silicon level in various entries and their degree of disease resistance. In resistant entry lignin deposition was more in vessel walls, cortical parenchyma cells and cuticle layer, while it was very less in susceptible entry at 50 days. Thus, peroxidase activity and silicon content can be useful traits in relation to downy mildew in pearl millet.

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