Variation in metabolites constituent in leaves of downy mildew resistant and susceptible genotypes of pearl millet

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ABSTRACT

In the present investigation biochemical characterization of pearl millet genotypes was carried at pre (45 DAS) and post-infection (57 DAS i.e. 7 days after infection) stage. Total soluble sugar was greater at pre infection than post infection in downy mildew resistant and susceptible genotypes of pearl millet. Total soluble sugar decreased in all genotypes at 7 days after infection (d.a.i.) except in 7042 S in which it increased 4.6 %. However, total soluble sugar was 2-3 folds more in highly susceptible genotypes (J-2296 and 7042 S) compared to resistant genotypes at 7 d.a.i. but it was decreased as compared to pre infection. The total amino acid content of all genotypes whether resistant or susceptible, finally increased as a result of infection. Moreover, susceptible genotypes registered 2-2.5 % higher amino acid, whereas resistant genotypes possessed 6.2-76 % higher amino acid than their constitutive level. Total chlorophyll and carotenoids content did not show any clear cut difference in resistant and susceptible genotypes at pre-infection stage. However, at post-infection stage a significant decrease in chlorophyll and carotenoids content occurred in susceptible genotypes from pre-infection. Amino acid profiling through HPTLC showed sulphur containing amino acids were higher in resistant genotypes. [Physiol. Mol. Biol. Plants 2009; 15(3) : 249-255] E-mail : maheshmahatma@gmail.com

Key words : Metabolites, Downy Mildew, Disease resistant, *Sclerospora graminicola*, *Pennisetum glaucum*

INTRODUCTION

Downy mildew disease of pearl millet (*Pennisetum glaucum* (L.) R.Br.) caused by the oomycetous, biotrophic fungus *Sclerospora graminicola* (Sacc.) Schroter, is one of the most devastating disease of the crop, causing yield losses of 10-60 % in various countries of Asia and Africa (Shetty *et al.* 1995, Deepak *et al.* 2003). Although, biotrophic pathogens rarely kills their host, the resulting loss of nutrients, increased respiration and decreased photosynthesis seriously reduce growth and yields. Disease development is likely to induce substantial changes in the carbohydrate content of host plants and metabolic alterations that may favour fungal development (Hwang and Heitefuss 1986). Differences between resistant and susceptible host plants are mainly related to the rate at which they express structural and chemical defenses (Dang and

Jones 2001), the signal transduction mechanisms involved in compatible and incompatible interactions being largely shared (Tao *et al.* 2003). Considering the above informations, the present study was conducted to characterize pearl millet genotypes resistant and susceptible to *S. graminicola*, on the basis of metabolite constituents in terms of changes in total soluble sugar, total free amino acids and their HPTLC profile, chlorophyll and carotenoids content.

MATERIALS AND METHODS

The seeds of four downy mildew resistance pearl millet genotypes (J-2290, J-2340, J-2405 and J-2480) and four susceptible genotypes (J-2296, J-2477, HB-3 and 7042 S) were procured from Main Millet Research Station, Jamnagar, Junagadh Agricultural University, Gujarat, India. Pearl millet genotypes were raised in net house to collect leaf samples for biochemical analysis. A pot mixture (Alfisol, sand and farm yard manure in a 2:1:1 ratio) with diammonium phosphate at 1 g kg⁻¹ of soil

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was used. Plants were thinned and five plants in each pot (26 x 24 cm/10 kg soil capacity) were kept 10 days after germination. The crop was well irrigated regularly to avoid any physiological stress and to maintain high relative humidity condition.

Inoculum was collected from infected leaves of 7042 S genotypes (national susceptible check for downy mildew), grown in the sick plot. Sporangia produced on the leaves were washed off into cold water (5–6 °C) in a beaker. The concentration of sporangia was adjusted using hemocytometer to 1×10^6 sporangia mL⁻¹. Plants (50 days old) of the eight genotypes were spray inoculated with the *S. graminicola* sporangia suspension.

All biochemical parameters were analyzed at two stages: (i) pre-infection [45 days after sowing (DAS)] and (ii) post-infection [7 days after infection (DAI) i.e. 57 DAS]. Fresh leaf samples for biochemical analysis at both stages were collected from second upper leaf and washed twice with tap water and then with milli Q water. Infected and uninfected leaves without mid rib were used for estimation of metabolite constituents.

The content of total soluble sugar, free amino acids, total chlorophyll was estimated following the methods described by Franscitt *et al.* (1971), Lee and Takahanshi, (1966) and Hiscox and Israelstam (1979), respectively. Total carotenoids content was determined using same extract (used for chlorophyll) and absorbance was recorded at 480 nm. Carotenoid content was calculated as described by Wellburn (1994). The data were recorded from two consecutive experiment in three replication and pooled to obtained average response. Statistical analysis of the results were analysed by simple completely randomized design (CRD) and the critical differences were calculated to assess the significance of treatment means wherever the “F” test was found significance at the 5 %.

Amino acid profiling through HPTLC (High Performance Thin Layer Chromatography)

Amino acids were extracted as described by Martino *et al.* (2003) with some modification. Leaf tissues (1 g) were ground in liquid N₂ to a fine powder that was mixed with 5 ml of 80 % ethanol and left for 5 min in water bath at 70 °C, collected and centrifuged at 5,000 rpm for 10 min. The extraction procedure was repeated 4 times on the pellet. The supernatants were pooled and made upto 25 ml with 80 % ethanol. The supernatants were evaporated in water bath at 70 °C to dryness and

residue was dissolved in 2 ml absolute ethanol. The alcohol extracts were depigmented with petroleum ether.

Sample application

Extracted samples (8 µl) standard amino acid mixtures (5 µl) were sprayed as 1x10 mm bands on an HPTLC aluminium plate 20x10 cm (silica gel 60 F₂₅₄ Merck) by CAMAG Linomat 5 applicator using nitrogen gas. The plates were activated at 100 °C for 20 min before sample application. Each of the (20) individual amino acid standard was sprayed on an HPTLC plate as mixture of 5 amino acids and divided into five group, mixture 1 (DL-alanine, arginine HCl, DL-aspartic acid and L-cystine), mixture 2 (L-glutamic acid, glycine, L-histidine HCl and DL-isoleucine) mixture 3 (L-leucine, L-lysine HCl, DL-methionine and L-proline) mixture 4 (DL-serine, DL-tryptophan, L-tyrosine and DL-valine) and mixture 5 (L-cysteine HCl, L-Hydroxy proline, phenyl alanine and threonine). Standard amino acids obtained from LOBA were used. Individual amino acids were dissolved in milli Q water at concentration of 1 mg/mL. Tyrosine and phenylalanine were dissolved in 0.05 N HCl and tryptophan was dissolved in 0.05 N NaOH.

Separation of amino acids

Solvent system and derivatization was performed as described by Sadasivam and Manickam (1996). The solvent system used was n-butanol : water : acetic acid (5:4:1). The lower aqueous layer was separated using separating funnel and the upper layer was filled in the CAMAG Twin Trough Chamber (20x10 cm) and left overnight for saturation. The plates were placed in chamber to separate amino acid until solvent reached 8 cm from origin.

Derivatization of amino acids

After separation of amino acids, plates were dried at room temperature and derivatized with 0.2 ninhydrin (dissolved in acetone) in a dipping tank. Plates were removed from the dipping tank just after dipping and put into oven at 100 °C for development of bands.

Qualitative and Area % analysis

HPTLC plates were photographed using CAMAG Reporstar 3 system and quantified by CAMAG SCANNER 3 at wave length of 546 nm.

RESULTS AND DISCUSSION

The total soluble sugar varied in resistant and

susceptible genotypes at pre-infection stage (Table-1). The total soluble sugar was up to 2 fold higher in resistant genotypes (J-2340 and J-2405) than susceptible (J-2477 and HB-3) genotypes. The total soluble sugar in resistant as well as susceptible genotypes was greater at pre-infection than post-infection stage. Total soluble sugar decreased in all the genotypes at 7 days after

infection (d.a.i.) except in 7042 S in which it increased 4.6 %. The reduction was more pronounced and significant in resistant genotypes. Reduction in total soluble sugar with the increase in age of crop observed in the present study may be correlated with the results of Kumar and Singh (1996) and Saharan and Saharan (2004).

All the above genotypes were also sown in field to record disease incidence and disease severity record. Highly susceptible genotype 7042 S (national check) was sown on every sixth rows and all eight genotype sown in five intervening rows of every sixth highly susceptible genotype's row. Disease incidence for each genotype was calculated as :

$$100 \times \frac{\text{Diseased Plants}}{\text{Total Plants}}$$

The severity record was taken on a 1-5 rating scale for reaction categories described by Singh *et al.*, 1993.

Where, 1 = No disease symptom

2 = Disease only on the nodal tillers of a plant infected

3 = Less than 50 % of the basal tillers of a plant infected

4 = More than 50 % of the basal tillers of a plant infected and

5 = No productive panicle produced

$$\text{Disease severity} = \frac{Y(1-1) + Y(2-1) + Y(3-1) + Y(4-1) + Y(5-1)}{N \times 4} \times 500$$

Where, Y = The number of plants in each reaction category (severity rating) and

N = The total number of plants in the genotype under test

Table : Downy mildew disease incidence and severity index in field grown pearl millet genotypes

No. and Name of genotypes		Number of plants with downy mildew reaction categories					Disease incidence (%)		Disease severity (%)
		1	2	3	4	5	30 DAS	60 DAS*	
Resistant									
1.	J-2290	100	0	0	0	0	0	0	0.00
2.	J-2340	92	2	0	0	0	0	2	0.50
3.	J-2405	97	3	0	0	0	0	3	0.75
4.	J-2480	100	0	0	0	0	0	0	0.00
Susceptible									
5.	J-2296	5	0	0	0	95	70	95	95.00
6.	J-2477	72	8	12	3	5	18	28	15.25
7.	HB-3	66	9	4	5	16	20	37	24.00
8.	7042 S	29	26	16	0	29	50	71	43.50

DAS – Days after sowing; *Disease severity was recorded at 60 DAS, because panicle emergence observed after 30 DAS. Reference: Singh SD, King SB and Werder J (1993). Downy mildew disease of pearl millet. *Information Bulletin No. 37.*, A.P. 502324, India.

The total soluble sugar was 2-3 fold more in highly susceptible genotypes (J-2296 and 7042 S) compared to resistant genotypes at 7 days after infection (57 DAS). The higher soluble sugar indicated that susceptible genotypes may have more efficient sugar synthesizing capacity which might be supporting the mycelium growth and sporulation. Moreover, availability of lower sugar content in the interacellular fluid of the resistant varieties may be responsible for the inhibition of the growth and multiplication of the pathogen (Borkar and Verma 1989).

Increase in sugar content has been linked to invertase activity during powdery mildew infection of barley and wheat (Scholes *et al.* 1994; Heisteruber *et al.* 1994) and pea leaves (Alked and Hall 1993). Furthermore, down regulation of the calvin cycle, resulting from such accumulation of sugar, decreases the availability of assimilate for export in powdery mildew infected barley (Scholes *et al.* 1994) and wheat (Heisteruber *et al.* 1994; Wright *et al.* 1995). The alterations in total soluble sugar content recorded here during *S. graminicola* infection of pearl millet leaves would be consistent with similar metabolic interactions.

Resistant genotype J-2405 possessed the highest concentration of amino acid at pre-infection stage followed by J-2340, J-2290 and J-2480 (Table 1). Although, the amino acid content was significantly higher only in the genotype J-2405, there appeared to exist no specific trend or correlation with inherent resistance or susceptibility to downy mildew. In the present study, the amino acid content of all genotypes whether resistant or susceptible finally hastened as a result of infection. However, susceptible genotypes registered 2-2.25 % higher amino acid whereas; resistant genotypes possessed 6.2-76.6 % higher amino acid than their constitutive level. These results are consistent with previous result (Chakrabarty *et al.* 2002). Total free amino acid content in susceptible cotton genotype increased by 280 % after infection. The rate of increase was only 22.95 % in resistant genotype of chickpea to grey mould (Mitter *et al.* 1997). It may be due to higher proteolytic enzyme activity in the susceptible genotypes due to pathogen or inhibition of protein synthesis in the host. Pesis *et al.* (1991) also detected the highest proteolytic activity of *Botrytis cinerea* in the infected parts of Kiwi fruit but no activity in the non-infected parts.

Table 1. Pre- and post-infectional changes of metabolites constituents in downy mildew resistant and susceptible genotypes of pearl millet

No. and name of genotypes	Total soluble sugar (mg g ⁻¹ FW)		Total free amino acids (mg g ⁻¹ FW)		Total chlorophylls (mg g ⁻¹ FW)		Total Carotenoids (mg g ⁻¹ FW)	
	Pre-infection	Post-infection	Pre-infection	Post-infection	Pre-infection	Post-infection	Pre-infection	Post-infection
Resistant								
1. J-2290	8.61	3.03	2.65	4.68	3.48	2.68	1.46	1.15
2. J-2340	11.06	3.27	2.71	3.85	4.18	3.88	1.80	1.59
3. J-2405	12.94	4.49	3.71	3.94	3.23	3.74	1.33	1.66
4. J-2480	11.13	4.52	2.59	3.99	3.32	2.70	1.37	1.13
Susceptible								
5. J-2296	8.93	8.50	2.58	5.20	4.00	0.73	1.02	0.226
6. J-2477	5.93	5.56	2.28	4.90	3.04	1.02	1.23	0.371
7. HB-37042	6.66	5.62	2.62	5.85	4.05	1.19	1.73	0.436
8. S	8.63	9.03	2.43	5.19	3.46	1.28	1.43	0.597
S.Em. C.D. at 5 % C.V. %	0.17	0.18	0.06	0.13	0.04	0.09	0.03	0.02
	0.52	0.54	0.17	0.38	0.11	0.27	0.08	0.07
	3.26	5.69	3.64	4.62	1.80	7.34	2.96	4.37

Pre-infection at 45 DAS, Post-infection at 57 DAS (7 days after infection), FW-Fresh weight

Total chlorophyll level did not show any clear cut difference in resistant and susceptible genotypes at pre-infection stage (Table-1). However, at post-infection stage large decrease ($0.73\text{--}1.28\text{ mg g}^{-1}\text{ FW}$) occurred in susceptible genotypes compared pre-infection ($3.04\text{--}4.00\text{ mg g}^{-1}\text{ FW}$). While in resistant genotypes total chlorophyll decreased only from 7.17 to 22.98% at post infection, except J-2405. This decrease may be due to increase in age of crop because resistant genotypes have incompatible interaction. The greater reduction of chlorophylls in compatible interaction (susceptible genotypes) might be due to reduced synthetic activity of the enzyme chlorophyllase, indicating a possible damage in the photosynthetic capacity of chloroplast. Hasabnis *et al.* (2004) recorded average coefficient of infection and chlorophyll content in wheat genotypes infected with leaf rust and found negative but significant correlation. At pre-infection, there was no specific trend observed in carotenoid content of resistant and susceptible genotypes (Table-1). A significant reduction in total carotenoids content was noticed in all genotypes except J-2405 at post-infection. The magnitude of decrease was more pronounced (2.39 to 8.05 fold) in susceptible genotypes. After infection, significant decrease in carotenoids content was observed in resistant genotypes J-2290, J-2340 and J-2480 (21, 12 and 17.5 %, respectively) over pre-infection. Higher content of carotenoids in resistant genotypes at both stages may suggest an antioxidant role.

A total 10-19 amino acids as detected according to R_F of standard amino acids by HPTLC CAMAG SCANNER 3, of which 5-13 were found unknown (Table-2). The 18 standard amino acids were detected in tested genotypes (Fig. 1), of which arginine, cystine, methionine, threonine and lysine were found only in J-2290, J-2405, J-2477 and 7042 S, respectively whereas aspartic acid was detected only in two genotypes J-2405 and J-2296. It is pertinent to note that the amount of the sulphur containing amino acid cysteine was higher in resistant genotypes (23.18-26.95 % area) compared to moderately susceptible genotypes J-2477 and HB-3 (18.17-20.31 % area) at pre-infection stage. Resistant genotype J-2405 contained cystine (63.14 % area covering arg, asp and cystine) instead of cysteine.

The analysis of leaves at post-infection (7 d.a.i.), showed an absence of cysteine in all genotypes except HB-3 and J-2405. In the HB-3 it was decreased from 18.17 to 7.51 % area. While in resistant genotype J-2405 cysteine was detected (23.18 %) only at post-infection stage but it was lower than cystine at pre-infection. Our results suggested that sulphur containing amino

acids (cysteine and cystine) were higher in resistant genotypes at pre-infection stage and might be responsible for restricting the fungal growth. Valine and isoleucine content were increased at post-infection stage in all genotypes in which these amino acids were found at pre-infection stage. Similar result has been found in chickpea genotypes resistant to grey mould (Mitter *et al.* 1997). Tyrosine content was increased after infection in resistant genotypes, whereas it was absent in susceptible genotypes at both stages of analysis except J-2296 in which it was found (3.33 % area) at pre-infection stage. Interestingly, serine and histidine were found only in resistant genotypes, while phenylalanine, glycine and leucine were detected in susceptible genotypes. Hydroxy proline content decreased in all the genotypes except 7042 S in which it not detected at post-infectional stage. Enrichment of the cell wall in

Fig. 1. Amino acid profiling of Pearl millet genotypes through HPTLC at pre (lane 1-8 without subscript p) and post (lane 1-8 with subscript p) infection stage. A. Lane; 1. J-2290, 2. J-2340, 3. J-2405, 4. J-2480 (Resistant genotypes). B. Lane; 5. J-2256, 6. J-2477, 7. HB-3, 8. 7042S (Susceptible genotypes). C. M₁- mixture 1 (DL-alanine, arginine HCl, DL-aspartic acid and L-cystine); M₂-mixture 2 (L-glutamic acid, glycine, L-histidine HCl and DL-isoleucine); M₃- mixture 3 (L-leucine, L-lysine HCl, DL-methionine and L-proline); M₄-mixture 4 (DL-serine, DL-tryptophan, L-tyrosine and DL-valine) and M₅-mixture 5 (L-cysteine HCl, L-hydroxy proline, phenyl alanine and threonine).

Table 2. Amino acid profiling of pearl millet genotypes at pre-infection (I) and post-infection (II) stage (Individual amino acid and % area was detected by CAMAG SCANNER 3 at 546 nm)

Geno- types	Stage	Total No. of Identified amino acid	% Area of known amino acid															No. of unknown amino acid
			Arg/Asp Cysteine	Cyst	Glu/His Gly	OH- Pro	Ileu	Leu	Lys	Met.	Pro	PAL	Ser	Thr	Trp	Tyr	Val.	
J-2290	I	15	22.7 Arg	24.89	-	20.46	5.81	-	-	-	0.36	-	-	-	7.89	5.19	8	
	II	11	-	-	-	7.28	16.58	-	-	-	0.25	-	43.29	-	13.93	14.69	5	
J-2340	I	12	-	26.95	-	22.25	7.58	-	-	-	-	-	28.93	-	7.76	-	7	
	II	13	-	-	43.37 Hist	6.40	18.01	-	-	-	0.18	-	-	-	11.07	14.30	7	
J-2405	I	10	63.14 Cys	-	-	15.35	3.71	-	-	-	-	-	-	-	3.85	3.39	5	
	II	14	0.24 Asp	23.18	-	9.55	24.28	-	-	-	-	-	21.19	-	5.64	-	12.8 7	
J-2480	I	14	-	24.49	12.62 Hist	13.02	6.35	-	-	-	0.23	-	23.74	-	6.54	5.89	5	
	II	13	-	-	22.69 Hist	8.41	13.25	-	-	-	0.16	-	20.94	-	3.56	11.72	13.68 5	
J-2296	I	14	-	-	53.84 Gly	23.50	2.38	-	-	-	-	6.87	-	-	3.33	-	9	
	II	18	2.4 Asp	-	42.04 Gly	6.52	8.58	-	-	-	-	9.57	-	-	-	8.84	12	
J-2477	I	19	-	20.31	31.66 Gly	16.20	4.51	6.24	-	1.17	-	-	-	-	-	4.83	12	
	II	15	-	-	-	11.41	-	21.86	29.33	-	16.63	-	-	-	-	8.01	10	
HB-3	I	18	-	18.17	-	13.74	5.95	39.34	-	-	-	5.80	-	-	-	4.13	12	
	II	19	-	7.51	-	12.53	6.92	46.48	-	-	-	6.12	-	-	-	4.43	13	
7042 S	I	17	-	-	60.67 Gly	22.65	-	4.55	-	-	-	-	-	0.26	2.93	-	13	
	II	17	-	-	24.49 Gly	-	23.58	-	-	-	-	-	16.02	2.47	-	-	13	

hydroxy proline rich glycoproteins were involved in the defense of muskmelon (*Cucumis melo*) seedlings to *Colletotrichum lagenarium*, the causative agent of anthracnose (Esquerre-Tugaye *et al.* 1979).

In the present study absence of these glycoprotein (hydroxy proline) in 7042 S at post-infection stage may be strictly correlated to an accelerated and more intense fungal growth on the host.

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