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BIOCHEMICAL, HISTOCHEMICAL AND ISOENZYME PEROXIDASE STUDIES ON PEARLMILLET [*PENNISETUM GLAUCUM* (L) R. BR.] SEEDLINGS INFECTED BY *CURVULARIA PENNESETI*

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Biochemical, histochemical and Native PAGE electrophoretic studies were conducted on healthy, moderate and heavily infected seedlings of pearlmillet with *Curvularia penneseti*. Histochemical observations revealed the presence of increasing amount of proteins, lignins, tannins, starch, phenols, cellulose and peroxidase enzyme in infected seedlings as compared to healthy(control). Biochemical examination also showed high level of proteins, total sugars, total starch, phenolics, and peroxidase activity in the infected seedlings as compared to healthy. The electrophoretic studies revealed the same observations of peroxidase isoenzymes at 10th day by Native PAGE. The drastic increase in IAA oxidase activity initially, followed by decrease and again increase showed the differential changes during progressive infection of *C. penneseti*. Which may play an important role in establisment of the infection.

Keywords : Biochemical; *Curvularia penneseti;* Histochemical; Isoenzyme peroxidase; Pearlmillet.

Introduction

Brown leaf spot disease caused by *Curvularia penneseti* occurs widely in pearlmillet seeds. The seed transmission of *Curvularia* species are frequently isolated from seed[1]. During infection the host plant defend itself against potential pathogens by means of number of physical and chemical factors which may already be present in the host, or may be produced in response to the infection[2]. The physical characterstics are mechanical barrier which prevents the entry and spread of pathogen. The chemical factors, which are toxic to the pathogen may produce certain compounds against the host. Plants are bestowed with various defence related genes. It is well known that the defense genes are sleeping genes and appopriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of biological inducer is thought to be a novel plant protection strategy. To see such reaction in the healthy (control), moderate and heavily infected seedlings, the biochemical, histochemical and electrophoretic studies were conducted in seedlings of pearlmillet at different stages after germination.

Material and Methods

The healthy and naturally (moderate and heavily) infected seeds with *Curvularia penneseti* were used for experimentation. The seeds were grown in petriplates and earthen pots. The emerging healthy, control and infected seedlings were excised for biochemical and histochemical studies at 1st, 5th, 10th, 15th and 20th day.

The histochemical tests were conducted for starch[3], phenols[4], total proteins[5,6], Cellulose[3,7], lignins[7], tannins[8],and peroxidase[9]. The stained preparations were observed under photolight trinocular microscope (Olympus) and photographed. Their qualitative increase or decrease was assesed in terms of intensity of metabolites as: nil(-), low(+), moderate(++), high(+++) and very high(++++).

In biochemical studies, the total sugar and starch were determined by Dubois *et al.*[10], total phenolics were estimated by Swain and Hillis[11], total proteins were measured according to Lowry *et al.*[12]. Peroxidase activity was measured by the method of Shanon *et al.*[13]. The IAA oxidase specific activity was determined by Sequeira and Minco[14]. One unit of enzyme activity was recorded as 0.01A min^{-1} mg^{-1} (protein).

The Native polyacrylamide gel electrophoresis for peroxidase isoenzymes were done at 10th day of germinating healthy, moderate, heavily infected seedlings and the seeds were treated with *Trichoderma viridae*, to show the effect of biocontrol agent on the activity of peroxidase isoenzymes. In multiple molecular forms of peroxidase alterations were examined by gel

electrophoresis[15] and were detected by the method of Seigel and Galston[16].

Results and Discussion

Histological studies -The histological stain reactions of penetration infection by *Curvularia penneseti* on pearlmillet seedlings (Table 1, Fig. 1) are listed as follows.

Proteins-The proteins were estimated by acid fuchsin reagent in germinated healthy and infected seedlings of pearlmillet. The proteins were stained magenta in colour, as observed in the host tissue. However, at the 5th , 10th , 15th, 20th day, the spore, germ tube and infection site / structure were also stained. It was interesting to note that the intensity of the stain was greater at the later stage of infection as compared to healthy control.

Phenolics-It was found that Nitroso reagent developed brown colour in the host, fungus and infection sites/ structures in seedlings at all the stages. The infected seedlings showed greater intensity after 15th day as compared to healthy seedlings.

Lignins-Phloroglucinol- HCl stained the host tissue and infected structures in red colour at 1st, 5th, 10th, 15th and 20th day. The intensity of the stain was increased with incubation time and was greater in infected seedlings as compared to healthy ones.

Tannins-Lugols iodine reagent stained the host tissue, spores and mycelium (infection structure). It was bright red in colour at all the stages of healthy and infected seedlings. The intensity was high throughout the period in infected seedlings as compared to healthy control.

Starch-The starch content was localized as blue to black in colour. It was high at all the stages in healthy control. In the infected seedlings the contents increased initially up to the 10th day and then decreased. The spores and infected structures were also stained.

Cellulose-Cellulose was stained dark blue to black. It was observed high in healthy and infected seedlings with spores and infected structures at all the respective stages.

Peroxidase- Peroxidase activity was observed to be higher in infected seedlings as compared to healthy ones. The spores and infected structures were also stained.

Biochemical studies-The biochemical evaluations of healthy and infected seedlings are outlined as follows at 1st, 5th, 10th, 15th and 20th day after germination (Fig. 2).

The protein contents in healthy, moderate and heavily infected seedlings were increased during infection in comparison with the healthy (control) tissue (except at 20th day).

The phenolics were also increased during the incubation time at all the respective stages in healthy, moderate and heavily infected seedlings. However, the phenolics were much higher in healthy control at 15th and 20th day.

The total sugar contents were continuously increased in moderate and heavily infected tissues along with healthy tissues from 1st to 15th day followed by decline at 20th day of germination.

The starch content was very high in the healthy control throughout the period of study. In moderate and heavily infected seedlings the starch contents were slightly increased up to the 10th day and followed by decrease up to the 20th day of infection.

The peroxidase enzyme specific activity showed a continuous increase in healthy, moderate and heavily infected seedlings at all the stages. However, the activity was quite high in healthy control as compared to infected seedlings. During the Native PAGE analysis, the isoenzyme bands showed more colour intensity during the infection and in the treated seedlings with biocontrol agent *Trichoderma viridae*. The two strong bands and two weak bands were more clear, which showed higher activity of peroxidase enzyme. The last band in case of treated seedlings by *T. viridae* showed the high Rm value as compared to infected seedlings (Table 2, Fig. 3).

The IAA oxidase specific activity was initially high at 1st day (24h) in healthy, moderate and heavily infected seedlings, followed by decrease up to the 10th day and again showed slight increase up to the 20th day after infection. However, the IAA oxidase activity was higher in healthy as compared to infected seedlings.

The positive histochemical stain reaction for proteins suggests the presence of glycoproteins, which may be involved in the infection process. Some reports indicate presence of proteihs in barley reaction sites[17]. Increase in proteins is due to the additional protein contributed by the fungal pathogen has also been reported in *Peronospora* infected pea leaves[18]. Phenolic compounds play a vital role in defence mechanism against various plant diseases. Certain enzymes such as phenylalanine- ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) cause deamination of respective aminoacids and help in synthesis of polyphenols and finally lignins. The higher levels of phenolics in the infected seedlings, as found in this investigation, have also been reported for *Helminthosporium maydis* and *H. sativum*[19].

The deposition of lignin has been implicated as defense response in wheat genotypes resistant to several diseases[20]. Lignin is one of the most abundant bioploymers, which provides resistance to plants against pathogens. It is thought to be formed in response to microbial penetration. It makes cell wall more resistant to

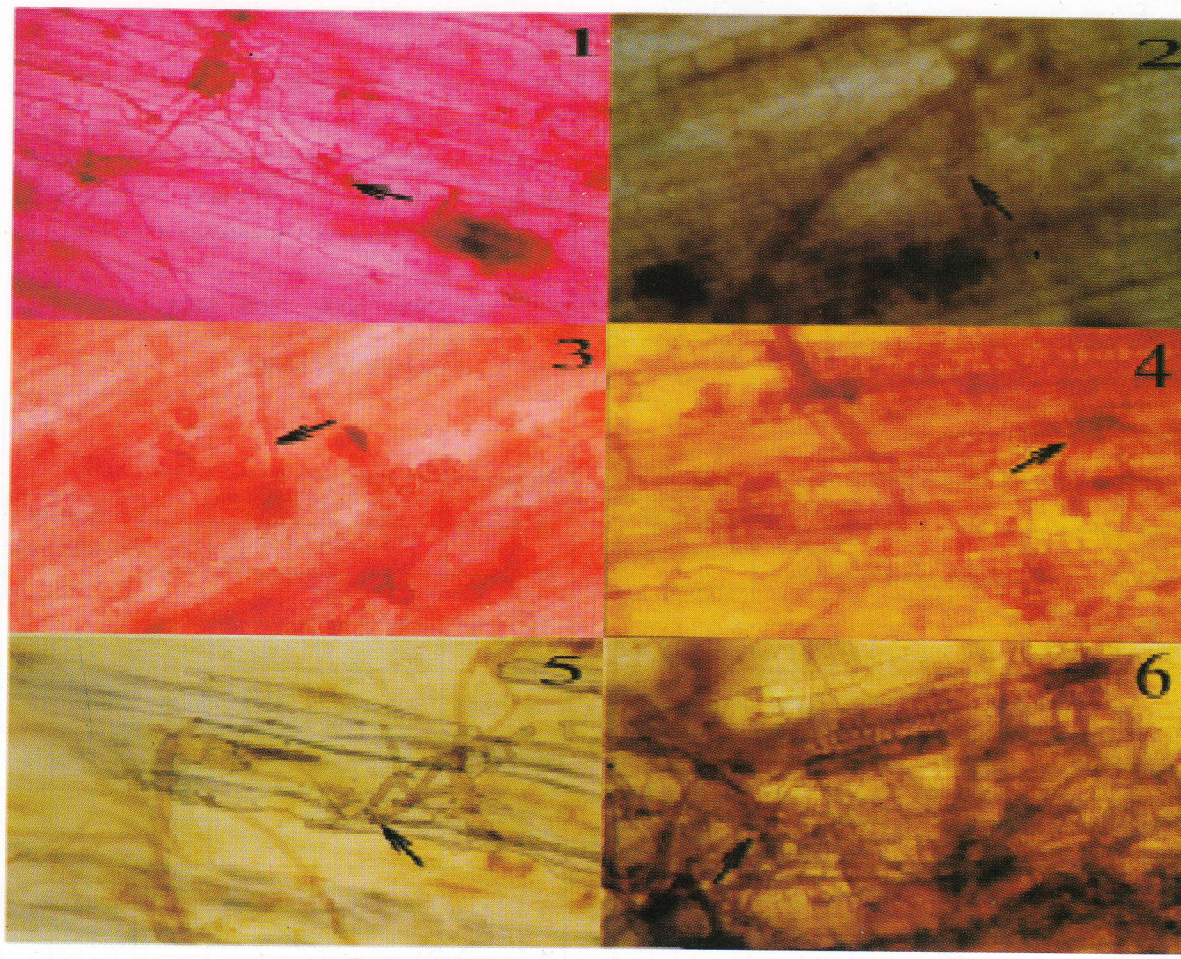


Fig.1. Photomicrograph of histochemical stain reactions of *Curvularia penneseti* – pearlmillet complex.
1. Heavily infected host tissue with spores and germ tubes localized intense proteins at 20th day by acid fuchsin reagent.; 2. Intense localization of starch in infected host, spores and germination.; 3. High localization of lignins in infected host with spores and mycelium at 20th day; 4. High localization of tannins in infected host with spores and germ tube; 5. Moderate localization of phenolics in host and infected part.; 6. Host, spores and germ tubes showing very high intensity of peroxidase activity at 20th day.

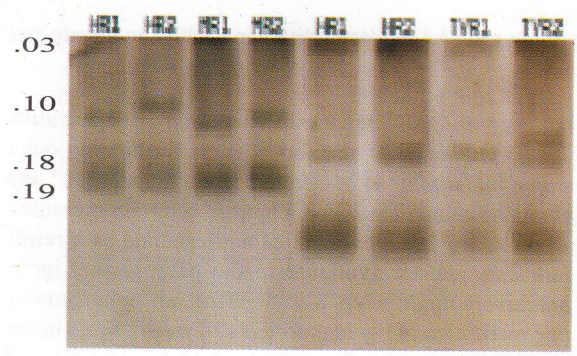
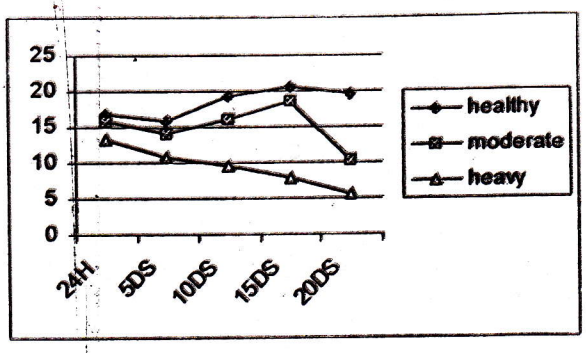
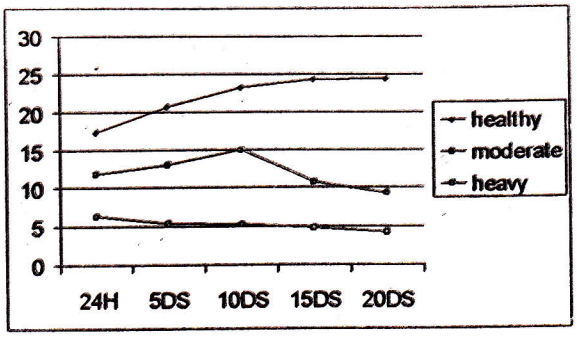


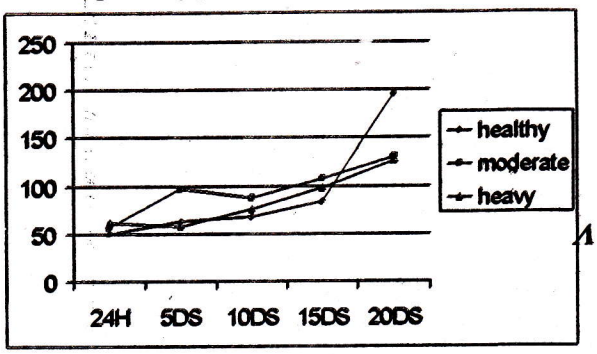
Fig. 3. Gel photograph of Native PAGE at the 10th day of germination for the separation of peroxidase isoenzymes in pearlmillet infected with *Curvularia penneseti* in healthy, moderate, heavily infected seedlings and seedlings treated with bioagent *Trichoderma viridae*.
HR_1 HR_2 = Healthy replicates; MR_1, MR_2 = Moderate Replicates; HR_1, HR_2 = Heavily Infected Replicates (HVR_1, HVR_2); TVR_1 TVR_2 = *T. viridae* treated Replicates.



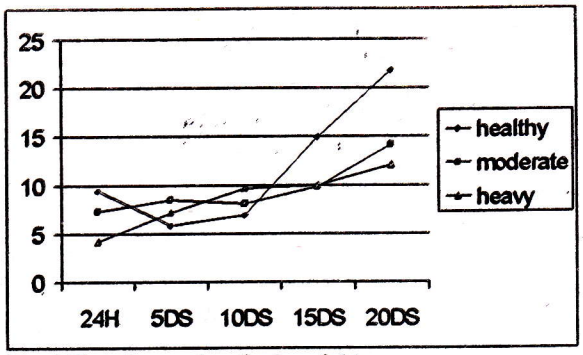
Total Sugars: mg/gm fresh weight.



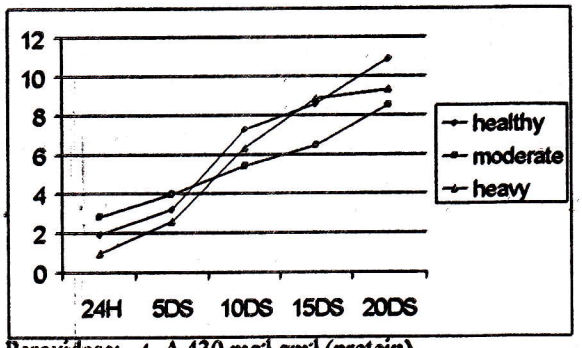
Total Starch: mg/gm fresh weight.



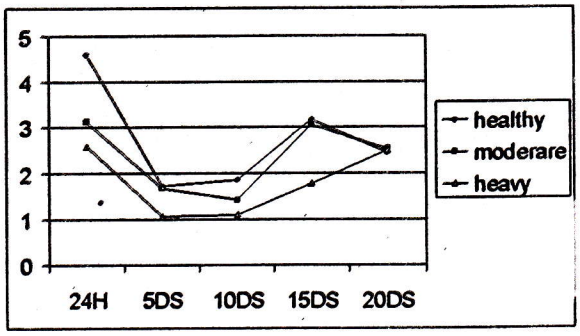
Total Proteins: mg/gm fresh weight.



Total Phenols : mg/gm fresh weight.



Peroxidase: Δ A 430 mg^{-1} gm^{-1} (protein).



IAO: Δ A 430mg^{-1} gm^{-1} (protein)

Fig. 2. Levels of total sugars, starch, proteins, phenolics, peroxidase and IAA oxidase activity in healthy, moderate and heavily infected genotypes of pearlmillet by *Curvularia penneseti.*

pathogen attack. To find out correlation between activation of enzymes and lignin production observations were made at different time intervals in healthy and infected seedlings. The increase in lignin production was observed at 10th day of germination in infected seedlings. The production of enzyme, time and trend of their maximum induction was shown by healthy genotypes, suggests a significant role in genotype governing plant resistance to this disease. The defense mechanism is activated in healthy genotype. The same mechanism may be operative in infected seedlings also, but it starts functioning quite slowly and reaches to its effective level only after 10th day of establishment of infection. Lignified cell walls constitute a barrier for free movement of nutrients and as a result nutrients are not available to the pathogens. Lignin precursors might exert a toxic effect on pathogens or polymerization of lignin precursors by free radicals in the intracellular space. It might lead to lignification of

Table 1. Histochemical stain reactions of *Curvularia penneseti* in heavily infected pearlmillet seedlings.

Components	Histochemical stain	Time of inoculation	Reaction to stain of					
			Host tissue		Spores		Germ tube	
			I	C	I	C	I	C
Proteins	Acid fuchsin	1D	++	++	++	-	++	-
		5D	++	++	++	-	++	-
		10D	+++	++	++	-	++	-
		15D	+++	++	++	-	++	-
		20D	+++	++	++	-	++	-
Phenolics	Nitroso reaction	1D	+	+	++	-	++	-
		5D	+	+	++	-	++	-
		10D	++	++	++	-	++	-
		15D	+++	++	++	-	++	-
		20D	+++	++	++	-	++	-
Lignins	Phloroglucinol-HCl	1D	+	++	++	-	++	-
		5D	++	++	++	-	++	-
		10D	++	++	++	-	++	-
		15D	++	++	++	-	++	-
		20D	++	++	++	-	++	-
Tannins	Lugols iodine	1D	+	+	++	-	++	-
		5D	+	+	++	-	++	-
		10D	++	+	++	-	++	-
		15D	++	+	++	-	++	-
		20D	++	+	++	-	++	-
Starch	I-KI	1D	++	+++	+	-	++	-
		5D	+++	+++	+	-	++	-
		10D	+	+++	++	-	++	-
		15D	+	+++	+	-	++	-
		20D	+	+++	+	-	++	-
Cellulose	I-KI-H_2SO_4	1D	++	++	++	-	++	-
		5D	++	++	++	-	++	-
		10D	++	++	++	-	++	-
		15D	++	++	++	-	++	-
		20D	++	++	++	-	++	-
Peroxidase		1D	+	++	++	-	++	-
		5D	++	+++	++	-	++	-
		10D	+++	+++	++	-	++	-
		15D	+++	+++	++	-	++	-
		20D	++++	+++	++	-	++	-

- = nil, + = low intensity, ++ = moderate intensity, +++ = high intensity, ++++ = very high intensity.
I = Infected, C = Control.

Table 2. Analytical observation of electrophoretic separation of peroxidase isoenzymes in healthy and naturally infected with *Curvularia penneseti* and infected seedlings of pearlmillet treated with *Trichoderma viridae* at 10th day of germination.

	Number of bands				mobilities of bands				
Category	Replicates	Total no. of bands	Prominent bands	Weak bands	Bands in first quarter	First band	Last band	Separation of first and last band	Most prominent band	Weaker band
Healthy	R1	2	-	2	2	.03	.13	.10	.03	.13
	R2	2	-	2	2	.03	.13	.10	.03	.13
Moderately	R1	3	2	1	2	.03	.18	.15	.18	.13
Infected	R2	3	2	1	2	.03	.18	.15	.18	.13
Heavily	R1	3	2	1	2	.03	.20	.17	.20	.13
Infected	R2	3	2	1	2	.03	.20	.17	.20	.13
T. viridae	R1	4	2	2	2	.03	.22	.19	.22	.16
treated	R2	4	2	2	2	.03	.22	.19	.22	.16

pathogen structures. Although the intercellular parts of the fungus become lignified, the intracellular haustoria remains unaffected[21].

The decline in starch contents in the infected seedlings may be due to the infection proceedings. The involvement of carbohydrates during pathogenecity, serving as constant energy source for the growing pathogen has been indicated in *Helminthosporium maydis, H. carborum* and *H. teres*[22].

It was noted that the ingress of the invading hyphae continued up to the cells that contained starch granules. Thus, it was not merely the total amount of carbohydrates but its pattern of distribution effected the depth of penetration in host cells by the pathogen.

Tannin contents of the healthy cv. was not only high but it was well distributed in the ray cells in which the pathogen usually colonized and blocked the passage of water.

Biochemical resistance was depended upon some pre existing or induced substance synthesized by plants in response to fungal infections. High level of phenol synthesis, rapid lignification and localized necrotization contributed resistance in plants against pathogen.

IAA metabolism was directly concerned with the expression of resistance by host cells. The high rates of decarboxylation of exogenous IAA might be only fortuitous expression of other metabolic activities those were actually concerned with resistance but that could govern simultaneouly metabolism of exogenous compounds unrelated to resistance or susceptibility. It had been assumed that IAA oxidase might control IAA concentration. The increase in IAA was associated with growth disturbances and reduction in IAA oxidase activity in homogenate of infected tissues. The drastic fall in IAA oxidase activity was the characteristic feature of the progressive brown spot infection. There might be some host parasite interaction at initial stage resulting in decline of auxin and protein contents and increase in IAA oxidase activity. Infection of the plant resulted in injury and other deformities which in turn causes high enzyme activity[23].

In the determination of the isoenzymic activities, the progressive infection was marked according to the bands which performed low electrophoretic mobility due to the higher mass.

The appearance of intense bands during electrophoretic separation was observed in healthy, moderate, heavily infected and *T. viridae* treated seedlings. It might be the resultant changes in expression of genes involved in healthy and infected category. Thus, it was proposed that higher activity of peroxidase in both the biochemical methods and by Native PAGE during the infection of *Curvularia penneseti* showed the multifacial involvement of peroxidase ranging from secondary phenol metabolism to lignin biosynthesis. In this respect such phenomenon was recognised as the primary reflection of brown spot disease establishment in young seedlings and later on at the maturity level. Such chemical changes degraded the quality of seeds during the storage. The increased activity of peroxidase in diseased tissues of lemon may attribute to the pathogens interaction with host[24].

The increased activity of peroxidase in infected seedlings was observed in pearlmillet seeds treated with biocontrol agent *Trichoderma viridae*. Kamalkanan *et al.*[25] also reported such activity in pretreated peppermint plants challanged with *Rhizoctonia solani. T. harzianum* and *T. hamatum* which were capable of antagonizing sensitive pathogenic fungi by producing antibiotics and lytic enzymes. It had been reported to induce systemic resistance in tomato, lettuce, pepper, bean and tobacco against gray mold, caused by *Botrytis cinerea*[26,27]. It is refered that *T. viridae* and *Curvularia penneseti* formulations consistently reduce the incidence of brown spot disease of pearlmillet and increase plant vigour index. Thus, it has been found that *Trichoderma viridae* and *Curvularia penneseti* shows induction of defence related enzymes and growth in pearlmillet against pathogens and can be utilized as an ecofriendly, inexpensive, effective and integrated pest management.

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