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Role of Isozymes in Pearl Millet Improvement (*Pennisetum glaucum)*

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Abstract

Crop improvement programmes based on phenotypic markers are generally slower than desired because phenotypic assessment does not always reflect true genotypic performance. Moreover, phenotypic markers are affected by environment and may have loose linkages with the target trait(s). In crop plants genetic markers are useful for the creation of genetic maps, for map based cloning, and for many breeding applications, including marker assisted selection, back cross conversion, and genotyping. They can also be used for population studies and genetic resource management. Allozymes exhibit characteristic differential mobility of the enzymes originating from different alleles of a particular gene.

Protein markers, including seed storage proteins, structural proteins, and isozymes were among the first group of molecular markers exploited for genetic diversity assessment and genetic linkage map development. These molecular markers have advantages over phenotypic markers in a way that these are easy to screen, not influenced by the environment, and show co-inheritance (i.e., have tight linkages) with the target trait(s). Due to such properties protein/enzyme markers are being extensively used in crop improvement programmes to speed up selection efficiency of breeding methods through marker-assisted selection (MAS).

This mini-review describes the contribution of isozymes toward various aspects of pearl millet improvement with particular reference to disease resistance, establishment of species relationships, study of genetic and cytoplasmic diversity, identification of introgressed gene(s) etc.

Key Words: Pearl Millet, Pennisetum glaucum, isozymes, downy mildew, ergot, smut, protogyny, d_2 dwarfing gene, phylogenetic affinities, gene introgression

Zusammenfassung

Ein Beitrag der Isoenzymanalyse bei der Perlhirsezüchtung

Auf phänotypischen Markern basierende Zuchtprogramme sind im Allgemeinen langsamer als erhofft, da die phänotypische Beurteilung nicht immer den wahren genotypischen Hintergrund reflektiert. Phänotypische Marker sind durch die Umwelt beeinflussbar und können ihre Koppelung mit den Zieleigenschaften verlieren. Bei Kulturpflanzen sind genetische Marker nützlich, um genetische Karten herzustellen und zur Unterstützung verschiedener Zuchtprogramme, z. B. markergestützte Selektion, Rückkreuzungsprogramme und 'genotyping'. Sie können auch für Populationsstudien und zum Management genetischer Ressourcen genutzt werden. Alloenzyme zeigen unterschiedliche Mobilitat, verursacht durch verschiedene Allele eines Gens.

Protein-Marker inklusive Samenspeicherproteine, Strukturproteine und Isoenzyme gehörten zu den ersten Markersystemen, die genutzt wurden, um genetische Diversität und Koppelung zu beurteilen und darzustellen. Diese molekularen Marker haben gegenüber phänotypischen Markern die Vorteile, dass sie leicht 'screenbar' sind. Sie werden nicht von der Umwelt beeinflusst sind eng mit den Zieleigenschaften gekoppelt. Wegen dieser Eigenschaften wurden Protein/Enzym-Marker sehr häufig in markergestützten Zuchtprogrammen genutzt, um die Selektionseffizienz zu erhöhen.

Diese Zusammenfassung beschreibt den Beitrag der Isoenzymanalyse bei der Perlhirsezüchtung in Bezug auf Krankheitsresistenz, Verwandtschaftsuntersuchungen von Arten, Studium der genetischen und cytoplasmatischen Diversität, der Identifikation eingekreuzter Gene etc.

Schlüsselwörter: Perlhirse, Pennisetum glaucum, Isoenzyme, Mehltau, Mutterkorn, Vorweiblichkeit, d_2-Verzweigungsgen, phylogenetische Verwandtschaft, Genintrogression

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1 Introduction

Crop improvement programmes based on phenotypic markers are generally slower than desired because phenotypic assessment does not always reflect true genotypic performance. Moreover, phenotypic markers are affected by environment and may have loose linkages with the target trait(s). Molecular markers have advantages over phenotypic markers in a way that these are easy to screen, not influenced by the environment, and show coinheritance (i.e., have tight linkages) with the target trait(s). Molecular markers are largely of two types, (1) protein/enzyme markers, and (2) DNA markers. In this paper, the role of enzymatic markers (isozymes) in pearl millet improvement is critically reviewed.

The term 'isoenzyme' was coined by Markert & MoIler (1959) to describe different molecular forms (i.e., having different molecular weights) of an enzyme with the same substrate specificity. Hunter & Markert (1957) proposed the term "zymogram" to refer to the lanes in which isozymes travel and their location is displayed according to their molecular weights. Detection of isozyme(s) using electrophoresis techniques has been extensively used for the characterization and identification of species, inbred lines, isogenic lines, and crosses in plant breeding studies. In pearl millet, enzyme diversity has mainly been studied in relation to disease resistance/susceptibility, as genetic markers for the construction of linkage maps, to assess taxonomic/phylogenetic affinities within the species, and to know the genetic variability in relation to geographical distribution and evolution. For brevity and systematic reading enzymes and their abbreviated symbols have been summarized in table 1.

ADH was the first enzyme system whose genetics has been worked out in pearl millet (Banuett-Bourillon & Hague, 1979; Banuett-Bourillon, 1982a,b). Another enzyme system subjected to a detailed study is that of EST (Sandmeier et al., 1981; Tostain & Riandey, 1984; Subba Rao et al., 1989) and MDH was studied by Tostain & Riandey (1985) and Lavergne *et al.* (1986). The inheritance pattern of some other enzymes 6-PGD, CAT, GOT, POX ,PGM, Phos and PGI have been studied by Trigui et al. (1986).

These enzyme systems have been used in pearl millet for various purposes as reviewed hereunder.

2 Disease resistance

EST and/or POX enzymes have been studied in relation to resistance/susceptibility to ergot *(Claviceps fusiformis)* and downy mildew *(Scherospora graminicola)* by various workers (Gupta et al., 1980; Chahal et al.,1986, 1988; Kumar et al., 1987; Shekhawat et al., 1984). Gupta et al., (1980) analysed EST and POX patterns of 8 downy mildew susceptible and resistant varieties during seedling stage. The number of POX isozyme bands of high molecular weight showed variation for number of bands and their intensity for both the enzymes in resistant and susceptible varieties. The number of POX bands were more in resistant varieties than in the susceptible ones; a reverse pattern was observed for ESTs. Satija et al., (1983) compared the amount of phenol and PPO isozyme activity in 1 2 genotypes having differential degree of resistance to downy mildew. Observations recorded at two growth stages (30 days and 50 days) particularly crucial for disease development revealed maximum amount of phenols at 30-days growth stage in the immune genotype L5 and minimum in susceptible genotypes, L10 and in A7. The ranking was also similar at 50-days growth stage but with much higher content. PPO activity had a linear relationship with resistance. The immune genotype (L5) was characterized by presence of an anodal band (Al) at 30-days stage whereas no single band difference was identified at 50-days stage however, the bands possessed differential intensity. Thukral et al., (1983) determined genetic and/or biochemical variation for POX and PPO enzymes among pearl millet lines showing differential reaction to downy mildew. Significant variation occurred for the pattern as well as intensity of bands. Even with within line variation it was possible to differentiate the resistant lines from the susceptible ones on the basis of certain bands, however, no single band clearly distinguished all the resistant genotypes from the susceptible ones. They suggested that the pattern and intensity of POX and PPO isozymes could be fairly correlated with resistance to downy mildew in pearl millet.

Tab. 1
Enzymes and their symbols used in the text

Name of Enzyme	Symbol Used
Beta-amylase	α-amylase
6-Phosphoglutamate dehydrogenase	6-PGD
Acid phosphatase	ACP
Alcohol dehydrogenase	ADH
Amylase	Amylase
Catalase	CAT
Esterase	EST
Glutamate dehydrogenase	GDH
Glutamate oxaloacetatetransaminase	GOT
Lactate dehydrogenase	LDH
Leucine aminopeptidase = Cytosolaminopeptidase	AMP
Malate dehydrogenase	MDH
Malic enzyme	ME
Peroxidase	POX
Phosphatase	Phos
Phosphoglucoisomerase	PGI
Phosphoglucomutase	PGM
Polyphenoloxidase	PP0
Shikimate dehydrogenase	SKDH
Superoxide dismutase	SOD

Subsequently, Kumar et al., (1987) studied five genotypes having differential response to downy mildew and correlated the degree of resistance with the presence or absence of a cathodal band (C2) and an anodal band (A4). Shekhawat & Arya (1979) reported highest POX activity in the suppressed earheads followed by green-ear initial stage, diseased half of half-deformed ear-heads, completely proliferated ear-heads and diseased leaves over their healthy counterparts. In another study Shekhawat et al., (1984) found that the number of POX isozymes followed the same line (Table 2). It is evident that POX banding pattern was similar in healthy panicle and healthy half deformed panicle; and between panicle at initial stage of disease and diseased half panicle. However, few bands appear only after the infection has started, thus indicating that the POX is not the cause of resistance but is the effect of susceptibility. Several other studies have also revealed that POXs are the effects rather than the cause of resistance (Seevers et al., 1971). The cause and effect relationship, however, can be studied only by analyzing the material (resistant and susceptible genotypes) grown under disease free as well as diseased conditions. Most of the studies discussed above do not clearly mention the disease conditions of the experimental plot.

Later on, Chahal et al., (1988) also studied the POX isozyme pattern in five downy mildew resistant, five susceptible inbred lines and downy mildew-free plants (from downy mildew sick plots) of susceptible lines of pearl millet. For isozyme analysis, samples comprising of top internodes from 30-days old plants were collected. POX isozyme pattern revealed six anodal (A1-A6) and 11 cathodal (C l - C l1) bands. Band C9 was specifically present in resistant plants of resistant lines and disease free plants of otherwise susceptible lines. The presence of the unique band suggested its possible involvement in disease resistance mechanism. These results were in accordance with the observations of Thukral et al., (1983) on pearl millet downy mildew, where the presence of an extra cathodal band in resistant plants has been reported. The involvement of two other isozymes (C5 and C6) was also indicated in the resistance mechanism.

Tab. 2
Peroxidase banding pattern in downy mildew affected plant parts of pearl millet

Band	HL	DL	HE	1/2D	IS	1/2E	CP	SH
A13	+	+	+	+	+	+	+	+
A12	+	+	+	+	+	+	+	+
A10	-	-	-	-	+	+	-	+
A9	-	-	-	-	+	+	-	+
A8	-	-	-	-	-	-	-	+
A7	-	-	-	-	+	+	-	+
A6	-	+	-	-	-	-	-	-
A5	-	+	-	-	-	-	-	-
A4	-	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+
A2	+	+	+	+	+	+	+	+
A1	-	-	-	-	+	+	+	+

HL = Healthy leaves, DL = Diseased leaves, HE = Healthy ear-head, 1/2D = Healthy half of deformed ear-head, IS = Green-ear initial stage, 1/2E = Diseased half ear-head, CP = Completely proliferated ear-head, SH = Suppressedear-head

Prepared from Shekhawat et.al. (1984)

Chahal et al., (1986) analyzed five each of the ergot susceptible and resistant pearl millet lines (field conditions not mentioned) for their POX isozyme patterns and observed considerable differences in the number and intensity of POX isozymes between the resistant and susceptible genotypes. The enzyme patterns in all the resistant lines were similar, except ICMPES-8 which showed only two cathodal isozymes, C3 and C5, and its mean ergot severity was also relatively high (15.5 % as compared to 0.8 to 1.9 % in resistant and 31.5 to 52.5 % in susceptible genotypes). Based on their and earlier results, Gupta et al., (1980) suggested that POX, if not the only factor, is one of the major factors related to disease resistance. Though, it is difficult to draw a definite conclusion based on different patterns of POX isozymes in these limited number of resistant and susceptible genotypes, further studies involving large number of lines are required to generalize these conclusions.

The above studies indicate that POX isozyme pattern may be utilized as selection criterion for screening downy mildew resistant plants in pearl millet because of the presence of cathodal band(s) in both, the resistant and the disease free susceptible plants. Liu et al., (1988) classified millet cultivars into resistant, susceptible and highly susceptible of smut based on peroxidase activities at seedling and heading stage. Poongodi (1996) based on his study also concluded that male sterile lines which has better expression of peroxidase may be used in hybridization to evolve disease resistant varieties of pearl millet.

3 Cytoplasmic diversity/cytoplasmic male sterility

A number of CMS sources have been identified in pearl millet. These have been characterized based upon fertility restoration in the field conditions, and also based on their phenotypic characters. These studies are not consistent over environments, thus, display a variable image of each CMS system in different environments. Various workers have tried to characterize these sources at enzyme level to get a more clear picture, for example, Phul et al., (1987) reported better expression of enzymes in sterile anthers. They emphasized the role of peroxidase in breaking down various metabolites that are important for the formation of fertile anthers. Mangat & Virk (1992) analyzed seven male-sterile lines [81A_1 (A_1 cytoplasm), Pb 310A_2(A_2 cytoplasm), Pb 31 1A_2(A_2 cytoplasm), ICMA88001 (A_V cytoplasm), Pb 406A_3 (A_3 cytoplasm), Pb 402A_1 (A_1 cytoplasm) and Pb 402A_3 (A_3 cytoplasm)) and their maintainers for POX, ACP and EST isozymes. The banding pat-

terns were different between and within cytoplasmic sources. Even the male-sterile and -fertile versions of the same cytoplasm also showed differences indicating that the cytoplasmic differences can be ascertained by isozyme analysis. To avoid the error due to nuclear-cytoplasmic interaction, Virk et al., (1993) involved five near-isonuclear versions of 81 A_1 and two of Pb 402A_3 CMS lines and their corresponding maintainer lines, 81 B and Pb 402B and recorded 14 agronomic, 2 disease-resistance traits, and 3 enzyme systems (P0, ACP and EST) of unbursted anthers and leaves. They observed significant differences for several agronomic traits among the lines, and also found variable POX banding patterns which up to some extent could reveal differences between cytoplasms. However, differences for isozymes of ACP and EST were not clear cut. They could differentiate different cytoplasms by looking at the number and/or intensity of band(s) but could not find unique band(s) which could characterize a particular cytoplasmic source.

Though Virk et al., (1993) reported the inability of ESTs to differentiate cytoplasmic sources, Thakur & Murty (1993) could discriminate and identify different cytoplasms on the basis of EST isozymes. They used leaf samples of three iso-nuclear lines containing the A_1, A_2 and A_3 cytoplasms (having nuclear genome of Li 10) and their maintainers [L 11O(A_1)B, L 110(A_2)B and L 110(A_3)B]. Leaf samples were analyzed at 4, 6 and 8 weeks stage after sowing and observed different isozyme patterns among the lines at all three growth stages but the discrimination and identification of cytoplasms was easiest at 6-week stage by individual or group of isozyme bands.

4 Genetic diversity and geographical distribution

Taxonomical and botanical data suggested four centers of diversity for pearl millet in Africa (Porteres, 1962), while Harlan (1971) and Brunken et al. (1977) suggested the existence of independent domestication centers. Tostain et al. (1987), Tostain & Marchais (1989) and Tostain (1992) made extensive studies of enzyme diversity in cultivated and wild pearl millet from Africa and India. Tostain et al. (1987) studied eight enzyme systems in 74 cultivated samples (37 samples each of early and late group) and 8 wild millets from West Africa and observed that cultivars of pearl millet formed three distinct groups (wild types, early and late maturing cultivars), which enabled them to put forward hypothesis on the evolution of pearl millet which states that the West African late cultivars were derived from a common cultivated early complex, which must have been distributed across the Sudanian zone. This complex might have been modified later by the limited gene flow with local early maturing cultivars. The highest enzyme diversity was shown by the early maturing group, whereas the late group showed the lowest.

Tostain & Marchais (1989) extended the survey of enzyme polymorphism by including 199 populations (including 74 populations studied earlier) from other regions of Africa and from India. These were studied for eight enzyme systems which included: ADH, β-EST, CAT, PGI, PGM, 6-PGD, GOT and MDH. Based on the results obtained, they proposed an evolutionary hypothesis which stated, multiple domestications in the Sahel, creation of early-maturing cultivars and their migration eastward to India plus a southward migration to Sudanian zone, and creation of late-maturing cultivars and their migration simultaneously westward, eastward, and southward to southern Africa. The Harlan's (1971) the non-center outline fitted very well with Tostain & Marchais (1989) enzyme data, and the evolutionary outline proposed above for pearl millet showed a remarkable parallelism with those proposed for *Sorghum* and finger millet, *Eteusine coracana* (Purseglove, 1976).

Furthermore, Tostain (1992) studied isoenzyme polymorphism for 8 enzyme systems in 188 accessions of wild millet, *P. glaucum* L. subsp. *monodii* [SYN *P. violaceum* (Lam) L. Rich.], representative of the species' geographic distribution in Africa. Variation in isoenzyme banding pattern corresponded to geographical zonation in five groups:

1. Western Group (Senegal, Mauritania, western Mali),
2. Central Group (eastern Mali and Niger),
3. Air Group (Air Mountains of Niger),
4. West Chad Group, and
5. Darfur Group (encompassing eastern Chad and western Sudan).

Overall Nei's diversity was equal in wild and cultivated millets but their locus by locus diversity was different. Wild millet, particularly populations growing far from the cultivated crop (allopatric wild accessions), shows most diversity from the cultivated millets for the alleles *Got A, Pgd A* and *Cat A,* whereas cultivated millets are the most diverse from each other for *Pgm A* and *Pgi A*. Based on enzyme allele distribution, Tostain further interpreted that the allopatric wild millet populations were more divergent from cultivated populations than sympatric wild millets and the cultivated millets from western Mali were closest to wild millets.

5 Reproduction behaviour

Schmelzer and Renno (1999) compared the type of reproduction in polyploid and diploid texa using isozyme electrophoresis for five enzyme systems on 1180 progeny from 118 genitors belonging to five taxa of *Pennisetum* sect. *Brevivaliuln.* A total of 112 different isozyme phenotypes were found, over all taxa. Genotypic variation was found among all progeny of the diploid populations of *P. polystachion* and *P. subangustum***,** as a consequence of their sexual reproduction system. At the polyploid level the type of reproduction appears to be predominantly agamic, but genotvpic variation in the progeny was not

rare: five tetraploid and one hexaploid *P. pedicellatum,* one pentaploid and one hexaploid *P. pedicillatum* and one hexaploid *P. hordeoides,* in a total of 90 genitors. Genetic relationships have been observed between the diploid sexual *P. polystachion* and *P. subangustum,* and, to a lesser extent, with the tetraploids of the same taxa as well. Tetraploid *P. polystachion* and *P. pedicillatum* share genotypes with most titer chromosomal taxa.

6 Phylogenetic relationships

Probably, the first report related to interspecific relationships using proteins, EST enzyme pattern and phenolic compounds in pearl millet is that of Sujatha (1984). In another study, observations recorded on the distribution pattern of 1 5 phenolics, 23 proteins and 1 9 ESTs using paper chromatography and PAGE revealed that 1 2 species grouped into 5 clusters with 7 species in one cluster, 2 in another cluster, and 1 each in three clusters (Rao et al., 1988). Saideswara Rao et al. (1986) studied EST variability in five *Pennisetum* species. Subba Rao et al. (1988) reported existence of closer affinities among the species belonging to x = 9 type and that *P. mezianum* (x=8) was closer to species of x=9 series than to those with x=7. Later on, an extensive study was carried out (Hussain et al., 1990) in 1 2 species with putative base numbers of x=7, 8 and 9 using 4 enzyme systems viz., ACP, amylase, POX, and GOT. Their zymogram patterns appeared to be in agreement with available cytological data (Hanna & Dujardin, 1982; Minocha & Singh, 1971; Patil & Singh, 1964; Raman 1965). For example, the similarities (11-25%) in ACP, amylase and GOT patterns between *P. glaucum* and *P. purpureum* were consistent with the genome homologies reflected by the extent of chromosome pairing between the genomes of these species representing the x=7, 8 and 9 basic types. *P. setaceum, P. c!andestinum, P. violaceum* and *P. squamulatum* (x=9) showed affinities with species of the x=7 and x=8 types, supporting their alloploid nature. Most of the species examined showed distinct species-specific patterns. The phylogenetic relationships among the *Pennisetum* species of three taxonomic genepools have been analyzed by Lagudah & Hanna (1989). Variation in leaf ESTs, 6-PGD, SKDH, AMP, PGM and MDH have been observed. In the primary genepool, polymorphism for EST, AMP and SKDH was very high, as compared to the near monomorphism for 6-PGD. Two loci controlling leaf ESTs, *Estl* and *Est2,* were identified in the primary genepool. Cultivated and the wild pearl millet species differed for the allelic frequency distribution of *EstI* locus and the prevalent alleles (in primary genepool) of *EstI* were absent in *P. purpureum* (secondary genepool). A monomorphic band of the α-EST-specific *Est2* locus was identified in most of the secondary genepool accessions, *P. squamulatum* and an accession of *P. pedicellatum.* Most of the tertiary genepool species differed in the SKDH and EST patterns. They also observed that species of the *Brevivalvula* section are closely related on the basis of the 6-PGD and α-EST pattern, but the same are highly divergent on the basis of AMP, thus emphasizing that the choice of an enzyme system may lead to variable interpretations and phylogenetic relationships.

Besides establishing interspecific affinity, species-specific isozyme patterns were also noticed for most of the enzymes, which may be useful in the identification of the individual species as well as for their identification in the hybrid progenies/ filial generations.

Kaushal & Sidhu (1993) conducted chemotaxonomic studies to detect species relationship of the genus *Pennisetum* using POX, EST and ACP. Based on similarity index values calculated from the isozyme bands, 3 phylogenetic groups were identified comprising

(1) *P. glaucum, P. glaucum* subsp. *violaceum* and *P. purpureum,*
(2) *P. squamulatum* and
(3) *P. orientale* and *P. setaceum.*

They also observed that EST isozyme patterns were most effective in differentiating between different *Pennisetum* species since each species exhibited at least one unique band. Genetic diversity among cultivars from different countries was also depicted at isozyme level in another study (Sedogo and Tostain 1996). Schmelzer and Renno (1997) correlated genetic diversity due to ploidy level with isozyme diversity in pearl millet.

7 Confirmation of the introgressed gene in the hybrid genome

In addition to morphological observations (phenotypic markers), enzyme- and DNA-based markers have been used to examine the extent of gene introgression and to identify and select the hybrids. In a study on the transfer of genes governing apomictic mode of reproduction from *P. squamulatum* to pearl millet, Ozias-Akins et al., (1993) followed molecular markers (RFLP and RAPD) assisted selection, and confirmed the presence of *P. squamulatum* DNA in BC_3 population. By further analysis of advanced backcross populations, they could depict the co-inheritance of apomictic mode of reproduction and two of the molecular markers. In a study including *P. glaucum, P. squamulatum,* their F_1, F_2 and BC_1 hybrids, five enzyme systems and chromosome numbers were used to characterize them. The parental taxa easily separated from the hybrids whose morphological characters extensively overlapped. However, F_1s possessed characteristics of both parents to varying degrees while BC_1s with *P. glaucum* as female parent showed closer resemblance to pearl millet than BC_1s obtained from crosses in which pearl millet was the male parent. The enzyme study revealed that AMP and 8-EST were better genetic markers for the two parental taxa and their hybrids than 6-PGD, PGM and SKDH. However, combining all five systems produced better separation of taxa than using any of the systems singly. Mar-

chais (1994) studied spontaneous introgression of wild genome into cultivated and vice-versa using morphometric and isozyme analysis of adjacent cultivated and spontaneous populations of pearl millet *(P. glaucum)* in Niger. The analysis revealed a unique continuous distribution of phenotypes ranging from a typical cultivated phenotype to one of a cultivated x wild hybrid. Based on the analysis, the natural population was subdivided into a major wild group and a hybrid (wild x cultivated) group. Cultivated millets displayed an equilibrium state between recombined domesticated and wild genes. The natural population, despite a high rate of immigration via pollen gene flow from cultivated plants, retained its genetic structure by some unknown method of isolation.

8 Cultivars identification

In pearl millet a number of different cultivars are available that include synthetic, composite, open-pollinated and hybrids. Discrimination between cultivars or lines by seed morphology is extremely difficult. Electrophoretic discrimination is, therefore, important for the efficient and precise operation of the seed certification schemes. Isoelectric focussing (IEF) has been used for distinguishing between and identifying cultivars and lines (HHB 67, H-77/833-2, 843A, HHB 50, H-90/4-5, and $81A_1$) of pearl millet (Varier & Cooke, 1992). The analysis of water-soluble seed ESTs was found to be potentially useful which showed clear differences between the eight cultivars studies and good replication of protein patterns (but complex banding pattern) from within each cultivar. This was the first reported demonstration of the use of IEF for discrimination between pearl millet cultivars/lines. It has been shown (Varier et al*.,* 1992) that the expression of pearl millet ESTs is unaffected by both, the site and season (year) of seed production in composite varieties. Shaista Halim (1997) reported that isozymes (esterase, glutamate oxaloacetate transaminase and peroxidase) were genotype-specific in pearlmillet hybrids, restorers and male sterile lines. Balma et al. (1996) conducted isozyme studies on sixty landraces of pearl millet representing genetic variability of eight countries of West Africa. Seed samples produced more variability and number of bands than leaf samples. Based on the results obtained, they discussed the importance of enzyme systems in detecting the variability among these species and also suggested further expansion of these studies to reach at concrete conclusions.

9 Assessment of degree of isogeneicity

Twelve tall and dwarf near-isogenic lines were developed at ICRISAT Asia Center, Patancheru with the objective of assessing the effect of d_2 dwarfing gene on grain yield and its component traits. Rai & Rao (1991) evaluated these pairs and reported that the d_2 gene or the genes linked to it affected many traits. It was not well understood whether the changes in plant characters other than height were due to environmental variation or pleiotropic effect of d_2 gene, due to linkage between the d_2 gene and other loci, or due to lack of isogeneicity. Chhabra et al*.,* (1996) used 12 enzyme systems (ADH, CAT, EST, GDH, GOT, LDH, MDH, ME, 6-PGD, PGI, SKDH & SOD) to answer this question by determining the degree of isogeneicity between isolines of each pair. Enzymatic data clearly showed that isolines of these pairs are still segregating for many loci even after nine generations of selfing, thus, emphasizing the need of more selfings to achieve complete isogeneicity except the gene(s) for plant height to precisely determine the effect of d_2 gene on plant traits of economic importance.

10 Protogyny

There is a wide range of gap between the time of first style emergence and the time of first anther emergence. This variation is genetically controlled and is also influenced by temperature fluctuations. This mechanism is advantageous as it ensures nearly 100 % cross pollination, but there are also some disadvantages associated with this. Firstly, the style in the tip region may dry up before anthesis causing tip sterility and secondly if there is heavy rainfall during flowering time, there is sever pollen wash. Consequently, there is very high incidence of ergot and smut. Protogyny in relation to POX and EST variation was reported by Gupta et al*.,* (1980). The isozyme patterns were studied at the stages of complete ear emergence, first style emergence and first anther emergence of the earhead. The objective was to identify biochemical markers for screening of short duration of protogyny lines that would lead to check of loss due to tip sterility, disease infection and poor seed set. The results indicated that the POX activity decreased from the stage of ear emergence to the time of anthesis while the activity of EST showed just the reverse trend, in genotypes with short duration of protogyny. The intensity of bands of POX at style emergence and its decline at anther emergence was more important while the availability of EST at anther emergence was most determining factor in determining the shorter duration of protogyny.

11 Chromosome depletion

Hybridizing tetraploid pearl millet with *P. squamulatum* yields a partially fertile, but unstable, hybrid which loses its chromosomes through its quasi-sexual progeny. Busri & Chapman (1992) studied reproductive and isozyme variation in an unstable *Pennisetum* hybrid (induced tetraploid pearl millet, 2n = 4x = 28 x *P. squamulatum,* 2n = 6x = 54) progeny. From embryosac analysis and isozyme (PGI, 6-PGD, and GOT) studies of 30 F_2 (22 selfed, 8 open-pollinated) plants, they concluded that the possibility of chromosome depletion may be linked with the

variation of isozyme band patterns which need to be further examined.

12 Tissue culture

Clonal propagation of plants is one of the major achievements through tissue culture methods. However, the presence of considerable genetic variation in the cultures as well as in the plants derived from them is a matter of serious concern in clonal propagation and genetic transformation. Shenoy and Vasil (1992) investigated the extent of biochemical and molecular variation in 63 plants of *P. purpureum* regenerated from 3- to 24-week-old embryogenic callus cultivars. The calli were derived from cultured basal segments of young leaves and immature inflorescence obtained from a single field-grown donor plant. Enzyme analysis (14 isozyme systems) and DNA (mitochondrial, plastid and nuclear DNA RFLP) studies revealed no variation in a representative sample of regenerated plants, thus confirming earlier reports of genetic uniformity of plants derived from somatic embryos and highlighting their value both for clonal propagation and genetic transformation.

13 Aging effect

Loss in viability due to ageing during seed storage is reported to result in loss of nucleic acid and protein synthetic capacity (Robert et al., 1973; Ghosh & Choudhury, 1984). This could result in differences in banding patterns of proteins/enzymes between seed lots of the same variety having different levels of viability or vigor. EST banding pattern has been studied to examine the effect of ageing [under ambient and high humidity (75 % RH)-high temperature (35° C) conditions] on this enzyme in pearl millet (Varier & Dadlani, 1992). They found that the banding pattern of EST isozymes changed both under natural and accelerated ageing conditions. Bands with higher molecular weight present in freshly harvested seeds could not be detected after ageing: instead, some additional bands having higher mobility (lower molecular weight) were detected. They suggested that additional bands which appeared after ageing might have resulted from the breakdown of lower mobility bands (high molecular weight proteins) or may have been synthesized in response to the shock caused by accelerated ageing.

14 Establishing linkage relationship(s) between d_2 gene and enzymatic markers

The genetic linkage relations between the d_2 dwarfing gene and seven enzymatic marker genes *(Adh A, Est A, Mdh D, Pec A, Pg/ A, Pgm A,* and *Skdh A)* were evaluated in three crosses between semi-dwarf and normal inbred lines of pearl millet (Tostain, 1985). All the eight genes segregated in Mendelian fashion and various linkages were observed between *Pgi A* and *Pgm A* [4±4 centimorgans (cM)], between *Skdh A* and *Adh A* (11±7 cM), between D_2 and *Skdh A* (9±5 cM) and between D_2 and *Ad/i A* (17±8 cM). The order of linkages is: *AdhA-11 cM-SkdhA-9cM-*D_2. He suggested that the linkage between d_2 gene and *Skdh-A* could be used in the separation of D_2D_2 and D_2d_2 at the seedling stage. Subsequently, Chhabra et al., (1996) also studied this linkage relationship using 12 enzyme systems. Although no definite trend was observed for the presence or absence of isozyme band(s) in tall/dwarf near-isogenic lines, but one ADH band of very light intensity was present in all the tall isolines of three most polymorphic pairs and absent in dwarfs, thus, indicating the possibility of its linkage with the d_2 gene.

One of the objectives of millet breeding since 1968 has been to reduce the quantity of stover by reducing plant height while maintaining the robustness of the traditional local varieties in west Africa and India. Several major genes causing substantial reduction in plant height (i.e. genes controlling the height of the first internodes) have been reported in pearl millet (Burton & Fortson, 1966; Appa Rao et al., 1986). Of these, the d_2 dwarfing gene has been more widely used than others for the development of hybrid parents (Lambert, 1983). The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) continue to work on this type of dwarfing plants. Twelve pairs of tall and dwarf near-isogenic lines developed at ICRISAT in the diverse genetic background of three composites were evaluated for grain yield and yield components (Rai & Rao, 1991). The d_2 gene or the genes linked to it, on an average, reduced plant height by 42 %, grain yield by 14 %, and head girth by 8 %, but increased head length and number of tillers per plant by about 5-6 %. Days to 50 % flowering and seed weight were least affected by the d_2 gene (Rai and Rao, 1991). Although the preliminary tests showed the absence of pleiotropic effects of d_2 gene on fertility (Thakare & Murty, 1972), yet it is not well understood whether the changes in plant characters other than height are due to pleiotropic effects of the d_2 gene, due to linkage between the d_2 gene and other loci, or due to lack of isogenicity. Tall and dwarf pairs look morphologically similar except for height, but other field observations on various morphological traits indicate differences within pairs. However, since quantitative traits are affected by the environment, further investigation is necessary to confirm the degree of isogenicity within these pairs using molecular markers which are least affected by the environment and reflect the true image of genotype, hence they can be used to look at the degree of similarity within the pair of these tall/dwarf near-isogenic lines.

15 Assessment of gametophytic competition

Sarr et al. (1988) investigated gametophytic competition using pollen mixture technique. They used five millet genotypes viz., Ligui, Massye, $23d_2B$, Chinese and Thiotande' with well defined characteristics. These were characterized for EST and ADH patterns. The genetics of these enzymatic markers was already known (Sandmeier et al., 1981; Trigui et al., 1986). They assessed relative competitive ability of pollen by isozyme electrophoresis of progeny plants and concluded that autopollen competed better than various types of allopollen in case of Ligui genotype.

16 Other uses

In addition to the uses of isozymes described above, there are few reports about other applications of isozymes in pearl millet. Isozymes of α-amylase were studied during germination (Sheoran & Wagle, 1981) and it was found that the number of bands increased up to 48 h of germination and then decreased.

Sandmeier (1993) used ADH (locus *A1A1)* as an marker to determine selfing rates in pearl millet, and found that selfing rates of nine test plants (homozygous for ADH locus *A1A1)* varied from 2.2 to 21.7 %. Selfing rates were not significantly different within spikes of the same plant, except for one individual.

Sidhu et al. (1984) differentiated primary trisomics of pearl millet from its disomic sib on the basis of its disomic sib using peroxidase band intensity.

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