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ESTERASE ISOZYMES IN DOWNY MILDEW RESISTANT PEARL MILLET CYTOLINES

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ABSTRACT

Downy mildew susceptible line Tifton 23A and Tifton 23B alongwith ten other resistant near isogenic derivatives of Tift 23A showed highly polymorphic esterase isozyme banding pattern by isoelectric focussing (pH 3.5-10.0) at three stages of growth. AT D2 all resistant near isogenic lines in both the sterile and fertile backgrounds were distinctly discriminated by a band with high intensity at pI 6.1 and low intensity at pI 6.9. All resistant lines except L111A and L111B were also characterized by intense band at pI 7.3 though band intensity in 126D2A and 126D2B was quite low. Discrimination of A and B lines of every pair was easily possible at D1 and D3 stages. The effect of sterile and fertile cytoplasms and nucleocytoplasmic interaction in estarase isozyme expression alongwith the role of isozyme in growth and development has been discussed.

Key words : Pearl millet, downy mildew, esterase isozyme, cytoplasmic male sterility, disease resistance.

The importance of electrophoresis and several isozyme systems including esterases has been studied and reviewed in relation to variety identification, genetics and evolution [1-4]. The aspect of isozyme polymorphism is the one which helps identification and discrimination of varieties and accessions. Isoelectric focussing though analogous to electrophoresis, is certainly a better technique for providing analysis of protein composition with very high resolution separating isozymes with pH differences of 0.01 as separate bands [5] and thus enhancing the possibility of showing polymorphism even between related lines. Villamil et al. [6] by isoelectric focussing found esterases to satisfactorily identify species, subspecies and cultivars in fescue without having any major influence of environment. There are reports of linkages between isozyme markers and different disease resistance genes [7-9] and differences in banding pattern of genic male sterility and fertility in flax [10]. Such information in millet, however, is scanty both for disease resistance and cytoplasmic male sterility.

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The development of several male sterile downy mildew resistant near isogenic lines (NILs) from susceptible line Tift 23A by back crossing or mutation by IARI group of researchers [11] provided a suitable genetic material for studying biochemical, ultrastructural and isozyme differences between susceptible and resistant and sterile versus fertile NILs. This material, therefore, has been subjected to esterase isozyme analysis by isoelectric focussing for finding out correlation between isozyme markers with resistance, cytoplasmic male sterility in specific and in growth and development in general.

MATERIALS AND METHODS

The materials for study consisted of 12 genotypes which were male sterile (A) and fertile (B) lines of Tifton 23, susceptible to downy mildew, and A and B of other resistant, 5054, 5141, (Back cross derivatives of Tift 23A), 5071m, a mutant of Tift 23A and L111 and 126D2 (almost immune and have Tift 23A cytoplasm) lines of pearl millet representing different sources of downy mildew resistance. Leaf samples were collected at 4 (D1), 6 (D2) and 8 (D3) weeks after sowing.

Esterase isozyme analysis was done by horizontal slab gel isoelectric focussing in thin layers of polyacrylamide gel. For this 0.5g material was ground in chilled pestle and mortar by using liquid nitrogen with 1.5 ml of 50 mM tris-HCl buffer (pH 7.6) containing 50 mM β-mercapto ethanol and 50 mM EDTA. Supernatant was got by centrifugation (10,000 rpm) for 15 minutes at low temperature. Polyacrylamide gel of 5 percent acrylamide containing two to three percent (v/v) carrier ampholite (pH 3.5- 1.0) was used in LKB Multiphore unit. Samples were applied by soaking uniform rectangular filter paper strips 10 × 5 × 1 mm in supernatant. Run was carried out for four hours at 500 volts, 10 mA current. Water was circulated for cooling the plate during run. After the run gel was stained by incubating in 50 ml of 50 mm phosphate buffer (pH 6_0) containing 1 ml of 1 percent napthyl acetate in 60% acetone and 25 mg of fast blue RR to room temperature, till the bands developed. The reaction was terminated by adding 7% acetic acid. The bands were scored on a trace paper by using a transilluminator making band marks corresponding to that present in the polyacrylamide gel soon after staining and photographs of D1 and D2 stages were also taken.

RESULTS AND DISCUSSION

Comparison of polymorphic isozyme pattern (Table 1 & 4, Fig. 1 & 2) revealed that at D1 and D3 stages it is not easy to differentiate between susceptible controls Tift 23A and Tift 23B and resistant NILs as a group. In contrast at D2 stage susceptible

Table 1. Intensity of different isozyme bands in a common female parent and the hybrids at D_1 and D_2 stages

At D_1 stage	5141A	BJ 104	BK 560	BD 111	BM 46
5.9	-	+	+	+	-
6.0	-	+	-	+	-
6.1		-	-	+	+
6.8	-	-	-	+	+
9.8	+	+	-	+	+
Total	17	18	16	20	18
Common 15 bands at pI = 4.2, 4.4, 4.7, 4.8, 4.9, 5.1, 5.2, 5.4, 5.5, 5.7, 5.8, 6.2, 6.3, 6.5, 6.7					
Extra in hybrids	-	2	1	3	1
Missing in hybrids	-	1	2	0	0
At D_2 Stage	5141A	BJ104	BK560	BD111	BM46
5.9	+	-	-	+	+
6.1	+++		-	+	+
6.5	+++	-	-	+	+
6.6	+++	-	-	+	+
6.9	+	-	-	+	+
7.3	+++	-	-	+	+
7.5	+	-	-	-	-
Total	20	14	13	19	19
Common	13 bands at pI = 4.2, 4.7, 4.8, 4.9, 5.1, 5.3, 5.4, 5.6, 5.7, 6.0, 6.3, 7.1, 9.8;				
Extra in hybrids	-	0	0	0	0
Missing in hybrids	-	6	7	1	1

- missing band/band not present; + faint band; ++ light band; +++ intense band

lines Tift 23A and Tift 23B could be discriminated by the additional intense band in resistant NILs at pI 6.1 and with a faint band at pI 6.9 (Table 3, Fig. 1). Similarly a band at pI 7.3 with discrenibly high intensity was seen in all resistant NILs except L111A and L111B. The band intensity at pI 7.3 in 126D2A and 126D2B was quite low. It has been argued [6] that absence of a band might reflect a low protein concentration rather than its complete absence within the extract prepared for assay. It is also to be noted that L111 and 126D2 are immune genotypes and the absence or low activity of isozyme at pI 7.3 in comparison to other resistant lines might be

Table 2. Intensity of esterase isozyme bands at different pI values in sterile (A) and fertile (B) lines of pearl millet at D_1 stage

pI	Tifton 23A	5054A	5141A	5071A	L111A	126D2 A	Tift 23B	5054B	5141B	5071B	L111B	126D2 B
4.5	+	-	-	+	+	-	+	+	+	+	-	-
4.7	+	+	+	+	+	+	+	+	+	+	-	+
4.8	+	-	+	+	+	-	+++	+++	+++	+	-	+
4.9	+	-	+	+	+	+	+++	+++	+++	+	+	+
5.1	+	-	+	+	+	+	+++	+++	+++	+	+	+
5.2	+	-	+	+	+	+	+++	+++	+++	+	+	+
5.4	+	+	+	+	+	-	+++	+++	+++	-	-	+
5.7	+	+	+	+	+	+	+++	+++	+++	+	-	+
5.9	+	-	-	-	-	-	+	-	-	+	-	-
6.0	+	-	-	-	-	-	+	-	-	+	-	-
6.1	+	-	+	-	+	-	+	+	+	+	+	+
6.2	+	+	+	+	+	+	+++	+++	+	+	-	+
6.3	+	+	+	+	+	+	+++	+++	+++	+	-	+
6.5	+	-	+	-	-	+	+++	+++	+	-	-	+
6.7	-	-	+	-	-	-	-	+	+	+	-	-
6.8	-	+	-	-	-	-	+	+	+	-	-	-
7.0		+	-	-	-	-	+	+	-	+	-	-
7.1	-	-	-	-	-	-	+	+++	+	-	-	-
7.4	-	-	-	-	+	-	+	+++	+	-	+	-
9.8	+	+++	+	+	+	-	+++	+++	+	+	-	+
Total	20	12	17	15	17	12	23	22	21	18	9	17
Common in all 4 bands at pI 4.2, 4.4, 5.5, 5.8												
Extra to 23A		1	1	0	1	0	3	4	4	0	1	0
Missing from 23A		9	4	5	4	8	0	2	3	2	11	4

- missing band, + faint band, ++ light band, +++ intense band

responsible for differential resistance mechanism in them. Since isozyme markers are generally stable, codominant and not influenced by pleiotropy and isozyme variations are due to amino acid sequence differences and could be generally parallel to nucleotide sequence changes of DNA of the isozyme genes [12], the bands at pI 6.1, 6.9, and 7.3 like the previous reports [7-9] could be correlated with downy mildew resistance. However, this needs to be confirmed by inheritance studies.

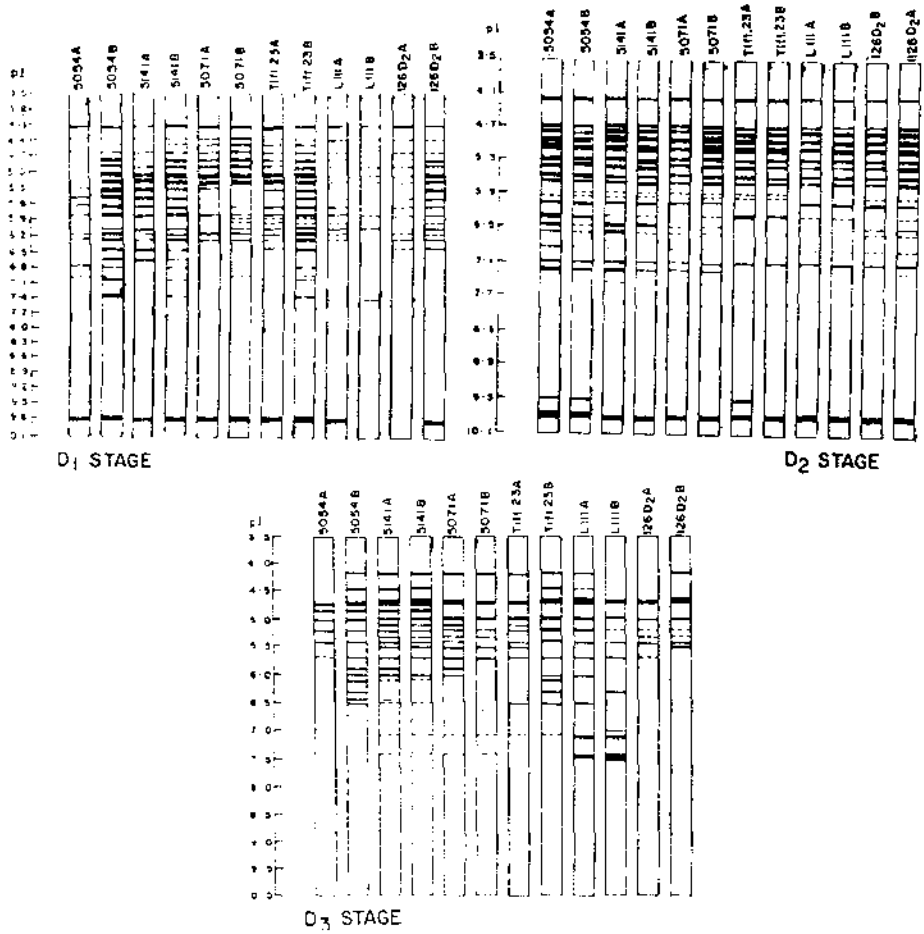

Fig. 1. Esterase isoenzyme pattern in different pearl millet genotypes

Table 3. Intensity of esterase isozyme bands at different pI value in sterile (A) and fertile (B) lines of pearl millet at D_2 stage

pI	Tifton 23A	5054A	5141A	5071A	L111A	126 D2A	Tift 23B	5054B	5141B	5071B	L111B	126D2 B
5.9	+	-	+	+	-	-	+	+	+	+	-	-
6.0	+	+	+	-	-	+	+	+	+	+	-	+
6.1	-	+	+++	+	+	+++	-	+	+	+	+	+
6.3	+	+++	+	-	-	-	+	+	+++	+	+	+
6.5	-	+	+++	+	+	+	-	+	+	+	+	+
6.6	-	+++	+++	+	-	+	-	+	+++	+	-	+
6.9	-	+	+	+	+	+	-	+	+	+	+	+
7.3	-	+++	+++	+	-	+	-	+	+	+	-	+
7.5	-	-	+	-	-	-	-	-	-	-	-	-
9.5	+	+	-	-	-	-	-	+	-	-	-	-
Total	15	19	28	17	14	17	14	20	19	19	14	18
Common in all 11 bands at pI 4.2, 4.7, 4.8, 4.9, 5.1, 5.3, 5.4, 5.6, 5.7, 7.1, 9.8												
Extra to 23A	-	5	6	5	3	5	1	5	5	5	3	5
Missing from 23A	-	1	1	3	3	3	1	0	1	1	3	2

- missing band; + faint band; ++ light band; +++ Intense band

The age dependent expression of isozymes has generally been reported [12] therefore, it can be concluded that D2 stage is the most suitable stage for discriminating lines susceptible or resistant to downy mildew by isozymes than D1 and D3. Generally maximum external manifestation of downy mildew during D2 also supports the possibility of maximum expression of factors for resistance during this stage. Apart from this, at D1 stage, bands at pI 5.9 and 6.0 with low intensities, present in susceptible lines, were absent in all the resistant lines except 5071B (Table 2). These bands, therefore, could also be used as discriminating markers.

Villamil *et al* [6] have stated that quantitative (band intensity) differences detected in samples subjected to identical experimental condition, such as those revealed in the same gel, provided a reliable comparison for the identification of otherwise similar cultivars. The presence of esterases in an appresoria of *Venturia inaequalis* has been suggested to be associated with cultivar penetration [13] and thus showing correlation with susceptibility or resistance. However, the reports regarding the exact role of esterase in resistance in different host pathogen systems are not available. So visibly higher band intensity, seen in most of the resistant NILs than susceptible lines at D2, could perhaps be used to discriminate susceptibles from the resistants in addition to the qualitative (band number) differences already discussed irrespective

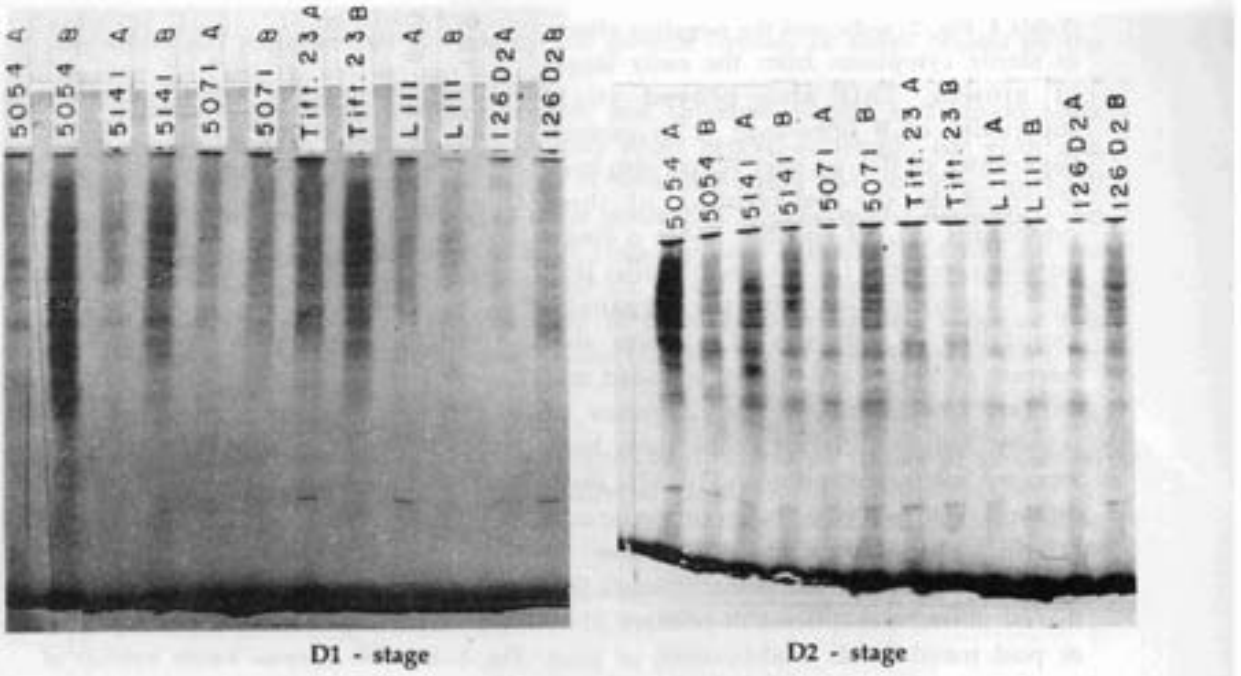

Fig. 2. Esterase isoensyme pattern in different pearl millet genotypes

of knowing the exact role played by this enzyme in mechanism of resistance. The presence or absence of some different isozyme bands of peroxidase and polyphenol oxidase in some downy mildew susceptible or resistant pearl millet lines have also been reported [14] but the nature of genetic material studied could not give any unambiguous correlation of bands with susceptibility or resistance. The higher esterase activity may reduce the metabolic imbalances in the pathogen attacked cells by providing quick energy by catabolizing esters and thus could perhaps be correlated with disease resistance.

On the basis of banding pattern at all the three stages of growth no common criterion of discrimination between sterile and fertile cytoplasm was observed. The individual pairs of A and B lines could, however, be discriminated by looking at the band pattern more easily at D1 and D3 stages than at D2 where differences between A and B lines of each pair were observed to be the least. Distinctly higher isozyme band numbers and intensity in B than A except L111 (Table 2, Fig. 2) at D1; equal or higher band number except Tift 23 and 5141 and slightly higher intensity of B over A in most of the pairs at D2 (Table 3, Fig. 2) and higher band number in half of the pairs and slightly higher intensity in B over A in most of the pairs

(Table 4, Fig. 2) indicated the negative effects of sterile cytoplasm from the early stages of growth. This also proved slight superiority of B lines over A in general. Superiority of B lines over A has also been observed in the background of three cytoplasms (S1, S2 and S3) under a single nuclear genotype, L110, in pearl millet [15].

Cytosolic but not the organelle localization of esterases [12] points that esterase isozymes are nuclear encoded and so far, in general, 4 to 12 zones of activity/loci in different crops have been reported for their expression [16, 17]. Thus, differences in isozymes between sterile and fertile cytoplasms might be due to differential post transcriptional changes like that of differential splicing of primary RNA or post translational modifications of gene products in the sterile or fertile cytoplasmic backgrounds. This perhaps indicated the main effect of cytoplasm in gene expression or nucleocytoplasmic interaction at biochemical level. However, information is wanted whether the organelle genes for esterases in pearl millet are present or not.

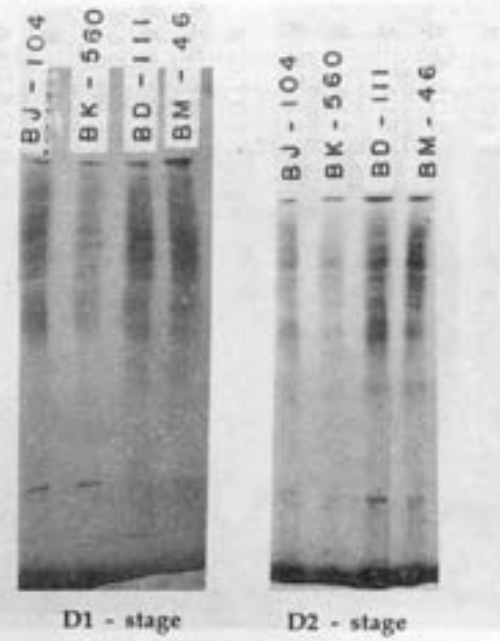

Fig. 3. Esterase isozyme bands hybrids of pearl millet

Contrary to the results of this study more band intensity in anthers of A lines than B in sorghum [18] and no differences in band number and intensity in anthers of maize [19] have been observed. In flax, difference was not observed in band pattern of esterases and peroxidases throughout the vegetative growth but significant difference was found in stamens of sterile and fertile plants [10]. Tissue specificity of gene expression for isozyme [12, 16] and differential effects of cytoplasmic backgrounds of different crop species could be the reasons for such contradictory results for sterile and fertile cytoplasms over crops.

There were no apparent differences between A and B lines in vigour and growth though B lines were superior to A in esterase isozymes and free amino acids [20] in leaves. A lines are invariably more susceptible to stress and diseases than B. Probably higher levels of esterases which catalyse variable substrates [21] under different metabolite concentrations and pH in different subcellular compartments [12] having differential role in growth and development, may be responsible in B lines for balancing the shock of stress and diseases than in A lines. The higher pool of free amino acids in B lines may help extra protein synthesis, needed to supplement

the additional requirements to cope up the general stresses or stress caused by the disease.

However, in maize in sterile anthers and spikelets, sugars and amino acids were higher than in fertil one [22]. Higher plant height, earlength, ear girth and tiller number in A lines of S1, S2 and S3 cytoplasms having common nucleus, L110 (isonuclear condition) and a group of other genotypes of pearl millet were observed than in their corresponding B lines [23]. Thus, perhaps nutrients not utilized for pollen formation are probably channelized for morphological attributes though no report of higher sugar and amino acids is there in pearl millet. Whether this channelization leads to higher biomass production and grain yield or not is the topic for investigation.

Table 4. Intensity of esterase isozyme bands at different pI values in sterile (A) and fertile(B) lines of pearl millet at D_3 stage

pI	Tifton 23A	5054A	5141A	5071A	L111A	126D2 A	Tift. 23B	5054B	5141B	5071B	L111B	126D2 B
4.2	+	-	+++	+	+++	-	+++	+++	+	+	-	+
4.5	-	-	+++	-	+	-	+++	+	+	-	-	+
4.8	-	+	+++	-	-	-	-	+	+	-	-	-
5.1	+	-	+	+	-	-	-	-	+	-	-	+
5.3	-	-	+	+	-	+	-	-	+	+	-	+
5.4	+	+	+	+	+	+	+	+	+	-	+	+
5.5	+	-	+	+	-	-	-	-	+	+	-	+
5.7	+	+	+	+	+	+	+	+	+	+	-	+
5.9	-	-	+	+	-	-	-	+	-	-	-	-
6.0	-	-	+++	+	+	-	+	+	+	-	-	-
6.1	-	-	+	-	-	-	+	+	+	-	-	-
6.3	-	-	-	-	-	-	+	+	-	-	+	-
6.4	-	-	+	-	-	-	-	+	-	-	-	-
6.5	+	-	+	-	+	-	+	+	+	-	-	-
7.0	-	-	-	-	-	-	-	-	-	+	-	+
7.1	+	-	+	+	+	-	+	-	+	+	+++	-
7.4	-	-	+	+	+	-	-	-	+	+	+++	-
Total	10	6	18	13	11	6	14	14	16	9	9	9
Common in all 3 bands at pI 4.7, 5.0 and 5.2												
Extra to 23A	-	1	7	4	2	2	4	7	6	2	4	2
Missing from 23A	-	5	0	1	2	5	2	3	0	3	4	3

- missing band, + faint band, ++ light band, +++ intense band

From breeding point of view biochemical inferiority of sterile cytoplasm as revealed in this study and also reported by Smirnova *et al.* [24] in maize for cholorophylls, structural abnormality of mitochondria as observed in the tapetal cells of microspore of sterile lines of sugarbeet [22] and in general higher susceptibility of sterile cytoplasm to stress and diseases will perhaps not favour limited cytoplasmic base even though there might be channelization of nutrients, not utilized for pollen formation, for morphological attributes. Therefore, cytoplasmic base of cms lines in hybrid seed production programme needs to be properly reviewed by the breeders from time to time for its successful exploitation.

The 12 genotypes under study showed at D1 stage a total number of 24 isozymes bands at various pI values followed by 21 at D2 and 20 at D3 (Table 1-4). Since all the resistant NILs are derived from Tift 23A, as expected, common bands among genotypes were found during all stages of growth, the highest being at D2 the peak growing period. In each genotype total isozyme bands varied at D1 and D2 with slightly higher or lower number but reduction was drastic at D3. In 5141A and 5141B the reduction was least over all the stages and so in this genotype isozyme expression was seen to be least influenced by age of the plant. Esterase isozyme gene activity as measured from band stain intensity also reduced drastically at D3 stage.

The qualitative and quantitative diversity was observed in sterile and fertile cytoplasms and between susceptible and resistant lines differentially over growth stages. Thus differential role of esterase isozymes in general growth and development is evident as has also been discussed by Herraman and Toole [25].

The growth stage specific bands in all or some of the genotypes were observed and bands at pI 4.4, 5.8, 6.2, 6.7 and 6.8 were present only at D1; at pI 5.6, 6.6, 6.9, 7.3, 7.5 and 9.5 at D2 and at pI 5.0 and 6.4 at D3 stage. The appearance of isozyme bands at specific pI in all or different genotypes at different stages of growth are perhaps required to catabolize different substrates for which these have the preferences [21] under different metabolic concentration and pH in different subcellular compartments [12] and thus have a role in growth and developments depending on genotype and age requirements of the plants. Thus perhaps the stage specific polymorphism is the physiological need of the species but at a particular stage it is least influenced by the environmental factors [6].

The variation of isozyme band number and intensity at a particular stage of growth among all sterile A resistant NILs and Tift 23A and all fertile B resistant NILs and Tift 23B (Table 1-4) suggested that differences among NILs are either due to effects of resistance genes in these lines which are from different sources or due to nucleocytoplasmic interaction. Nucleocytoplasmic interactions have also been observed by other researchers [23, 27-28] and such interactions are to be looked

properly for their exploitation in breeding for superior trait combinations. Hence growth stage and cytoplasmic background are important while interpreting biochemical parameters, like isozymes in relation to resistance or may be to other traits, depending on the genotypic materials used for exploiting the traits for crop improvement by selection.

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