COMPARISON OF MOLECULAR MARKERS FOR DEVELOPING PEARL MILLET GENETIC LINKAGE MAPS

By

I. MURALI KRISHNA

A thesis submitted to the

FACULTY OF TECHNOLOGY

in partial fulfilment of the requirements for the award of the degree of

MASTER OF TECHNOLOGY
in
BIO TECHNOLOGY

ANNA UNIVERSITY
CHENNAI - 600 025

1997

BONAFIED CERTIFICATE

Certified that this thesis entitled "**COMPARISON OF MOLECULAR MARKERS FOR DEVELOPING PEARL MILLET GENETIC LINKAGE MAPS**" is the bonafide work of Mr. I. MURALI KRISHNA who carried out the research under my supervision. Certified further, that to the best of my knowledge the work reported here in does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion on this or any other candidate.

Signature of supervisors

Dr. N. Seetharama, Senior Scientist, CMBD, ICRISAT, HYDERABAD.	Prof. P. Kaliraj, Director, Centre For Biotechnology, Anna University, CHENNAI.

ACKNOWLEDGEMENT

I take this opportunity to express my deep sense of gratitude to my supervisor, Dr. N.Seetharama, senior scientist at ICRISAT for his valuable guidance during my investigation and in writing the thesis.

I express my sincere gratitude to Prof. Kunthala Jayaraman, Dean, Faculty of Technology, Anna University and Prof. P. Kaliraj, Director, Centre For Biotechnology for allowing me to work on this project.

I am also grateful to Dr. Manjula Sritharan and Dr. R.B.Narayanan for their help in getting me this project.

I like to express my gratefulness to Dr. Diwakar, Program Leader, TAFP and to Mr. K.D.V Prasad, Design and Development Division, ICRISAT for their help in carrying out my work at ICRISAT.

I wish to express my heartfelt gratitude and indebtedness to Leela, Research Associate, and Mr. Yosuf, CMBD, ICRISAT without whose help this work might not be in its present form.

I wish to specially thank Shyamala, Dr. Sairam, Dr. Hiren, Srinivas, Anand and Sravan for their help and valuable suggestions.

I specially thank Hanumantha Rao, Prashanth, Laxminarayana, Malla Reddy, and others in CMBD lab.

I also thank my friends Erik, Raj, Leela Prasad and other classmates for their help.

Abstract

Downy mildew (DM) disease caused by the fungus *Sclerosprora graminicola* (Sacc.) Schroet is the major yield reducer of pearl millet cropped in India and Africa. DM resistance is a complex, quantitatively - inherited trait. The available RFLP map of this crop developed at the Cambridge laboratory at Norwich, UK is useful for breeding for DM resistant pearl millets by marker-assisted selection and backcrossing. Further, the quantitative trait loci (QTLs) identified for resistance are also useful for searching for newer sources of resistance. The present work deals with identification of newer sources and markers for DM resistance in pearl millet. For this various types of molecular markers were compared. The marker types being compared includs Isozymes, RFLP, RAPD, and AFLP.

Among the five accessions of pearl millet selected for the study three (7042 DMR P_2 P_1, IP 18298, and IP18292) are downy mildew resistant and the other two accessions (843B P_5 P_2, and 23 D_2B) are downy mildew suseptible. Out of the eight isozyme systems tried three (peroxidase, esterase, alcohol dehydrogenase) detected polymorphism between the test entries. RFLP analysis of these five accessions with the homologous genomic clones (PSM) in combination with 2 to 4 restriction enzymes (*DraI, HindIII, EcoRI, EcoRV*) have revealed several polymorphic bands. RAPD assays with the same pearl millet accessions and 16 random primers showed that 50% of the RAPD primers identified one or more polymorphic bands. AFLP assay of two accessions 843 B P_5 P_2 and 7042 DMR P_2 P_1 revealed several bands in a 5% denaturing polyacrylamide gel. All the seven primers tested showed polymorphism. AFLP seem to be technique of choice for further characterization of power of resistance and genetic linkage mapping.

CONTENTS

CHAPTER NO	TITLE	PAGE NO
	LIST OF FIGURES	
	LIST OF TABLES	
1.	INTRODUCTION	1
	1.1 PEARL MILLET	1
	1.2 DOWNY MILDEW	2
	1.3 MOLECULAR MARKERS	3
	1.4 MAPPING IN PEARL MILLET	8
	1.5 OBJECTIVES OF THE PRESENT STUDY	9
2.	MATERIALS AND METHODS	10
	2.1 PLANT METERIAL	10
	2.2 ISOZYMES	10
	2.3 EXTRACTION AND PURIFICATION OF GENOMIC DNA	13
	2.4 RFLP ANALYSIS	15
	2.5 RAPD ANALYSIS	18
	2.6 AFLP ANALYSIS	20
3.	RESULTS AND DISCUSSION	22
	3.1 ISOZYME ANALYSIS	22
	3.2 RFLP ANALYSIS	24
	3.3 RAPD ANALYSIS	36
	3.4 AFLP ANALYSIS	41
4.	CONCLUSIONS	45
	REFERENCES	47

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
1	Zymogram of peroxidase	23
2	Agarose gel elsectrophoresis of recombinant pUC 18plasmid DNA	26
3	Agarose electrophoresis of PCR-amplified insert DNA	27
4	Agarose gel electrophoresis of restriction digested genomic DNA of 5 Pearl millet accessions	28
5	RFLP profile of 5 pearl millet accessions with PSM 858 probe	30
6	RFLP profile of 5 pearl millet accessions with PSM 634 probe	31
7	RAPD profile of 5 pearl millet accessions	37
8	RAPD profile of 5 pearl millet accessions	38
9	AFLP profile of pearl millet accessions in combination with E-AAC and different Mse primers	42

LIST OF TABLES

Table No	Title	Page No
3.1	Isozyme data of Pearl millet accessions	24
3.2	RFLP data of Pearl millet accessions	32
3.3	RAPD data of Pearl millet accessions	39
3.4	AFLP data of two Pearl millet accessions	43

CHAPTER 1

INTRODUCTION

1.1 PEARL MILLET

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] is the world's sixth most important cereal crop, occupying an area of 26 million hectares and having an annual production of 13 million tonnes (Govila 1994). In western Africa and northeastern India, it is the principle grain crop of the semi-arid and arid tropics, while in the USA it is one of the best annual summer forage crops for the drier regions. Pearl millet is the most drought tolerant domesticated cereal (de Wet 1987), and grow under a wide range of agroclimatic conditions, ranging from the tropics to hot areas of temperate zones. It's capacity to grow and yield reasonably well on sandy marginal soils, or on soils of low pH, high salinity or low fertility, makes it a staple food crop in many low-input, drought-prone agricultural areas of the tropics. In addition, under favorable environmental conditions it has nearly the potential to perform in per with other cereals, such as sorghum or maize with respect to both grain and fodder yield. Pearl millet is an indispensable food for millions of people inhabiting the semi-arid and arid tropics especially the poor. Flour and whole grains of pearl millet are used to prepare staple foods like unfermented bread (*roti*), cooked whole grains "rice", and thin or thick porridge. It is also used to prepare fermented bread, couscous, beer and popped and roasted snacks. The fodder can be used as feed for animals, building and fencing material, and fuel.

Pearl millet is the forth most important grain and fodder crop in India after rice, wheat and sorghum. It is cultivated over an area of 11-12 million hectares with an annual production of 5-6 million tones. The crop is grown in the arid and

semi-arid regions of the country mainly in the states of Rajastan (4.9 million ha), Maharastra (1.8 million ha), Gujarat (0.9 million ha), Uttar Pradesh (0.7 million ha) and Haryana (0.6 million ha). It is also grown in parts of Karnataka and Tamilnadu.

Pearl millet is an excellent species to research on, especially for genetic study. It is a diploid with 2n =2x=14 chromosomes and has a protogynous habit of flowering which allows it to be easily cross-pollinated and genetically manipulated (Hanna 1987). It has a haploid (1C) DNA content of about 2.5 pg (Bennet 1976), and possess abundant phenotypic variation (Brunken 1977).

1.2 DOWNY MILDEW

Downy mildew (DM), caused by *Sclerospora graminicola* (Sacc.) Schroet., is the most widespread and destructive pearl millet disease. *S.graminicola* is an obligate, diploid, biotrophic oomycete of the family Peronosporaceae (Shaw,1981). The disease has been reported in more than 20 countries (Safeeulla 1976) and is a major factor limiting the full exploitation of the high-yielding improved cultivars in India (Singh et al., 1987). Before the introduction of hybrids, downy mildew was rarely reported as being a problem because of the genetic diversity in the traditional land races. In late 1960s pearl millet cultivation was revolutionised by the development of male-sterile lines that allowed the production and large scale cultivation of high-yielding hybrids with very narrow genetic base of seed parents. By 1971, over two million ha out of twelve million were planted with hybrids when the first of several major epidemics of downy mildew occurred causing devastating losses. In 1971, DM epidemic combined with drought reduced annual production of pearl millet grain in India from 13 to 3 million metric tonnes (Anand Kumar,1983). The epidemics are continued and heavy losses are reported in 1974, 1983 and 1984 (Singh et al., 1987). Losses of 10-60% of the pearl millet harvest have also been reported in countries, such as Nigeria (King and Webster,1970) and Tanzania (Dogget 1970).

The life cycle of *S.graminicola* comprises both sexual and asexual phases. The sexual cycle produces numerous oospores in the infected leaves towards the end of the growing season which are perennating structures. The oospores produced in the leaves get mixed with soil or seeds to initiate the disease in the next season. The asexual stage produces abundant sporangia which germinate indirectly to give zoospores that act as secondary infectious propagules. Zoospores can infect pearl millet plant through roots, root hairs, coleoptiles and the base of young and emerging leaves still present with in the whorl.

The downy mildew infection results in various types of symptoms In pearl millet. The disease appears at all stages of plant development, from seedling to the earhead stage. Systemic infection is observed early in the seedling stage when the plants attains 5-15 cm height. Infection follows invasion of the apical meristem and colonisation of the undifferentiated tissue. Typical symptoms are partial chlorotic mottling on first leaf to develop symptoms and an increased area of chlorosis on subsequent leaves according to the extent that the leaf tissue was undifferentiated at the time the pathogen invaded the apex. The abaxial surface of the chlorotic tissue are heavyly loaded with the asexual sporangia produced on the sporangiophores. Plants infected before panicle initiation produce no inflorescence whilst later infection results in grossly malformed inflorescence with various degree of transformation of the gynoecia which is termed phylloidy. This lends to downy mildew, or the common name `green-ear'.

1.3 MOLECULAR MARKERS

In 1865 Mendel determined that genetic factors behave as discrete particles when passed from parent to offspring. His studies on pea plants marked the beginning of the discipline concerned with the segregation of genes. Over 40 years later, Morgan (1911) discovered that the genetic factors

described by Mendel could be linked, and the chromosomal distance between the genetic factors could be estimated from the frequency of their co-segregation. Phenotypic differences between parents were often co-inherited but do not co-segregate in every case, depending on the markers concerned. Even some characters were physically linked as linkage groups, they occasionally split by exchange of genetic material (recombination) between parental chromosomes. The extent of such recombination between markers was taken as a measure of the distance between them, a rate of one recombination in 100 meioses being called a centiMorgan (cM).

Genetic linkage maps of crop species were among the first to be constructed, and predate the demonstration of DNA as the hereditary material (Tanksley et al., 1989). However, these maps are of limited use due to fewer polymorphic morphological markers available and environmental influences on expression of these markers. It is also not possible to determine the genotype of an individual from its phenotype. morphological characters (markers) also have several disadvantages in plant genetic studies or breeding schemes. The recessive alleles of genes for morphological characters may be deleterious when homozygous

1.3.1 Protein markers

With the advent of new technologies, the concept of molecular markers has been developed. First in this context are protein markers (Isozymes). Enzymes are attractive for direct genetic study as they are primary products of structural genes and they can be separated and visualized on gels based up on their migration (size) under the influence of electric field. The alleles at most Isozyme loci are co-dominant in nature, and therefore cause no deleterious effects in plant phenotype through recessiveness or pleiotrophy. This co-dominance allows heterozygotes to be distinguished from homogygotes an

advantage shared by few morphological markers. Isozymes rarely exhibit epistatic interactions, so that theoretically a genetic stock containing an infinite number of markers could be constructed (Thanksley and Orton 1983).

Polymorphic isozyme systems have been investigated for use in germplasm characterisation of a wide range of crops including rice (Glaszmann 1987), wheat (Cooke 1987), maize (Cardy and Kamenberg 1982), and sorghum (Morden et al., 1989) . Isozyme characters can also provide a valid biochemical basis of varietal identification. Tostain et al., (1992) used isozymes to classify Asian and African pearl millet varieties, and to examine the relationship between electrophoretic variability and the geographic distribution of pearl millet genotypes. Use of isozymes to tag single gene or loci conferring disease resistance will have immense value in plant breeding. In tomato, acid phosphatase locus tightly linked to genes for resistance to nematode *Meloidogyne incognita* (Rick and Fobes, 1974) has been identified. In garden pea, an esterase locus associated with the gene conferring resistance to *Fusarium* wilt (Hunt and Barnes,1982) and in bean yellow mosaic virus resistance tightly linked to phosphoglucomutase (Weeden, 1984) were reported. Changes in isoenzyme pattern of peroxidase during downy mildew of pearl millet were also reported (Shekhawat et al.,1984). Since these isozymes alleles can be established by electrophoretic separations from embryo or leaf tissue, this co-domenant genetic markers can be used in breeding programs to screen progenies for resistance.

1.3.2 Restriction Fragment Length Polymorphism (RFLP)

The beginning of the 1980 saw emergence of restriction fragment length polymorphisms (RFLPs) that exploited variation at the DNA level (Paterson et al.,1991b). The first map of the human genome based on molecular markers (Botstein et al.,1980) fueled the development of genomic maps in other

organisms. This new technique of molecular markers and their variation at the level of the DNA sequence provide the base for the analysis of the inheritance of quantitative traits (Beckmann and Soller 1988). These markers serve the need for markers that describe the genotype of the individual directly and are unaffected by environmental bias.

RFLP technology exploited restriction enzymes synthesised by bacteria that cut genomic DNA at specific palindromic recognition sequences, generating thousends of fragments of defined length. If a recognition sequence is present at a distinct genomic location in one individual but not in the other, the enzyme generates differently sized restriction fragments of this locus. This length polymorphism is detected by a radioactively labeled, complementary DNA probe derived from the same locus. In principle, RFLP probes are single copy markers that detect only one defined genomic fragment, although multilocus probes, such as repetitive DNA that detect several fragments at a time are also used. As crop plants have about 10^8-10^9 nucleotides of DNA there was the potential for a large number of markers to be generated. RFLP markers are co-dominant and are very reliable markers for linkage analysis and marker-assisted selection (MAS), since they make it possible to determine if a linked trait is present in hetero- or homo-zygous state in an individual.

RFLP markers have allowed comprehensive linkage maps to be constructed for many crop species. The information generated from such maps would have immense value in selection and to refine the breeding strategies in crop improvement programs. In hybrid seeds production, parent-specific RFLP markers can distinguish true F_1 hybrid seeds from contaminants (Soller and Beckmann,1983). Use of molecular markers to tag single genes or loci controlling economically important traits has already become an important tool for selection. In tomato, RFLP markers that are tightly linked to genes for resistance to tobacco mosaic virus (Young et al., 1988), root knot nematode

(Klein et al., 1991), and genes controlling plant habit and fruit ripening properties (Paterson et al., 1988) have been identified. Similar tightly linked RFLP markers in rice for genes conferring resistance to bacterial leaf blight, blast and white plant hopper (Tanksley et al., 1990), thermo-sensitive genic male sterile gene, and root penetration ability (Ray et al., 1996) were also reported.

1.3.3 Randomly Amplified DNA Polymorphism (RAPD)

The analysis of nucleotide sequence variability has been revolutionized by the development of the Polymerase Chain Reaction (PCR). PCR provides selective amplification of a specific DNA sigment many times, this capability of PCR can be used to analyse DNA that would be difficult with conventional molecular biology techniques. In a large variety of plants and animals it has been shown that single arbitrary primers, 8 to 10 nucleotides in length, will produce one to few amplification products (Williams,1990). The primers are enerated with at least >50% (G+C) content to ensure efficient annealing, and with sequences that are not capable of internal pairing that can produce PCR artifacts. The PCR procedure allows the specific amplification of DNA fragments ranging from 200bp to 3000bp in length, that can be visualized after electrophoresis by staining with ethydium bromide. RAPD technology is much faster and requires fewer resources than RFLP analysis (Williams ,1990).The major limitation of RAPD technology is that most markers are dominant as opposed to the co-dominant nature of RFLP markers. Despite this disadvantage, due to its speed and ease RAPD analysis has found application in population studies (Welsh,1991), taxonomic studies (Halward,1991), detection of genome specific markers (Quiros,1991) and in gene mapping (Mudge,1996).

1.3.4 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is a novel PCR based assay, which selectively amplifies DNA fragments to detects large number (20-100) of

loci in a single reaction. It requires no prior knowledge of genomic DNA sequence unlike in microsatellite marker assay. AFLPs are faster than either RFLPs or RAPDs as multiple analysis can detect thousands of genetic loci in a short period of time, and theoretically produce large number of informative loci (Maughan,1996).

In AFLP, genomic DNA is digested with two restriction endonucleases. Site specific adapters are then ligate to the DNA fragments. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification. Primers complementary to the adapters and to the restriction sites that match the selective nucleotides of the primer. Resolution of the resulting amplified DNA fragments on denaturing polyacrylamide gels will produce polymorphic bands. The AFLP technique is robust and reliable because of stringent reaction conditions used for primer annealing. It combines the reliability of RFLP with the power of PCR based techniques.

The polymorphism's identified using AFLPs will inherit in Mendelian fashion and segregate independent of each other. So it can be used for a variety of purposes including generation of genetic fingerprints and linkage maps. Due to its speed and reliability, AFLP analyses have wide applications such as determining the genetic structure of the populations (Tohme 1996), Constructing high density genetic maps (Vos,1995), and gene tagging (Moughan,1996).

1.4 MAPPING IN PEARL MILLET

A detailed RFLP based pearl millet genetic map was constructed by Liu et al., (1994) using 200 probes from the *Pst* I pearl millet genomic library. This map comprises seven linkage groups with total length of 303 cM. The average map distance between loci was about 2 cM. Further, the quantitative treat loci identified for resistance to downy mildew by Jones (1994) using RFLP probes

1.5 OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were to identify the molecular markers which show polymorphism between the downy mildew susceptible and resistant pearl millet accessions, and to compare various marker types.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

Following genotypes were used:

1. **843B P_5P_2** : downy mildew susceptible
2. **7042 DMR P_2P_1** : downy mildew resistant
3. **IP18292** : downy mildew resistant
4. **IP18298** : downy mildew resistant
5. **23D_2B** : downy mildew susceptible

2.2 ISOZYME ANALYSIS

2.2.1 Sample preparation

Five-days old actively growing seedlings were collected and frozen in liquid nitrogen (LN_2). Frozen tissue (1 gm) was crushed into fine powder in a mortar with LN_2 to facilitate maceration and to keep the temperature very low. Immediately, 1.0 ml of extraction buffer was added and homogenised at 4°C for 30 min. The resulting solution was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and stored in multiple aliquots at -20°C for further use.

Extraction Buffer (pH 7.5) Composition For Isozymes (100 ml)		
Ascorbic Acid	5 mM	88 mg
Sodium phosphate, dibasic	42.3 mM	600 mg
Sucrose	0.21 mM	7.2 mg
Ethylenediaminetetraacetic acid (EDTA) disodium salt	1.55 mM	52 gm
Polyvinylpyrrolidone (PVP)-40	5% w/v	5 gm
2-Mercaptoethanol	0.014 mM	100 ul

2.2 Electrophoresis procedure

Samples were run in 10% polyacrylamide gels prepared according to Davis (1964). Tris-Glycine (pH8.3) was used as running buffer. Samples were loaded into wells after a prerun of the gel at 300 v for 1h. Electrophoreses was carried out using *BIORAD Protean* IIxi cell electrophoresis unit at constant voltage of 300 v for 5 h.The temperature of the gel system during entire run was kept at 4°C by circulating cold water. At the end of electrophoresis the gels were stained for enzyme activity.

10% Polyacrylamide gel composition (50 ml)	
Water	20.5 ml
Acrylamide 30%	16.6 ml
Tris 1.5 M pH 8.8	12.5 ml
APS 10% fresh	0.5 ml
TEMED	20.0 ul

Running buffer - pH 8.3 (2 lit)	
Tris	3.03 gm
Glycine	14.41 gm

2.3 Staining for enzyme activity

After electrophoresis, the gels were removed and stained with appropriate reagents for enzymatic activity. Regions of enzymatic activity (bands) were detected after incubation with staining solution at specified conditions as described for the enzyme under study.

Staining solutions for individual isozyme systems

1. **Acid phosphatase:**

Sodium-∝-naphthyl acid phosphate (dissolved in 1 ml acetone/1 ml water)	100 mg
Fast garnet GBC salt	75 mg
1 M Mg Cl_2	0 .25 ml
0.2 M Sodium acetate buffer (pH 5.5)	100 ml

2 **Alcohol dehydrogenase**

Analar ethanol	3 ml
NAD	30 mg
Nitro-blue-tetrazolium	25 mg
Phenazine mithosulphate	2 mg
0.1 M Tris.Cl buffer (pH 8.0)	100 ml

3 **Esterase**

∝-Naphthyl Acetate (dissolved in 2 ml acetone)	20 mg
Fast blue BB salt	50 mg
0.1 M Phosphate buffer (pH 7.2)	100 mg

4 **Glutamate oxaloacetate transaminase**

L-Aspartic acid	100 mg
∝-Ketoglutaric acid	100 mg
Fast blue BB salt	150 mg
Pyridoxal-5-Phosphate	5 mg
0.1 M Tris.Cl	100 ml

Assay in dark at room temperature.

5. **Leucine aminopeptidase**

L-Leucyl-β-Naphthyl amide (in 4 ml dimethyl formamide)	20 mg
Black-K salt	32 mg
0.2 M Tris Malate buffer (pH 6.8)	100 mg

Assay in dark at 37°C

6. **Malic enzyme**

L-Malic acid	280 mg
N.A.D.P	20 mg
Nitro Blue Tetrazolium	30 mg
Phenazine Methosulphate	5 mg
1 M Mg Cl_2	0.1 ml
0.1 M Tris.Cl (pH 8.6)	100 ml

7. **Superoxide dismutase**

Riboflavin	3 mg
E.D.T.A.	75 mg
Nitro blue tetrazolium	10 mg
0.05 M Tris.Cl (pH 8.2)	100 ml

Incubated in dark for 20 min and then exposed to strong light source.

8. **Peroxidase**

3-Amino Ethyle Carbazole (in 4 ml dimethyl formamide)	100 mg
30% H_2O_2	4 drops
0.2 M Sodium acetate (pH 5.0)	100 ml

2.3 EXTRACTION AND PURIFICATION OF GENOMIC DNA

Genomic DNA was extracted from the young leaves of pearl millet plants following the method described by Sharp et al. (1988). Freshly harvested young leaves were harvested, frozen in liquid nitrogen and stored at -70°C. Frogen leaf

tissue was ground, suspended in 20 ml lysis buffer (1M Tris.Cl pH 8.5, 5 M NaCl, 0.5 M EDTA, 2% SDS pH 8.0) and treated with 100 ul of proteinase K (0.05 mg ml^{-1}). The samples were incubated at 65°C for 60 min with occasional gentle mixing. An equal volume of phenol and chloroform (1:1 ratio) was added, mixed gently to form an emulsion and centrifuged at 6000 rpm for 20 min at 2°C in a swing bucket rotor using *Sorvall RC5* centrifuge. This step was repeated after adding equal volume of chloroform to the supernatant. The supernatant was collected and DNA was precipitated by adding 0.6 volumes of cold isopropanol. DNA was spooled out with bent ends of pasture pipette washed with 70% ethanol, and dried under vacuum. Later DNA was dissolved in 2 ml of 1xTE (10 mM Tris.Cl, 1 mM EDTA pH 8.0) containing RNase (250 ug ml^{-1}).

The polysaccharide impurities were removed by treating the sample with 1/10 volume of 5 M NaCl for 20 min at 4°C, followed by centrifugation at 6000 rpm for 20 min at 4°C. DNA was purified by extracting with equal volume of Chloroform, and precipitating by the addition of 1/10 volumes of 3 M Sodium acetate and 2 volumes of chilled (-20°C) absolute ethanol. The precipitated DNA was spooled, washed, with 70% ethanol, dried under vacuum and dissolved in 200 ul of 1x TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA pH 8.0).

The quantity and purity of the DNA samples were determined spectrophotometrically by measuring the absorbance at 230, 260 and 280 nm with a *SHIMADZU UV 160A* spectrophotometer. DNA was quantified considering that one OD unit at 260 nm is equivalent to 50 ug of DNA (Sambrook et al. 1989).

2.4 RFLP ANALYSIS

2.4.1 DNA probes

Cloned pearl millet fragments that were used as probes were derived from a pearl millet *Pst I* genomic library. This genomic library was constructed by Gale group at Cambridge laboratories, Norwich, UK. A library, containing about 1000 clones (Pg PSM 1 - Pg PSM 1000), was constructed with total plant DNA extracted from leaves of the pearl millet genotype 7042(S).The *Pst I* digested fragments in the size range 500 to 3000 bp were cloned in to the *Pst I* site of *pUC 18* plasmid and transformed into *E.coli* bacterial stain *DH 5*α (Liu et al. 1994). A subset of these probes available at ICRISAT Asia center were used in the present study.

Culture conditions : The recombinant *E.coli* clones were selectively cultured in 5 ml of LB medium containing 10 ug of ampicillin. The cultures were grown overnight at 37^{0}C in water bath shaker rotating at 175 rpm.

2.4.2 Plasmid Isolation

Plasmid DNA was extracted following the rapid isolation method of Holmes and Quigley (1981) as described by Sambrook et al. (1989). The cells were harvested from overnight grown bacterial culture and the pellet was resuspended in 350 ul STET (8%sucrose, 0.5% Triton x-100, 50 mM EDTA, 10mM Tris.Cl) buffer. The cell debris and chromosomal DNA was precipitated after lysing the cell wall with 25 ul lysozyme solution (10 mg ml^{-1} in 10 mM Tris.Cl, pH 8.0). The plasmid DNA was precipitated by adding 40 ul sodium acetate (2.5 M) and 420 ul isopropanol to the supernatant, followed by cooling at -20^{0}C for 15 min and centrifugation. The plasmid DNA pellet was washed with

ethanol (70%), dried under vacuum and dissolved in 40 ul of TE (10 mM Tris.Cl, 1 mM EDTA) containing RNase (5 mg ml^{-1}). The quality of DNA was checked on 0.8% agarose gel, quantity and the purity were determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm with a SHIMDZU UV 160 A spectrophotometer.

2.4.3 PCR amplification of genomic inserts in plasmids

The PCR reaction mixture consisting of 50 ng of recombinant plasmid DNA, a final concentration of 250 uM each of dNTPs, 50 nM M13 forward primer, 50 nM M13 reverse primer (17 bp in length each; supplied by *Life Technologies*), 2.5 units of *Taq* polymerase (*Promega*), 3 mM $MgCl_2$, 0.4x PCR buffer (last 2 from *USB*) and sterile H_2O to make volume 50 ul. Following a first denaturation step for 2 min at 94°C, amplification was carried out for 38 cycles with following temperature profile. Denaturation for 1 min at 94°C, primer annealing for 1 min at 40°C, primer extension for 2 min at 72°C, then one additional cycle with the same except for a 5 min extension time. The amplified DNA inserts were analysed by electrophoresis on 2.5% agarose gel using 1xTAE buffer. The PCR reaction was carried out using *Perkin Elmer Gene Amp PCR system 9600.* The amplified inserts were purified with Sepharose CL-6B spin columns.

2.4.4 Labeling of probes

Random primer labeling of inserts ware performed as described by Feinberg and Vogelstein (1983). A total of 20 ng purified insert DNA used as the probe was denatured for 5 min in a boiling water bath, flash cooled, a final concentration of 60 uM dNTP mix (*New England, Biolabs*), 50 uCi of [α-^{32}P] dCTP (*Amersham)*, 2.4 units of Klenow *enzyme (New England, Biolabs*), 1x oligolabeling buffer, and sterile distilled water to make volume 50 ul was added,

and incubated at 37°C for 3 h. The reaction was stopped using 0.5 M EDTA and the sample was diluted to 200 ul with distilled water. The unincorporated radioactive material was removed by using spin columns packed with Sephadex G-50. The radioactivity of 2 ul aliquot was monitored before and after purification to calculate the percent of incorporation.

2.4.5 Restriction digestion of genomic DNA and Southern transfer

The DNA samples extracted from the leaf tissues of genotypes under study were digested with 5 units each of *EcoRI, EcoRV, Hind III,* and *Dra I* enzymes per ug DNA and incubated at 37°C for overnight. The digested DNA was size-fractionated on 0.8% agarose gel for 12 h at 25 v, and vacuum blotted using *BIO-RAD* Model 785 Vacuum Blotter.

Blotting procedure : The size fractionated DNA was depurinated by rinsing the gel in 0.25 N HCl for 15 min followed by denaturation in 0.4 N NaOH for 45 min. The DNA was transferred on to nylon membrane *(Zeta-Probe® GT* Blotting Membrane, *Bio Rad)* at 5 inches Hg vacuum for 2 h using 20xSSC (3M sodium chloride, 0.3M trisodium citrate) as transfer buffer. After completion of transfer, the blots were rinsed in 6x SSC and UV crosslinked using UV crosslinker (*Stratagene*).

2.4.6 Prehybridization and hybridization

Prehybridization was carried out in boxes containing prehybridization solution (200 ml 20x SSPE, 5gm SDS, 50 ml 100x Denhart's reagent, 20ml salmon sperm DNA (10 mg ml^{-1}), distilled water to make volume 1000 ml) at 65°C for 4 h. The labeled probe was denatured for 5 min in boiling water bath, flash-cooled and added to the same prehybridization solution. The hybridization was carried out in hybridization oven for 16 h at 65°C with constant agitation (rotation).

20x SSPE (1000 ml)		
NaCl	3 6 M	210 gm
$Na_2HPO_4 7H_2O$	0 2 M	53 6 gm
EDTA	0 02 M	7 44 gm

100x Denhart's reagent (1000ml)	
Ficoll 400	20 gm
Polyvinylpyrrolidone (PVP)	20 gm
BSA fraction V	20 gm

2.4.7 Posthybridization processing of Southern blots

After 16 h hybridization the excess probe was removed by washing the blots in solution I (2x SSC, 0 5% SDS) for 15 min and in solution II (0 1x SSC, 0 1% SDS) for 15 min at 65°C with constant agitation The blots were wrapped in (*Saran wrap*) and exposed to x-ray film (*Kodak*) at -80°C for 4-5 days, before developing the film

2.5 RAPD ANALYSIS

The RAPD assay was performed following the method of Williams et al (1990) PCR reaction was performed with 20 ul of a reaction mixture containing a total of 25 ng of genomic DNA, 0 5 uM of arbitary decamers, (*Operon Primers* Inc), 2 5 mM $MgCl_2$ (*Promega*), 150 uM dNTPs (*Sigma* chemicals), 0 4x PCR buffer (*Promega*) and 1 5 units of *Taq* polymerase *(promega*) and sterile distilled water to make volume 20 ul per reaction

PCR reaction was carried out using *Perkin Elmer Gene Amp PCR system 9600* for 40 cycles with the following temperature profile:

First cycle :	Denaturation at 94°C for 2 min. Primer annealing at 40°C for 1 min. Primer extension at 72°C for 2 min.
Next 38 cycles :	Denaturation at 94°C for 1 min. Primer annealing at 40°C for 1 min. Primer extension at 72°C for 2 min
last cycle :	Denaturation at 94°C for 1 min. Primer annealing at 40°C for 1 min Primer extension at 72°C for 5 min.

A control without template DNA was included in each set of reactions with a single primer. Reaction products were resolved by electrophoresis on gels consisting of 1.5% *FMC Nu-Sieve* agarose.

Primer Id.	Sequence (5'→3')	(G+C)%
OPE1	CCCAAGGTCC	70
OPE2	GGTGCGGGAA	70
OPE3	CCAGATGCAC	60
OPE4	GTGACATGCC	60
OPE5	TCAGGGAGGT	60
OPE6	AAGACCCCTC	60
OPE7	AGATGCAGCC	60
OPE8	TCACCACGGT	60
OPF1	ACGGATCCTG	60
OPF2	GAGGATCCCT	60
OPF3	CCTGATCACC	60
OPF4	GGTGATCAGG	60
OPF5	CCGAATTCCC	60
OPF6	GGGAATTCGG	60
OPF8	GGGATATCGG	60
OPF9	CCAAGCTTCC	60

2.6 AFLP ANALYSIS

AFLP analysis was performed by using AFLP analysis system I kit (*Life Technologies* Inc.) following manufacturer's instructions.

2.6.1 Restriction digestion and Adapter ligation

Genomic DNA (250 ng) was double-digested with *EcoRI* and *MseI* using 2.5 units each of *EcoRI* and *MseI* and 5 ul of 5x reaction buffer made to a final volume 25 ul with AFLP-grade water. The mixture was incubated at 37°C for 2h and then denatured the enzymes at 70°C for 15 min. The DNA fragmants are ligated using 1 unit of T4 DNA ligase and 24 ul of adapter ligation solution (provided along with the kit) at 20°C for 2 h.

2.6.2 PCR amplification for AFLP study

The ligation mixture was diluted 10-fold with sterile distilled water and the fragments were preamplified for 20 cycles using *Perkin Elmer 9600 Gene Amp* system. A total of 5 ul of double-digested and adapter ligated DNA was amplified in a final volume of 50 ul containing 40 ul of pre-amp primer mix (*EcoRI* + A and *MseI* + C), 5 ul of 10 x PCR buffer for AFLP (both provided in kit) and 1 unit of *Taq* polymerase (*Promega*). The cycle profile was 94°C for 30 sec, 56°C for 60 sec, and 72°C for 60 sec. Selective amplification was achieved by PCR using aliquots of preamplified fragments diluted 50-fold, and γ-^{32}P labeled *EcoRI* + 3 and unlabeled *MseI* + 3 primers. PCR temperature profile used was, one cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 60 sec, followed by lowering the annealing temperature during each cycle by 0.7°C for twelve cycles. Then the reaction was amplified for 23 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec.

2.6.3 Electrophoresis procedure

Following amplification, reaction products were mixed with an equal volume of formamide dye (98% deionised formamide, 10mM EDTA (pH 8.0), 0.025% bromo phenol blue and 0.025% xylene cyanol as tracking dyes). The resulting mixtures were heated for 3 min at 90°C, and then quickly cooled on ice, Each sample (2 ul) was loaded on a 5% denaturing (sequencing) polyacrylamide gel (20:1 acrylamide:bis; 7.5 M urea; 1x TBE buffer). Electrophoresis was performed at constant power of 50 W for 3h. After electrophoresis, gels were dryed and exposed to x-ray film.

CHAPTER3

RESULTS AND DISCUSSION

3.1 ISOZYMES

The seeds allowed to germinate on folded blotting paper wicks in the absence of light gave enough coleoptile tissue 5 days after germination. The enzymes extracted as described by Morden et al (1987) showed good activity. Various isozyme alleles were well resolved and exhibited clear distinct bands on native 10% polyacrylamide gels. Out of the eight enzyme systems studied (Acid Phosphatase, Alcohol Dehydrogenase, Esterase, Glutamate Oxaloacetate Transaminase, Leucine Aminopeptidase, Malic enzyme, Superoxide Dismutase, Peroxidase), three enzymes (peroxidase, esterase and alcohol dehydrogenase,) showed polymorphism among the five accessions (843B P_5 P_2, 7042 DMR P_2 P_1, 23 D_2B, IP 18298, IP18292) of pearl millet. Staining for peroxidase showed polymorphism at two isozyme loci between the 843B P_5 P_2 (DM susceptible) and 7042 DMR P_2 P_1 (DM resistant). Esterase showed only one loci which is polymorphic between the 843BP_5P_2 and 7042 DMR P_2 P_1, where as ADH loci are monomorphic between these accessions.

Isozymes are distinguishable forms of an enzyme whose presence can be determined by electrophoresis and specific staining. The pattern, represented by discrete bands in the gel, indicates localized areas of enzyme catalyzing a particular reaction. The success of producing zymograms mainly depends on the incubation conditions such as pH, temperature and light. Most staining procedures requires incubation at room temperature and a few requires 37^{0}C. Many stains are extremely sensitive to light, tetrazolium salts, deazonium salts such as fast blue BB can be photoreduced and results in undesirable back ground staining.

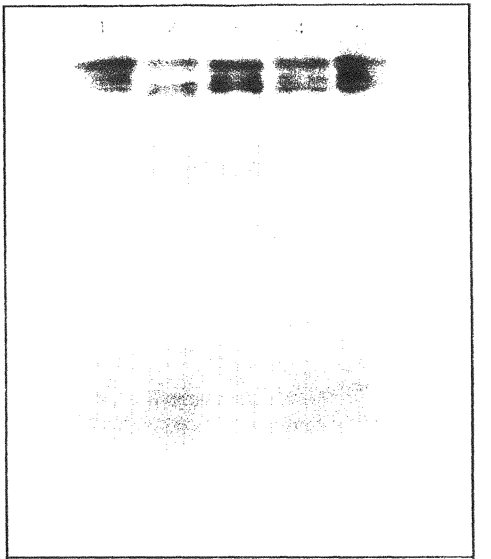


Figure 1
Zymogram of Peroxidase:
1.843B P_5 P_3; 2.7042DMR P_2 P_1; 3.IP18292; 4.IP18298; 5.23D_2B.

Enzyme	Locus	Genotypes				
		G1	G2	G3	G4	G5
	Prx-1	+	+	+	+	+
	Prx-2	+	+	+	+	+
Peroxidase	Prx-3	-	+	+	+	-
	Prx-4	-	-	-	+	-
	Prx-5	+	+	+	+	+
	Prx-6	+	+	+	+	+
	Prx-7	+	+	+	+	+
Esterase	Est-1	+	-	-	+	-
	Est-2	-	+	-	+	+
	Est-3	-	-	+	-	-
	Est-4	-	-	-	+	-
Alcohol dehydrogenase	Adh-1	+	+	+	+	+
	Adh-2	+	+	+	-	-
	Adh-3	+	+	+	+	+
	Adh-4	+	+	+	+	-
	Adh-5	+	+	+	+	+
	Adh-6	+	+	+	+	+

Table 3.1 Isozyme data of five pearl millet accessions G1. 843B P_5 P_2 ; G2. 7042 DMR P_2 P_1 ; G3. IP18292 ; G4. IP18298 ; G5. 23 D_2B

3.2 RFLP ANALYSIS

3.2.1 Genomic DNA extraction

The aim of any genomic DNA preparation is to isolate DNA of high molecular weight and sufficient purity. The method described by Sharp et al., fulfilled both the criteria. Two factors affect the size of the plant DNA isolated were shear and nuclease activity. In order to reduce the shear the sample was handled gently throughout the preparation. To reduce the nuclease activity after harvesting the samples were handled with dry ice and stored at -70°C and treated with extraction buffer containing detergent and EDTA immediately after twawing.

An aliquot of genomic DNA run on 0.8% agarose electrophoresis conformed that the DNA was of high molecular weight. The purity of DNA was

confirmed after spectrophotometric analysis at 230, 260 and 280 nm. The absorbance ratio at 260 and 280 nm were between 1.6 to 1.9 which suggests that the genomic DNA preparation is reasonably pure. The yield of DNA obtained by this method is about 150 ug per gm of the fresh leaf tissue.

Further the DNA obtained by this method could be easily digested well with all the 4 restriction enzymes tried (*DraI, HindIII, EcoRI, EcoRV*). Complete digestion of the DNA was evident from the appearance of smear of DNA (shown in figure 4) when analysed on 0.8% agarose gels,. Molecules of linear, duplex DNA travel through gel matrices at a rate that is inversely proportional to the $\log_{10}$ of their molecular weight.

Plasmid DNA isolated using the protocol of Holmes et al.,(1981) yield around 50 - 60 ug of DNA per ml of over night grown culture (considering 1 OD at 260 nm = 50 ug of DNA). Ethedium bromide stained 0.8% agarose gel (Fig. 2) on which isolated plasmid DNA samples were analysed showed clear bands characteristic of plasmid DNA without any RNA contamination. The OD ratio of 260/ 280 nm which is around 1.8 conforms the purity of the DNA sample isolated. The plasmid DNA was isolated from 60 different clones. Further, insert from 8 clones were used for RFLP analysis. The insert DNA fragments were amplified using M13 primers for 40 cycles. The PCR amplified and sepharose CL-6B spin column purified inserts showed a single band on 2.4% agarose gel as represented in the Fig. 3. The estimated size of the insert was corresponded to what was reported earlier at Cambridge laboratory.

Nylon membranes were used for blotting the restriction digested genomic DNA. Nylon membranes are quite durable and can be exposed to basic solutions for extended periods of time. This eliminating the need for the neutralization step present in standard southern protocols, which in turn prevents the reannealing during the transfer, there by increasing the sensitivity. Nylon membranes are adventitious as DNA can be covalently linked to membrane by UV irradiation, hybridized probe can be completely stripped from the membrane by denaturation and stringent washing.

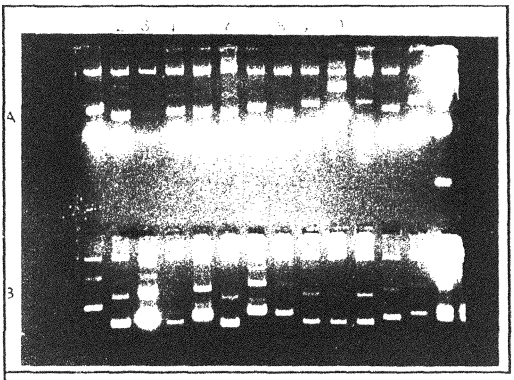


Figure 2
Agarose gel electrophoresis of recombinant pUC 18 plasmid DNA

A1 PSM 341	A8 PSM 568	B1 PSM 717	B8 PSM 81
A2 PSM 345	A9 PSM 592	B2 PSM 727	B9 PSM 87
A3 PSM 366	A10 PSM 612	B3 PSM 710	B10 PSM 9
A4 PSM 410	A11 PSM 616	B4 PSM 6 4	B11 PSM 347
A5 PSM 428	A12 PSM 648	B5 PSM 657	B12 PSM 295
A6 PSM 464	A13 PSM 713	B6 PSM 658	B13 PSM 349
A7 PSM 575	A14 λ HindIII marker	B7 PSM 7 5	B14 λ HindIII ma

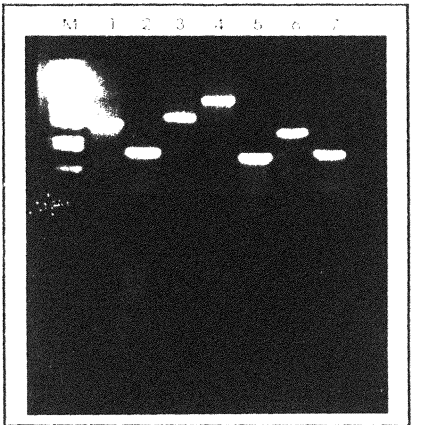


Figure 3
Agarose electrophoresis of PCR-amplified insert DNA
M. λ HindIII marker; 1. PSM 223; 2. PSM 320; 3. PSM 84; 4. PSM 330; 5. PSM 409; 6. PSM 634; 7. PSM 227.

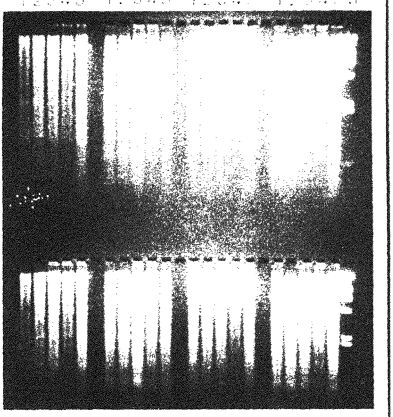


Figure 4 : Agarose gel electrophoresis of restiction digested genomic DNA of 5 Pearl millet accessions
1.843B P5 P2; 2.7042 DMR P2 P1; 3. 23D2B; 4.IP18298; 5.IP18298; M.λ Hind III marker.

Eight probes from PstI genomic library of pearl millet were used for RFLP analysis of five pearl millet (843B P_5 P_2, 7042 DMR P_2 P_1, 23 D_2B, IP 18298, IP18292) accessions. The RFLP patterns of these accessions showed high degree of polymorphism. Probe PSM 330 was used in combination with two restriction enzymes *EcoRI* and *EcoRV* whereas probes PSM 320 and PSM 409 were used in combination with *DraI* and *HindIII*. Probes PSM 84, PSM 223, PSM 416, PSM 858 and PSM 634 were used in combination with four enzymes *DraI, HindIII, EcoRI* and *EcoRV*. Out of the eight probes used in the study PSM 84 alone is found to be in single copy, resulting in single banding pattern.

Analysis of the five accessions with 26 enzyme-probe combinations revealed high degree of polymorphism and 90% of the enzyme probe combinations are polymorphic and only three enzyme-probe combinations (*DraI* -320, *DraI*-84, *EcoRI*-84) are monomorphic. The probes PSM 858 and PSM 634 revealed several polymorphic fragments with all the 4 enzymes tried as shown in the Fig.5. The probe PSM 320 could not find any polymorphism in combination with *DraI* , whereas it revealed polymorphism in combination with *HindIII*. The probe PSM 84 failed to reveal polymorphism in combination with *DraI* and *EcoRI* but showed polymorphic bands in combination with *EcoRI* and *HindIII*. A total of 20 enzyme probe combinations showed unique bands in at least one of the 5 accessions. A total of 39 polymorphic fragments are identified between DM susceptible 843B P_5 P_2 and DM resistant 7042 DMR P_2 P_1 in 13 enzyme probe combinations (*EcoRI*- PSM 330, *EcoRV*- PSM 330, *HindIII*- PSM 320, DraI- PSM 409, *HindIII*- PSM 409, *DraI*- PSM 223, and *EcoRV*- PSM 223, *DraI*-PSM 856, *HindIII*-PSM 856, *EcoRI*-PSM 856, *EcoRV*-PSM 856, *DraI*-PSM 634, *HindIII*-PSM 634, *EcoRI*-PSM 634, *EcoRV*-PSM 634).

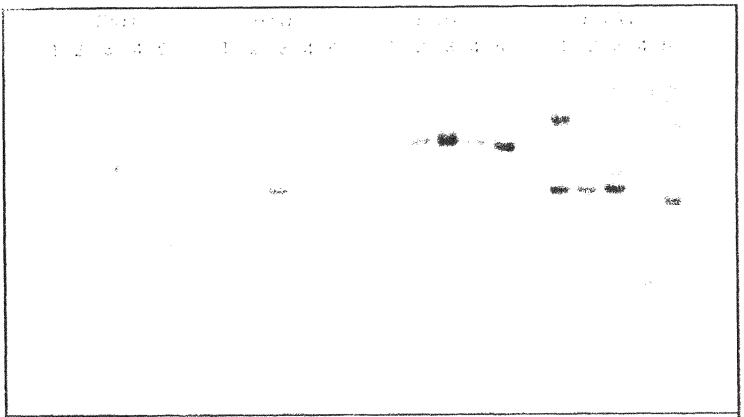


Figure 5
RFLP profile of 5 Pearl millet accessions with PSM 2/3 probe
1.843B P_x P_2; 2.7042 DMR P_2 P_1; 3. 23D_B; 4.IP18298; 5.IP18298.

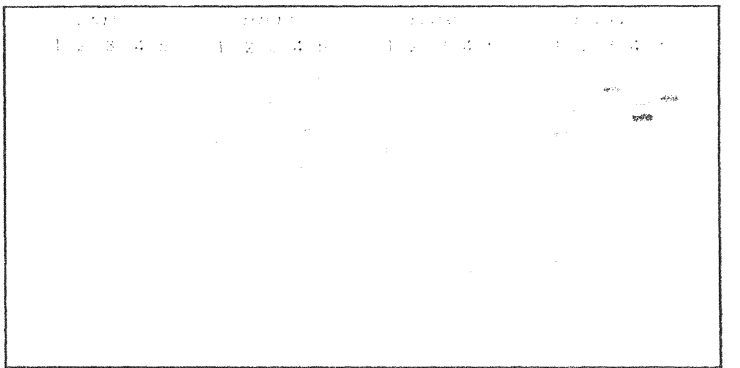


Figure 6
RFLP profile of 5 Pearl millet accessions with PSM 634 probe
1.843B P5 P2; 2.7042 DMR P2 P1; 3. 23D2B; 4.IP18298; 5.IP18298.

PSM Probe	Enzyme	No of band s	Distance (cm)	Pearl millet accessions				
				G1	G2	G3	G4	G5
330	*EcoRI*	10						
			1	-	+	-	+	-
			1.8	-	-	-	+	+
			2.2	-	+	-	-	-
			2.4	+	-	+	-	+
			2.9	+	-	+	-	+
			3.2	+	+	-	-	-
			3.9	+	+	+	-	-
			4.5	+	+	+	+	+
			5.2	+	+	+	+	+
			6.8	+	-	+	+	+
330	*EcoRV*	7						
			1.1	+	-	+	-	-
			2.1	-	-	-	-	+
			2.4	+	-	+		+
			3.5	+	+	+	+	+
			4.2	+	+	+	+	+
			4.9	+	+	+	+	+
			5.6	+	+	+	+	+
320	*DraI*	5						
			2.6	+	+	+	+	+
			5.2	+	+	+	+	+
			5.8	+	+	+	+	+
			7.5	+	+	+	+	+
			8.1	+	+	+	+	+
320	*HindIII*	6						
			2.6	+	+	+	-	+
			2.9	-	-	-	+	-
			3.8	+	+	+	+	+
			4.7	+	+	+	+	+
			5.9	+	-	-	-	-
			6.2	-	+	+	+	+
409	*DraI*	6						
			2.75	+	-	-	-	-
			2.9	-	+	-	-	-
			3	-	-	+	+	+
			4.5	-	-	+	-	+
			4.8	+	-	-	-	-
			5.5	+	+	+	+	+

Table 3.2 : RFLP data of pearl millet accessions1. 843B P_5 P_2 ; 2. 7042 DMR P_2 P_1 ; 3. 23 D_2B ; 4. IP 18298 ; 5. IP18292

PSM probe	Enzyme	No of bands	Distance (cm)	pearl millet accessions				
				G1	G2	G3	G4	G5
409	*HindIII*	7						
			1.75	+	+	+	+	+
			2.8	-	+	-	-	-
			3.6	+	-	-	+	-
			4.1	-	+	-	-	-
			4.2	-	-	-	-	+
			4.7	-	-	+	-	+
			5.2	+	+	+	+	+
84	*DraI*	1						
			0.7	+	+	+	+	+
	HindIII	2						
			1.5	+	+	+	-	+
			1.7	-	-	-	+	-
	EcoRI	2						
			2	-	-	-	-	+
			4.7	+	+	+	+	-
	EcoRV	1						
			1.6	+	+	+	+	+
223	*DraI*	3						
			0.7	-	+	+	-	-
			3.3	+	+	+	+	+
			5	+	+	+	+	+
223	*HindIII*	3						
			0.6	+	+	+	+	+
			0.8	-	-	-	+	+
			1.2	-	-	+	-	-
223	*EcoRI*	3						
			0.5	+	+	+	-	-
			1	+	+	+	+	+
			2.2	+	+	+	-	+
223	*EcoRV*	3						
			0.5	-	-	-	+	+
			2	-	+	-	-	-
			2.4	+	-	+	+	+
416	*DraI*	6						
			2.7	+	+	+	-	+
			3.2	+	+	+	+	+
			3.9	-	-	+	-	-
			4.3	-	-	-	+	-
			4.5	-	-	+	-	-
			5	+	+	+	+	+

Table 3.2 : RFLP data of pearl millet accessions G1. 843B P_5 P_2 ; G2. 7042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP 18298 ; G5. IP18292

PSM probe	Enzyme	No. Bands	Distance (cm)	pearl millet accessions				
				G1	G2	G3	G4	G5
416	*HindIII*	8						
			1.5	+	+	+	+	+
			2	+	+	+	-	+
			2.4	-	-	-	+	-
			3	+	+	+	+	+
			3.3	-	-	-	+	-
			3.6	-	-	-	+	-
			3.9	-	-	-	+	-
			5	+	+	+	+	+
416	*EcoRI*	4						
			2.7	+	+	+	+	+
			4.3	+	+	+	+	-
			4.6	+	+	+	+	+
			5.3	+	+	+	+	+
416	*EcoRV*	6						
			1.2	+	+	+	+	+
			2.5	+	+	+	+	+
			3	-	-	+	-	-
			3.3	-	-	+	-	-
			3.7	-	-	+	-	-
			4.7	+	+	+	+	+
858	*DraI*	8						
			3.3	+	-	+	-	+
			3.6	+	-	+	+	+
			4.1	+	+	-	+	-
			4.6	+	+	+	+	-
			5	-	-	-	+	+
			5.5	-	-	-	-	+
			5.8	+	-	-	-	-
			6.2	-	+	+	+	+
858	*HindIII*	8	2.7	-	-	+	+	+
			2.9	-	-	+	+	+
			3.5	-	-	-	-	+
			3.9	-	-	+	-	+
			4.8	-	-	+	-	-
			5.2	+	+	+	-	-
			5.4	-	-	-	+	+
			7.4	-	-	-	+	-

Table 3.2 : RFLP data of pearl millet accessions G1. 843B P_5 P_2 ; G2. 7042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP 18298 ; G5. IP18292

PSM probe	Enzyme	No. Bands	Distance (cm)	pearl millet accessions				
				G1	G2	G3	G4	G5
858	*EcoRI*	7	2.5	+	+	+	+	+
			3.2	-	-	+	-	-
			3.7	+	+	+	+	-
			4	+	+	+	+	+
			4.3	+	-	-	-	-
			5.6	+	-	-	-	-
			6	+	-	+	-	+
858	*EcoRV*	9	2.6	+	+	+	+	+
			2.8	-	+	+	+	-
			3.1	+	-	-	-	+
			3.5	+	+	-	-	+
			3.8	-	-	+	-	-
			4.1	+	+	+	-	+
			4.7	-	-	+	-	-
			5.1	+	+	+	+	+
			7.3	-	-	-	+	-
634	*DraI*	6	0.6	-	-	+	-	-
			1.1	-	+	-	-	
			1.5	-	-	-	+	-
			1.7	+	-	-	-	-
			2.2	-	-	-	+	-
			3.2	+	-	-	-	+
634	*HindIII*	5	0.5	-	-	+	-	-
			0.7	+	-	-	-	+
			0.9	-	-	-	+	-
			1.1	-	+	-	+	-
			1.5	+	-	-	-	-
634	*EcoRI*	6	1.1	-	+	-	-	-
			1.4	-	-	-	+	-
			1.7	+	-	-	+	-
			2	+	-	+	-	-
			2.5	-	-	-	-	+
			2.7	-	-	-	-	+

Table 3.2 : RFLP data of pearl millet accessions G1. 843B P_5 P_2 ; G2. 7042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP 18298 ; G5. IP18292

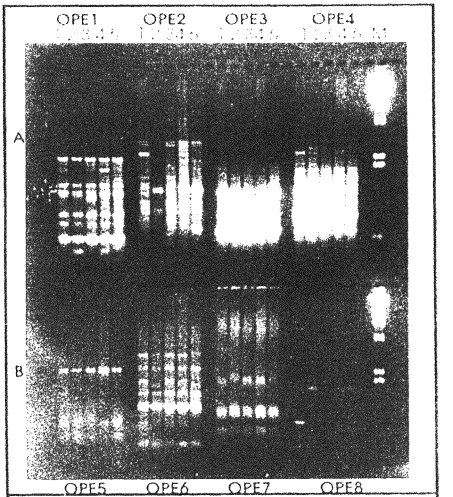
PSM probe	Enzyme	No. Bands	Distance (cm)	pearl millet accessions				
				G1	G2	G3	G4	G5
634	*EcoRV*	6	0.4	-	-	+	-	-
			0.6	+	-	-	-	+
			0.8	-	-	-	+	-
			1	-	+	-	-	-
			1.2	-	-	-	+	-
			1.4	+	-	-	-	-

Table 3.2 : RFLP data of pearl millet accessions G1. 843B P_5 P_2 ; G2. 7042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP 18298 ; G5. IP18292

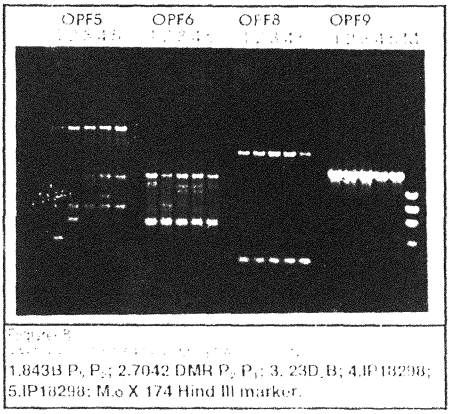
3.3 RAPD results

RAPD analysis is simple and fast, it involves PCR amplification followed by gel electrophoresis of genomic DNA. It requires very minute amount of genomic DNA (25 ng per reaction) and analysis is free from the radioactive materials. As the primers used are of 10 bp length, the conditions for the PCR amplification such as annealing temperature, $MgCl_2$ concentration, dNTPs concentration, the G+C content of primers are very crucial to get reproducible results.

Five pearl millet lines 843B P_5 P_2, 7042 DMR P_2 P_1, 23 D_2B, IP 18298, IP18292 were screened with 16, ten-base pair long primers. As shown in figuers 7 and 8 a total of 90 amplified fragments were identified when PCR amplified products were run on 1.5% agarose gels out of them 42 were polymorphic . An average of 5.6 fragments ranging from 300 bp to 3000 bp in size were amplified and 46 percent (2.6 fragments per primer) of them showed polymorphism. All the primers except OPF8 produced amplified products. Two primers OPF4 and OPF9 produced single high intensity bands where no polymorphism was identified. Of the 16 primers tested OPE5, OPF4, OPF8, OPF9 did not produced any polymorphism. Out of the 16 primers tested 8 primers (OPE1, OPE2, OPE4, OPE6, OPF2, OPF3, OPF5, OPF6) showed polymorphic bands as shown in the figuers 7 and 8. Primers OPE1, OPE2, OPE4, OPF2, OPF3 produced higher number of polymorphic bands. A total of 23 polymorphic bands were detected between DM susceptible 843B P_5 P_2 and resistant 7042 DMR P_2 P_1.



1.843B P_6 P_2; 2.7042 DMR P_2 P_1; 3. 23D$_2$B;
4.IP18298; 5.IP18298; M.λ Hind III marker



1.843B P_1, P_2; 2.7042 DMR P_2, P_1; 3. 23D_B; 4.IP18298; 5.IP18298; M.o X 174 Hind III marker.

primer-band	G1	G2	G3	G4	G5
E1-B1	+	+	+	+	+
E1-B2	+	-	+	+	-
E1-B3	+	-	+	+	-
E1-B4	+	+	+	+	+
E1-B5	+	+	+	+	+
E1-B6	-	-	+	-	-
E1-B7	+	+	+	+	+
E1-B8	+	+	+	+	+
E1-B9	+	+	+	+	+
E1-B10	-	+	-	+	-
E2-B1	+	+	+	+	+
E2-B2	+	-	+	+	+
E2-B3	+	-	-	+	-
E2-B4	+	-	+	+	-
E2-B5	+	-	+	+	+
E2-B6	-	+	-	+	+
E2-B7	+	+	+	+	+
E2-B8	-	-	-	-	+
E3-B1	+	+	+	+	+
E3-B2	-	+	+	+	-
E3-B3	+	+	+	+	+
E4-B1	-	+	-	-	-
E4-B2	+	+	-	+	-
E4-B3	+	+	+	+	+
E4-B4	+	+	+	+	+
E4-B5	+	+	+	+	+
E4-B6	+	-	+	+	+
E4-B7	+	+	+	+	+
E4-B8	-	-	-	-	+
E4-B9	+	-	+	+	-
E4-B10	+	+	+	+	+
E4-B11	-	+	-	-	-
E5-B1	+	+	+	+	+
E5-B2	+	+	+	+	+
E6-B1	+	+	+	+	-
E6-B2	+	+	+	+	+
E6-B3	+	-	+	+	-
E6-B4	+	+	+	+	+
E6-B5	+	+	+	+	+

Table 3.3 : RAPD data of five pearl millet accession G1. 843B P_5 P_2 ; G2. 70 42 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP18298 ; G5. IP18292

primer-band	G1	G2	G3	G4	G5
E7-B1	-	+	-	-	-
E7-B2	+	-	+	+	+
E7-B3	+	+	+	+	+
F1-B1	+	+	+	+	+
F1-B2	+	+	+	+	+
F1-B3	+	+	+	+	+
F1-B4	+	-	-	-	-
F2-B1	+	+	+	+	+
F2-B2	+	+	+	+	+
F2-B3	+	-	+	+	-
F2-B4	+	+	+	+	+
F2-B5	+	+	+	+	+
F2-B6	+	+	+	-	+
F2-B7	+	+	+	+	+
F2-B8	+	+	+	+	+
F2-B9	+	+	+	+	+
F2-B10	-	+	-	-	-
F3-B1	+	-	+	+	-
F3-B2	+	+	+	+	+
F3-B3	+	+	+	+	+
F3-B4	-	+	-	-	+
F3-B5	+	+	+	-	+
F3-B6	-	-	-	+	-
F3-B7	+	+	+	+	+
F3-B8	+	+	+	+	+
F3-B9	+	-	+	+	+
F3-B10	+	+	+	+	+
F3-B11	-	+	-	+	-
F3-B12	+	+	-	-	+
F4-B1	+	+	+	+	+
F5-B1	+	+	+	+	+
F5-B2	-	-	-	+	+
F5-B3	-	-	+	-	-
F5-B4	+	+	+	+	+
F5-B5	-	-	+	-	-
F5-B6	+	-	-	+	-
F5-B7	+	+	+	+	+
F5-B8	-	+	-	-	-
F5-B9	+	-	-	-	-

Table 3.3 : RAPD data of five pearl millet accessions

G1. 843B; G2. 7 P_5 $P_2$042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP18298 ; G5. IP18292.

primer-band	G1	G2	G3	G4	G5
F6-B1	+	+	+	+	+
F6-B2	+	-	-	-	+
F6-B3	+	+	+	+	+
F6-B4	-	-	+	+	-
F6-B5	-	+	+	-	-
F6-B6	+	+	+	+	+
F6-B7	+	+	-	+	+
F6-B8	+	+	+	+	+
F8-B1	+	+	+	+	+
F8-B2	+	+	+	+	+
F8-B3	+	+	+	+	+

Table 3.3 : RAPD data of five pearl millet accessions

G1. 843B P_5 P_2; G2. 7042 DMR P_2 P_1 ; G3. 23 D_2B ; G4. IP18298 ; G5. IP18292

3.4 AFLP ANALYSIS

AFLP technique is a very effective tool as, it displays high rate of polymorphism compared to that obtained by RFLP and RAPD. The fragment patterns developed by the AFLP are reproducible and reliable due to the stringent conditions used in the analysis. The genomic DNA was digested with two restriction enzymes *MseI* and *EcoRI*. *MseI* is a frequent cutter which recognises a 4 bp recognition site, *EcoRI* is a rare cutter detects a 6 bp sequence. The primers for amplification are selected such that only those fragments which are digested by both enzymes are amplified in subsequent reactions.

Amplified fragment length polymorphism (AFLP) kit supplied by *Gibco - BRL* worked well for detecting the polymorphism in pearl millet accessions. Analysis of the 2 accessions of pearl millet 843 B P_5 P_2, 7042DMR P_2 P_1 with 8 AFLP primer pairs identified a total of 260 fragments, of which 66 (25%) are polymorphic shown in fig.9. Polymorphic fragments are generated by all the seven primers used. Tomato DNA supplied along with the kit was used as control.

The average number of fragments detected by individual primer pairs ranged from 28 to 45. The number of polymorphic fragments for each primer pair varied from 6 for E-AAC, M-CTC to 12 for E-AAC, M-CAG with an average of 9.4 polymorphic fragments per primer pair.

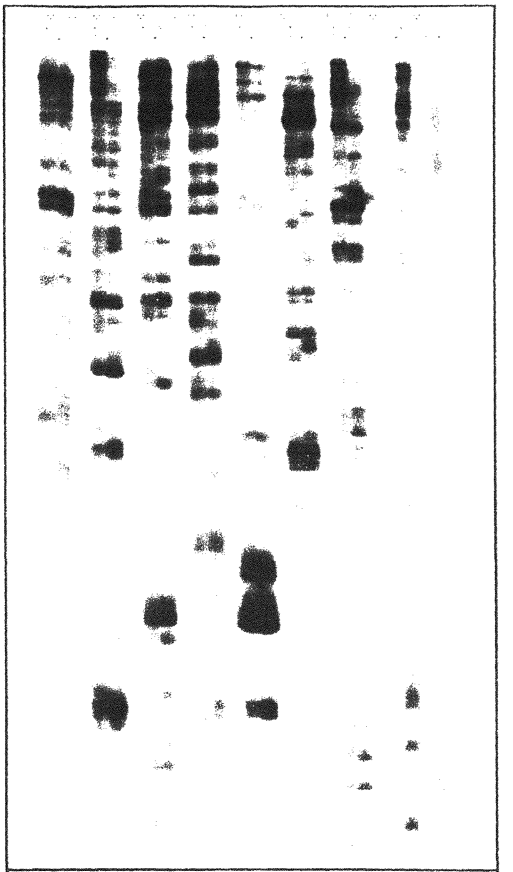


Figure 7
AFLP PROFILE OF PEARL MILLET ACCESSIONS
IN COMBINATION WITH E-AAC / M-CTT PRIMERS
1.643B P_5 P_6; 2.7042 DMR P_2 P_3; C. Tomato control.

EcoRI primer	*Mse I* primer	No.poly. bands	distance (cm)	843B	7042 DMR
E-AAC	M-CAC	11	4.6	-	+
			7.6	+	-
			9.2	-	+
			10.2	-	+
			10.9	-	+
			11.1	-	+
			13.5	+	-
			14	-	+
			15	-	+
			20	-	+
			25.8	-	+
E-AAC	MCAG	12	2.9	+	-
			6.1	+	-
			6.9	-	+
			7	+	-
			11.5	+	-
			14.2	-	+
			15	+	-
			17	+	-
			21.5	-	+
			24	-	+
			26.4	-	+
			27.8	+	-
E-AAC	M-CAT	7	10	-	+
			10.4	+	-
			13	+	-
			15.3	+	-
			16.4	+	-
			20	+	-
			27	+	-
E-AAC	M-CTC	6	5	+	-
			7.8	-	+
			8.8	-	+
			9.2	+	-
			27.8	-	+
			28.1	-	+

Table 3.4 : AFLP data of two pearl millet accessions

EcoRI primer	*Mse I* primer	No.poly. bands	distance (cm)	843B	7042 DMR
E-AAC	M-CTG	12	3.2	-	+
			4.8	+	-
			5.2	-	+
			5.7	-	+
			6.2	+	-
			6.8	+	-
			7.3	-	+
			10.5	-	+
			12.4	+	-
			16.9	-	+
			17.5	-	+
			23	-	+
E-AAC	M-CTT	9	0.2	+	-
			6.3	+	-
			10.5	-	+
			14.6	-	+
			15.5	+	-
			16.4	+	-
			19.5	-	+
			21.3	-	+
			25.5	-	+
E-AAC	M-CAA	9	4.2	+	-
			7.4	-	+
			9	+	-
			9.2	-	+
			9.7	+	-
			11.4	-	+
			11.9	-	+
			12	+	-
			14.9	+	-

Table 3.4 : AFLP data of two pearl millet accessions

CHAPTER 4
CONCLUSIONS

All the molecular markers tried (isozymes, RFLP, RAPD, and AFLP) revealed polymorphism between the pearl millet accessions studied. However, each technique has its own advantages and disadvantages.

Isozyme analysis is simple, fast and reliable, but the number of polymorphic markers that can be identified were limited and shows developmental and tissue specific variations. It can be used best in screening F_1 generation for testing hybridity.

RFLP analysis is very reliable and is independent from developmental and tissue specific variations. But it is expensive, time consuming, and requires skilled workers and generally involves hazardous radioactive material.

RAPD is simple, fast, free from hazardous material, and needs only small amounts of DNA. Although it has above said advantages over isozymes and RFLP, the uncertainty about reproducibility of RAPD markers and their dominant nature limits its use.

AFLP combines the advantages of RFLP and RAPDs, it requires less amount of DNA and is faster than RFLP. It reveals several polymorphic fragments in a single reaction. These markers are reliable like RFLP markers. However, the AFLP analysis is expensive and requires highly skilled workers.

The markers which revealed polymorphism between the DM susceptible and resistant accessions 843B P_5P_2 and 7042 DMR P_2P_1 can be used further for screening the F_2 population. The segregation pattern of these markers in F_2

generation can be linked with the pathological data and the markers that are tightly linked to the DM resistance can be identified.

REFERENCES

Anand Kumar, K., Jain, RP.,and Singh, SD (1983). "Doeny mildew reactions of Pearl millet lines with and without cytoplasmic male sterility." Plant Disease **67**: 663-665.

Beckmen, J., Soller, M., (1988). "Selection of linkage between marker loci and loci affecting quantitative traits in cross between segregating population." TAG **76**: 228-236.

Bennett , M. (1976). "DNA amount, latitute, and and crop distribution." Environ Exp Bot **16**: 93-108.

Botstein, D., White, RL., Skolnik, M., Davis, RW (1980). "Construction of a genetic linkage map in man using restriction fragment length polymorphisms." American Journal of Human Genetics **32**: 314-331.

Brunken, J. (1977). "A systematic study of pennisetum sect. pennisetum (Gramineae)." Amarican Journal of Botany **64**: 161-176.

Cardy, B., Kannenberg, LW., (1982). "Allozyme variability among mize inbread lines and hybrids: Applications for cultivar identification." Crop Science **22**: 1016-1020.

Cooke, R. (1987). "The classificattion of wheat cultivars using a standark reference electrophoresis method." Journal of the National Institute of Agricultural Botany **17**: 273-281.

Dogget, H. (1970). "Downy mildew in east Africa." Indian phytopathology **23**: 350-355.

Glaszmann, J. (1987). "Isozymes and classification of Asian rice varieties." TAG **74**: 21-30.

Govila, O. (1994). Pearl millit breeding stratagies in India : Their relevance in molecular mapping. Use of molecular markers in sorghum and millet breeding for developing countries. D. R. Witcombe JR. London, Overseas Developmint Administration (ODA): 96-98.

Hanna, W. (1986). Utilization of wild relatives of pearl millet. Proceedings of the International pearl millet work shop, ICRISAT, Patancheru, AP 502324, India, ICRISAT.

Jones, E. (1994). Mapping quantitative trait loci for resistance to downy mildew in pearl millet. School of Biological sciences. Bangor, University of Wales.

King, S., Webster, OJ. (1970). "Downy mildew of sorghum in Nigeria." Indian phytopathology **23**: 342-349.

Klein -Lakhorst, R., Rietveld,P., Machiels,B., Verkerk, R., Weld, R., Gebhardt, C., Korneef, M. (1991). "RFLP markers linked to the root knot nematode gene Mi in tomato." TAG **81**: 661-667.

Liu, C., Witcombe,J., Pittaway, T., Nash, M., Hash, C., Busso,C., and Gale, M. (1994). "An RFLP based genetic linkage map of pearl millet (Pennisetum glaucum)." TAG **89**: 481-487.

Maughan, P., Saghai Maroof, MA., Buss, GR., Huestis,GM. (1996). "Amplified fragment length polymorphism (AFLP) un soymean species diversity, inheritance, and near-isogenic line analyses." TAG **93**: 392-401.

Morden, C., Doebley,J., Schwartz,KF., (1990). "Allozyme variation among the spontaneous species of Sorghum section sorhghum(poaceae)." TAG **80**: 296-304.

Morgan, T. (1911). "Randum segregation versus coupling in Mindelian inheritance." Science **34**: 384-389.

Paterson, A., Lander, ES., Hewitt, JD., Peterson, S., Lincoln, SE., Tanksley, SD (1988). "Resolution of quantitative traits in to mendelian factors by using a complete linkage map of RFLPs." Nature **335**: 721-726.

Paterson, A., Tanksley, SD., Sorrells, ME. (1991). "DNA markers in plant improvement." Advances in Agronomy **46**: 40-48.

Ray, J. (1996). "Mapping quantitative trait loci associated with root penetration ability in rice *(Oryza Sativa.* L.)." TAG **92**: 627-636.

Rick , C., Fobes ,JF. (1974). "Association of allozyme with nematode resistance." Tomaato gentics cooperative Report **24:25**.

Safeeulla, K. (1976). Biology and control of the Downy mildew of pearl millet, sorghum and finger millet. Mysore, India, Wesley press.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular cloning, a laboratory manual. NY, Cold spring harbor laboratory.

Sharp, P., Kreis, M., Shewry, PR., Gale MD. (1988). "Location of B-amylase sequences in wheat and its relatives." TAG **75**: 286-290.

Shaw, C. (1981). Taxonomy and evolution. The Downy mildews. D. Spencer. London, Accademic press: 147-160.

Shekhawat, N., Purhodit,SD., Arya,HC. (1984). "Changes in isozymes of peroxidases in green ear of pearl millet." Current Science **53**(21): 1157-1158.

Singh, S., Ball, S., Thakur, DP. (1996). Problems and strategies in the controle of downey mildew. Proceedings of the international pearl millet work shop, ICRISAT centre, Patancheru, AP, 502324 , India, ICRISAT.

Tanksley, S., Orton,TJ. (1983). Isozymes in plant genetics and breeding. Amsterdam, Elsevier science.

Tanksley, S., Young, ND., Paterson, AH., and Bonbierbale, MW. (1989). "RFLP mapping in plant breeding: New tools for an old science." Biotechnology **7**: 257-264.

Tohme, J., Gonzalez,DO., Beebe, S., Duque,MC., (1996). "AFLP analysis of gene pools of a wild Bean core collection." Crop Science **36**: 1375-1384.

Torres,A., Weeden,NF., Martin, A. (1993). "Linkage among isozyme, RFLP and RAPD markers in Vicia faba." Theor Appl Genet **85**: 937-945.

Tostain, S. (1992). "Enzyme diversity in pearl millet 3. Wild millet." TAG **83**: 733-742.

Vos, P., Bleeker, M., Reijans,M., vande Lee, T., Furjters, A., Pot, J., peceman J., Kuper, M ,Zebeau, M (1995). "AFLP: Anew technique for DNA finger printing." Nucleic Acid Research **23**: 4407-4414.

Welsh, J., Mc Clelland,M. (1990). "Figerprinting genomes using PCR with arbitary primers." Nucleic Acids Research **18**: 7213-7218.

Wet de, J. (1986). Pearl millet in Africa and India. Proceedings of the international pearl millet workshop, ICRISAT centre, Patancheru, AP, 502324, INDIA, ICRISAT.
Williams, J., Kubelic, AR., Livak, KJ., Rafalski, JA., Tingey , SV. (1990). "Dna polymorphism amplified by arbitrary primers are usefulas genetic markers." Nucleic Acid Research **18**: 7213-7218.

Young, N., Zamir RD., Ganal MW, Tanksley, SD., (1988). "Use of isogenic lines and simultaneously probing to identify DNA markers tightly linked to the Tm-2a gene in tomato." TAG **81**: 661-667.