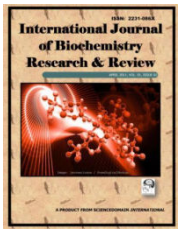

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Biochemical Constituents in Malformed Tissues of Pearl Millet Cultivars Caused by Aggressive Pathotype of *Sclerospora graminicola* Causing Downy Mildew Disease

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Research Article

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ABSTRACT

Downy mildew (DM) of pearl millet [*Pennisetum glaucum* (L.) R. Br.] caused by *Sclerospora graminicola* is the most widespread and destructive disease. In DM affected plants disease symptoms appear suddenly with the emergence of green ear, which exhibits all possible degrees of proliferations and malformation of the panicle. The pathogen population at Jodhpur, India is more virulent among other prevalent pathotypes as highly resistant pearl millet lines turned susceptible at this location. Virulence of pathotype rapidly changes host physiology producing varied symptoms in leaves and ear heads. Biochemical components including carbohydrates, phenols, free proline, photosynthetic pigments and enzymes like polyphenol oxidase (PPO), peroxidase (POX), IAA oxidase (IAAO) and catalase were found considerably deranged in malformed tissues. Results indicated that in two highly susceptible cultivars (*Nokha local and Eknath*) high soluble sugars were recorded in DM necrotic/chlorotic leaves and malformed ear heads, whereas starch contents were reduced in infected ear heads. Total and O-dihydroxy phenols were higher in DM infected leaves as well as in the malformed ear heads. Free proline contents were increased manifold in DM infected leaves and in proliferated panicles. Total chlorophyll contents reduced drastically in DM infected leaves. In ear heads showing tufting and complete malformation, total chlorophyll and carotenoids were low when compared to healthy and diseased leaves. Activities of PPO, POX, IAAO and catalase were higher in DM affected leaves and suppressed and completely malformed ear heads in comparison to their healthy counterparts. The study

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suggests that accumulation of total phenols caused the hyperphenolicity in infected host tissues despite increased activities of POX, PPO, catalase and IAA oxidase.

Keywords: Pearl millet; downy mildew; Sclerospora graminicola; new aggressive pathotype; biochemical analysis;

1. INTRODUCTION

Bajra, the pearl millet (*Pennisetum glaucum*) is the principal staple food crop grown in arid India. Downy mildew (DM) is the major limiting factor of pearl millet production in Rajasthan and all other millet-cultivating tracts in India. DM infected plants develop severe disease syndrome from the very beginning and succumb even before reaching maturity. The disease normally appears in the form of chlorosis at the base of infected leaf followed by production of sporulation on the lower side of leaves (Fig. 1).

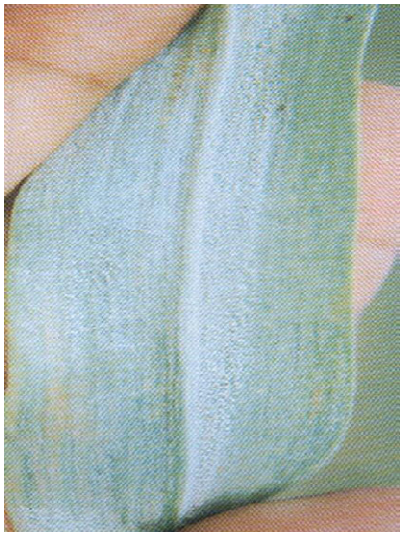

Fig. 1. Downy mildew affected leaf showing profuse downy white growth on the abaxial (lower) surface

Symptoms appear on ear head with all possible degrees of proliferations and malformations. In malformation the florets are converted into leafy structures of diverse appearance. Primary source of infection arrive from soil and systemically infect seeds. The invasion of the fungus (*Sclerospora graminicola* [Sacc.] Shroet.) in floral primordia plays a crucial role in deciding the extent of malformation. Generally four types of malformations have been observed (Fig. 2) : a) lower half of the ear-head is proliferated; b) the entire ear-head is transformed; c) only bristles become long and no malformed leafy structures are formed, and d) leafy tufts and frenching where the shoots remain stunted and produce leafy tufts at the top (Arya and Arun-Kumar, 1976). During 1995-97 a trial under International Pearl Millet Downy Mildew Virulence Nursery (IPMDMVN, 1998) programme was conducted at Central Arid Zone Research Institute (CAZRI), Jodhpur, India as one of the sites for understanding

variability in the pathogen (*S. graminicola*) populations. Results of IPMDMVN provided ample evidences for the existence of variable pathotypes (International Pearl Millet Downy Mildew Virulence Nursery (IPMDMVN, 1998)' which was supported by Thakur et al. (1998 and 2001) and tentatively christened it known as a new virulent (Jodhpur) pathotype.

The Jodhpur pathotype has been identified in changing host physiology producing varied symptoms as shown in Fig. 2. In the present study different pathogen-induced host metabolites and oxidizing enzymes in DM affected malformed tissues of pearl millet cultivars infected with this pathotype were analysed to understand the biochemical nature of the pathogen.

Fig. 2. Green ear heads showing types of malformations with a healthy ear head (extreme left)

Figs. a to k:
a. Healthy ear head
b, f and g. Partially proliferated ear head with bracts
c, d and e. Completely proliferated ear head
h, i and j. Completely proliferated ear head with suppressed proliferation (tufted)
k. Leafy tufts

2. MATERIALS AND METHODS

To estimate biochemical constituents such as metabolites and enzymes, fresh samples of leaves and ear-heads were collected from the DM infected and normal plants of two highly susceptible cvs. Eknath and Nokha local grown in experimental field of Central Arid Zone Research Institute, Jodhpur in the rainy season. Following types of samples were drawn from DM infected plants: a) DM necrotic/chlorotic leaves; b) suppressed ear heads (Tufting) and c) completely malformed ear heads. Corresponding healthy plant parts were taken as control.

2.1 Metabolites and Enzyme Estimations

The total soluble sugars and starch were estimated by the method of Yemm and Willis (1954), reducing sugars were analysed using method suggested by Nelson (1944). Chlorophyll and carotenoids were analysed by the method described by Mali *et al.* (2000).

Free proline was determined after Bates et al. (1973). Total phenols and O-dihydroxy phenols (ODP) were analysed by adopting methods given by Bray and Thorpe (1954) and Mahadevan and Sridhar (1986). Activities of peroxidase (POX) and polyphenol oxidase (PPO) enzymes were estimated using the method suggested by Kar and Mishra (1976). IAA oxidase and catalase were estimated as reported by Mahadevan and Sridhar (1986).

Data from four replicates were analyzed for each experiment and subjected to LSD values.

3. RESULTS

3.1 Metabolites

3.1.1 Carbohydrates

Results presented in Table 1 showed that in cv. Nokha local total sugar content in DM infected leaves and ear-heads was higher than their healthy counterparts. Among infected leaf samples maximum increase was recorded in necrotic leaves (44.7%). Results further showed an increase in reducing sugars in diseased leaves and ear-heads. Reducing sugar contents were highest (88.1 mg g^{-1} dry wt.) in suppressed ear-heads (tufting), followed by completely malformed ear-heads (67.1 mg g^{-1} dry wt.).

In case of cv. Eknath total soluble sugars were significantly higher in DM necrotic leaf tissues as compared to healthy leaves (Table 1). Similar propensity was also noticed in case of diseased and healthy ear heads. High amount of total soluble sugars were recorded in completely malformed ear heads followed by tufting when compared with the healthy counterparts. The same was also found true in case of reducing sugars where significantly higher contents were recorded in necrotic leaves (24.4 mg g^{-1} dry wt) and completely malformed green ears (73.4 mg g^{-1} dry wt). In case of starch contents DM necrotic leaves showed 142.0 mg g^{-1}dry wt., whereas it was less (93.1 mg g^{-1}dry wt.) in the healthy leaves. As far as ear head is concerned tufting symptoms had more (131.9 mg g^{-1}dry wt.) starch contents in comparison to the completely malformed ear heads (130.0 mg g^{-1}dry wt.). However, healthy ear head showed much higher starch contents (723.6 mg g^{-1}dry wt.) due to presence of grains.

3.1.2 Phenols

Increased total phenols were observed in DM infected and chlorotic leaves (8.4 and 8.8 mg g^{-1}dry wt., respectively) of cv. Nokha local in comparison to healthy counterparts (4.4 mg g^{-1}dry wt.). Likewise phenols were higher in tufting and completely malformed ear-heads (8.8 and 7.1 mg g^{-1}dry wt., respectively) than normal tissues. O-dihydroxy phenols (ODP) were recorded maximum in suppressed ear-head (tufting) followed by completely malformed ear-heads. However, it was lesser by about 4% in DM infected leaves and chlorotic leaves (Table 1).

There was significant difference in the total phenolic contents of diseased and healthy leaves of cv. Eknath. It was much higher (94.1%) in the DM necrotic leaves in comparison to healthy ones.

Table 1. Changes in metabolites of healthy and downy mildew/green ear infected malformed tissues of pearl millet

Metabolites	Cultivar	Pearl millet tissues					LSD (P≤ 0.01)
		Healthy leaves	DM infected necrotic / chlorotic leaves	Healthy ear - head	Tufting	Completely malformed ear - head	
Total soluble sugars (mg g^{-1} dry wt)	Nokha local	38.2	55.2 (+ 44.7)*	36.3	95.4 (+ 162.8)	69.1 (+ 90.3)	9.7
	Eknath	60.6	73.4 (+ 21.2)	20.4	80.0 (+ 291.9)	97.5 (+ 377.6)	4.6
Reducing sugars (mg g^{-1} dry wt)	Nokha local	11.5	43.9 (+281.0)	22.0	88.1 (300.4)	67.1 (205.0)	9.1
	Eknath	60.5	73.4 (+21.3)	3.4	66.1 (+94.8)	73.3 (+95.3)	3.4
Starch (mg g^{-1} dry wt)	Nokha local	86.6	183.7 (+112.2)	496.1	144.0 (-71.0)	135.1 (-72.7)	29.3
	Eknath	93.1	142.0 (+52.5)	723.6	132.0 (-448.1)	130.0 (-456.6)	41.9
O-dihydroxy-phenols (mg g^{-1} dry wt)	Nokha local	1.74	1.66 (-4.59)	1.08	1.78 (+ 64.8)	1.43 (+32.4)	0.21
	Eknath	1.5	1.9 (+ 30.9)	0.5	0.7 (+ 49.7)	0.8 (+ 52.3)	0.16
Free proline (µ g g^{-1} dry wt)	Nokha local	578.7	894.5 (+ 54.6)	316.1	2668.6 (+ 744.2)	1823.7 (+ 476.9)	219.2
	Eknath	279.0	432.0 (+54.8)	153.0	1368.2 (+794.2)	1168.0 (+663.3)	243.9

**Figures in parentheses are percent changes in DM infected tissues*

In ear heads the phenolic contents were low (1.6 mg g^{-1} dry wt.) when compared with the tufting (4.3 mg g^{-1} dry wt.) and completely malformed ear heads (4.7 mg g^{-1} dry wt.). Similar observations were recorded in case of O-dihydroxy phenols where it was around 31% more in DM necrotic leaves when compared to healthy leaves. In case of malformed ear head maximum ODP contents (0.76 mg g^{-1} dry wt.) were recorded followed by tufting (0.74 mg g^{-1} dry wt.) and healthy ear head (0.50 mg g^{-1} dry wt.) (Table 1).

3.1.3 Free proline

Data presented in Table 1 showed that free proline contents increased manifold in diseased chlorotic leaves (894.5 µg g^{-1} dry wt) of cv. Nokha local in comparison to healthy leaves (579.0 µg g^{-1} dry wt). However, the percent change over healthy counterparts was more in tufting (744.2%) than completely malformed ear-heads (477.0%).

In cv. Eknath manifold increase of free proline in DM necrotic/ chlorotic leaves (55%) and tufting (794.2%) and completely malformed ear heads (663.3%) in comparison to their healthy counterparts was observed (Table 1). The difference was much higher in tufting in comparison to malformed ear heads. This confirms that under the biotic stress production of proline increases.

All the data presented in the tables were significant at 1% level except for chlorophyll b, which were found non-significant (ns) at 1% level.

3.1.4 Photosynthetic pigments

Biochemical analysis of photosynthetic pigments revealed that total chlorophyll and carotenoid contents reduced drastically (97.7%) in DM infected leaves of cv. Nokha local. Detailed analysis of chlorophyll contents showed that Chlorophyll-a was reduced by 59% whereas chlorophyll-b was absent. Similarly, reduction of chlorophyll content was also observed in infected bushy leaves however, chlorophyll-a content was found in traces. Chlorophyll estimations in ear heads of completely malformed and suppressed (tufting) tissues showed highest increase (250.0%) of chlorophyll-a followed by tufting (100.0%). The carotenoid contents were higher in both types of malformations.

Results presented in Table-1 showed that chlorophyll-a, chlorophyll-b and total chlorophyll contents were reduced by 47.1%, 97.8% and 58.8%, respectively in necrotic leaves in comparison to healthy leaves of cv. Eknath. The reverse was found true in tufting and completely malformed ear heads, where total Chlorophyll increased by 354% and 321%, respectively in tufting and completely malformed ear heads. However, carotenoids were significantly higher (mean 160%) in both types of malformations.

3.1.5 Oxidizing enzymes

Results revealed that in cv. Nokha local activity of polyphenol oxidase (PPO) was higher in diseased leaves and completely malformed ear-heads. Highest activity of peroxidase (PO) and IAA oxidase (IAAO) was recorded in DM infected leaves, followed by chlorotic leaves in comparison to the healthy ones. In case of ear-head, tufting showed higher activity of PPO and POX, whereas the maximum IAAO activity was observed in completely malformed tissues (Table 2).

Table 2. Changes in photosynthetic pigments in different malformed tissues of pearl millet infected with downy mildew/green ear disease

Metabolites (mg g^{-1} dry wt)	Cultivar	Pearl millet tissues					LSD (P≤ 0.01)
		Healthy leaves	DM infected necrotic / chlorotic leaves	Healthy ear - head	Tufting	Completely malformed ear - head	
Chlorophyll a	Nokha local	3.00	0.01 (-99.6)*	0.32	0.21 (+233.3)	0.13 (+102.0)	0.09
	Eknath	2.15	1.14 (-47.0)	0.07	0.34 (+386.0)	0.31 (+343)	0.19
Chlorophyll b	Nokha local	1.24	0.00	0.00	0.00	0.00	ns
	Eknath	0.65	0.01 (-98.4)	0.00	0.00	0.00	ns
Total chlorophyll	Nokha local	4.10	1.16 (- 71.6)	0.06	0.21 (+ 232.2)	0.12 (+ 100.9)	0.11
	Eknath	2.80	1.15 (- 58.8)	0.07	0.34 (+ 354.1)	0.31 (+ 321.6)	0.27
Carotenoids	Nokha local	1.12	0.70 (-7.2)	0.39	0.53 (+36.2)	0.43 (+13.0)	0.14
	Eknath	0.74	0.76 (+2.7)	0.14	0.37 (+164.2)	0.35 (+150.0)	0.12

**Percent changes in DM infected tissues*

Table 3. Activities of oxidative enzymes with percent change in downy mildew/green ear infected malformed tissues of pearl millet

Enzyme	Cultivar	Pearl millet tissues					LSD (P≤ 0.01)
		Healthy leaves	DM necrotic leaves	Healthy ear -head	Tufting	Completely malformed	
Peroxidase (POX) (Δ OD min^{-1} mg^{-1} protein)	Eknath	1.69	4.83 (+ 185.4)*	1.13	11.32 (+ 896.7)	7.22 (+ 535.5)	2.04
	Nokha local	1.41	3.35 (+ 137.6)	2.53	7.64 (+ 201.9)	3.77 (+ 49.2)	1.39
Polyphenol oxidase (PPO) (Δ OD min^{-1} mg^{-1} protein)	Eknath	0.002	0.009 (+ 340.0)	0.007	0.022 (+ 208.4)	0.014 (+ 91.5)	0.004
	Nokha local	0.003	0.009 (+ 210.3)	0.004	0.008 (+ 92.8)	0.012 (+ 185.7)	0.003
IAA oxidase (Δ OD h^{-1} mg protein)	Eknath	0.028	0.052 (+ 87.1)	0.015	0.024 (+ 58.3)	0.019 (+ 23.8)	0.008
	Nokha local	0.010	0.041 (+ 306.9)	0.014	0.032 (+ 127.8)	0.043 (+ 203.5)	0.009
Catalase	Eknath	1.734	4.832	1.135	11.316	7.215	0.589
	Nokha local	0.002	0.004	0.004	0.008	0.012	0.002

***** *Figures in parentheses are percent changes in DM infected tissues*

Interestingly, the catalase activity was not detected in both healthy and diseased leaves but its activity was found maximum in tufting (0.31 OD min^{-1} mg^{-1} protein) followed by completely malformed ear heads (0.28 min^{-1} mg^{-1} protein) than healthy ear-heads (0.19 min^{-1} mg^{-1} protein).

Activities of polyphenol oxidase (PPO), peroxidase (POX), IAA oxidase (IAAO) and catalase (CA) were estimated in DM necrotic and healthy tissues of leaves and ear heads of cv. Eknath. Results presented in Table 2 showed that PPO, POX and IAAO activities were much higher in the DM infected necrotic leaves (340; 185 and 87%, respectively). Maximum increase in PPO and POX was recorded in tufting (208 and 897%, respectively) in comparison to healthy ear heads. In case of IAAO the activity of the enzyme was almost doubled in the necrotic leaves when compared to healthy leaves. In ear heads the highest activity of IAAO was observed in tufting followed by completely malformed ear head and the least in healthy ear head.

The catalase activity was almost negligible in case of healthy and diseased leaves. In case of ear heads it was maximum in completely malformed ear heads (205%) followed by tufting (133%).

5. DISCUSSION

Downy mildew of pearl millet is a ubiquitous pathogen causing severe loss to yield and produces variety of symptoms on growing vegetative and flowering tissues of plants. Attack of *S. graminicola* on developing plants disturb the host physiology particularly host metabolites and oxidizing enzymes. The consequences of biochemical changes during host pathogenesis lead to various types of malformations.

Present study indicated significant changes in photosynthetic pigments, carbohydrate, proline and phenol contents in the course of *S. graminicola* pathogenesis. The Jodhpur pathotype of *S. graminicola* and susceptible hosts played major role in deranging host morphology and physiology.

Loss of chlorophyll in diseased leafy tissues of pearl millet indicates below normal photosynthetic activities. Considerable decrease in chlorophyll content (chlorophyll-a, chlorophyll-b and carotenoids) has been reported in *Plantago ovata* caused by *Peronospora alta* (Rathore et al., 2001). Increase in the total chlorophyll contents in the malformed and suppressed (tufted) ear-heads is supposed to be due to conversion of panicle into leafy structures during DM pathogenesis.

Higher sugar contents in diseased ear-heads may adversely contribute in inhibiting grain formation in the infected plants. There are reports of higher soluble sugars and glucose in DM susceptible cultivars (Muthuswamy *et al.,* 1983). The non-alternation of sugar in the DM colonized plants suggests the possibility of sugars finding their way into fungal tissues. An attempt on the qualitative resolution may throw light on the changes in sugar quality as a result of DM infection, its influence on pearl millet and reaction of pearl millet colonized by the DM pathogen.

Proline is reported to modulate certain functions, which are essential for plant recovery from stress (Szabados and Savoure, 2009). Proline accumulation has been reported during biotic stresses (Haudecoeur, 2009).Though, studies are needed in elucidating precise role of

proline in improving defenses against pathogens but there are reports that increase in free proline contents in diseased tissues may be due to reduced protein synthesis (Van Andel, 1966), however, it has been observed that apart from inducing protection against downy mildew, proline was also found effective in enhancing vegetative and reproductive growth of pearl millet plants (Raj et al., 2004; Sudisha et al., 2011). Increased free proline content has been observed in various types of red rot pathogenesis in sugarcane. It has been suggested that consequent upon fungal pathogenesis proline accumulation occurs in diseased tissues to meet the energy requirement (Raj Bhansali et al., 1983; Sinha et al., 1983).

In present study the hyperphenolicity was observed in diseased leaves and malformed ear-heads confirmed that the infection resulted in aromatization of host tissues as also reported by previous workers (Shekhawat et al., 1984; Arun-Kumar et al., 2010).

Change of reproductive parts into leafy structures also depicted in form of abnormal activities of oxidizing enzymes in DM infected pearl millet. Polyphenol oxidase and POX oxidize phenolic compounds to quinines and polymerize lignin-like substances. These transformed compounds are toxic to microorganisms and impart resistance to DM infections. A low activity of PPO and POX observed in the present studies in healthy leaves and ear-heads indicates the role of these enzymes in case of disease resistance. Effect of *S. graminicola* infection on the accumulation of phenol content of pearl millet at different stages of plant growth of healthy and diseased leaves, stems and roots was observed (Bhatia and Thakur, 1992). Total phenols increased during the early stages of plant growth (30 DAS) but decreased with plant age and increase in infection, whereas total soluble sugars and total chlorophyll decreased at all the plant growth stages in highly susceptible and moderately resistant genotypes (Yadav et al., 1998). Activity of POX in the extracts of leaves and ears at different stages of development in infected plants of cv. NHB-3 (susceptible) was higher than healthy plants. This may be ascribed to acceleration of host senescence by the pathogen (Arora *et al*., 1986). However, the DM resistant populations of pearl millet showed higher enzyme activities, while lower activities of the enzymes were recorded in the susceptible populations (Shetty et al., 2001). Formation of some new isoenzymes of peroxidase in green ears of pearl millet was not able to oxidize auxins due to high phenolicity (Shekhawat et al., 1984). Activity of IAAO was also higher in infected plants suggests quick utilization of auxins like compounds, which are responsible for induction of malformation. In case of maize downy mildew caused by *Peronosclerospora sorghi* biochemical changes were observed in the form of low chlorophyll, total and reducing sugars and phenols in susceptible cultivars (Setty et al., 2001).

5. CONCLUSION

Present study has thus illustrated the drastic metabolic changes expressed into varied abnormal growths in downy mildew infected pearl millet plants by the aggressive Jodhpur pathotype. In the diseased tissues carbohydrate build-up was possibly caused by sugar transportation from healthy to diseased tissues. Conversion of starch of the seed to sugars may be one of the reasons for carbohydrate accumulation. Accumulation of phenols and ODP along with higher activities of oxidizing enzymes might be the result of imbalance in the hormonal status of the plant, which leads to the abnormal growths.

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