BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ISABGOL

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BHARTI MITTAL
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DEPARTMENT OF BIOCHEMISTRY
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GUJARAT AGRICULTURAL UNIVERSITY
ANAND CAMPUS, ANAND - 388 110.

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Regn. No. : 04-04042-98

Dr. R. Bhatnagar
Associate Professor
Department of Biochemistry
B. A. College of Agriculture
Gujarat Agricultural University
Anand Campus, Anand – 388 110
Gujarat (India)

CERTIFICATE

This is to certify that the thesis entitled "**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ISABGOL**" submitted by **BHARTI MITTAL** in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** of the Gujarat Agricultural University is a record of bonafide research work carried out by her under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

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(R. BHATNAGAR)
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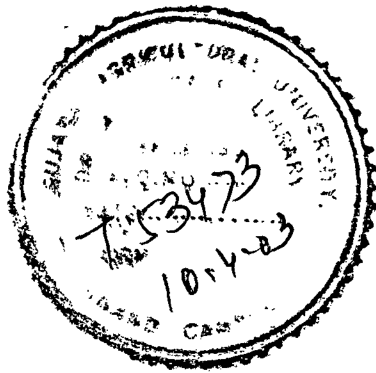

DEDICATED

TO

DR. C.G. JOSHI

Abstract

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ISABGOL

Name of Student **Major Advisor**

Bharti Mittal **Dr. R. Bhatnagar**

Department of Biochemistry
B. A. College of Agriculture
Gujarat Agricultural University
Anand Campus, Anand.

ABSTRACT

Plantago ovata Forsk, commonly known as "Isabgol" belongs to the family Plantaginaceae. It is a native of Mediterranean region and is cultivated for its valuable husk. Although, it has various medicinal uses but less exploited for research at biochemical and molecular level. India is the sole country in the world, which commonly cultivates the Isabgol and is the largest exporter for the same.

In addition to husk, Isabgol contains sufficient amount of essential amino acids (Lysine and Methionine), which are deficit in cereals and pulses. The seeds of Isabgol can be used as supplement to cereals and pulses. The study showed that the highest amount of oil, husk, total carbohydrates, cellulose and protein are present in the genotypes DM3 (4.06%), EC124345 & JI65 (36%), EC124345 & JI65 (63%), EC124345 (7.7%) and DM3 (18%), respectively and present in very low amounts in the genotypes JI10 (3.25%), DM3 (31%), DM3 & DM1 (51%), DM1 (4.2%) and EC124345 & JI65 (15%).

It was also observed that the activity of peroxidase is more in genotype EC124345, which is found to be resistant to downy mildew. On the other hand, the activity of polyphenol oxidase is more in the genotype DM3, which is found to be susceptible to downy mildew.

Phenotypic variations are very less in Isabgol and the genotypes are mostly classified on the basis of Disease Index. because in Isabgol downy mildew disease is very severe and causes high yield loss. The markers include biochemical constituents (eg secondary metabolites in plants) and macromolecules, viz. proteins and DNA. Analysis of secondary metabolites is, however, restricted to those plants that produce a suitable range of metabolites, which can be easily analyzed and distinguished between cultivars. The metabolites, which are being used as markers, should be ideally neutral to environmental effects or management practices. Hence, amongst the molecular markers used, DNA markers are more suitable and ubiquitous to most living organisms.

Genetic maps comprising closely spaced DNA markers are useful for genome analysis. Molecular markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. These markers are not environmentally regulated and are, therefore, unaffected by the conditions in which the plants are grown and are detectable in all stages of growth.

RAPD, CAPS, ISSR and AFLP markers were used in fingerprinting and to examine genetic diversity among twelve genotypes of the *Plantago ovata* Forsk. RAPD and ISSR generated unique profiles for each genotype. CAPS could not distinguish between three of the genotypes DM1, JI10 and GI2, whereas, AFLP could not distinguish between DM1 and JI10. Similarity matrices and dendrograms illustrated the genetic similarities between the different genotypes except some clustering variations, which depend upon the type of marker used.

Similarity and variation among the Isabgol genotypes were observed by cluster analysis and dendrograms were constructed, which were compared with the

dendrogram from biochemical characters and finally the clustering was done using the plant/per cent disease index (PDI), which formed cluster between the genotypes originated at the same place eg JI10, JI42, JI53 and JI56 (developed at Jagudan) except JI65 which showed the similarities with the genotypes developed at Anand (DM1, DM2, DM3 and DM4).

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Introduction

CHAPTER – I
INTRODUCTION

Human beings have a long history of the use of higher plant extracts for the therapy of diverse maladies. Medicinal plants play a major role in the lives of many people worldwide, and their usage has increased significantly. Epidemiological studies have associated a reduced risk of infectious diseases and cancer with a diet high in fruits and vegetables. Finding additional agents for human or agricultural use based on higher plant extracts contribute to increase in the number of plant compounds of potentially beneficial applications (Ebadi, 2002).

Among the various known medicinal plants *Plantago ovata* Forsk, commonly known as **Isabgol**, belonging to the Plantaginaceae family of the order Plantagenales, has gained a tradition of commercial cultivation in India. It is native to Mediterranean region, especially south of France (Rastogi and Mehrotra, 1993). It is an irrigated medicinal crop grown in North Gujarat, Madhya Pradesh and parts of Southern Rajasthan (Kanbi and Chakraborty, 1989).

The roots and the herbaceous parts are generally stringent and slightly bitter. Seeds are sweet, acrid, mucilaginous, astringent to the bowel, useful in kapha, biliousness, leprosy and chronic dysentery. The cooling effect of seeds, demulcent are used in inflammation of digestive organs, rheumatic and goutry swellings. The seeds are taken in large quantities and as they swell up in contact with water they increase the bulk of the intestinal content and in this way relieve chronic constipation by mechanically stimulating the intestinal peristalsis (Kirtikar and Basu, 1935). The recent findings of its potentiality to lower cholesterol levels in blood serum (Swega *et*

al., 1998) and its ability to act as a gelling agent for microbial culture media (Neeru *et al*., 1997) has increased its importance further.

It is the first ranking foreign exchange earner for India. Although it has several advantages, very less work has been done so far. No prior information is available regarding nutrient value of seeds as well as genome. The research in this crop is oriented mainly in the direction of higher yield and to obtain better quality drug, as husk is the main constituent of Isabgol and is medicinally important. Till date only few varieties have been released viz. GI1, GI2, Haryana Isabgol-5 etc. The main reason for the release of very few varieties is that, it has very less phenotypic variation. In view of wide economic importance of Isabgol, biochemical and molecular characterization holds a prominent role. In the Patent acts law (under UPOV convention, Geneva, Switzerland) advanced biochemical and molecular biological techniques like High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR), and Amplified Fragment Length Polymorphism (AFLP) should be used for characterizing the plants and for varietal identification (Gill *et al*., 1996).

A variety of marker systems have been developed over the years to facilitate the analysis of plant and animal genomes. Detailed genome investigation using classical genetic phenotypes and isozymes as markers which are the first genetic markers, posed severe limitations, including problems with phenotypic penetrance or heritability, difficulties involved with constructing multiple marked lines and low map resolution. Thus DNA based markers are potentially limitless in number and their

identification which are unaffected by phenotype (Vogel *et al.*, 1996) and other environmental conditions.

Application of molecular marker system has significantly advanced the plant genome system, as plants have always been looked upon as a key source of energy for survival and evolution of the animal kingdom, thus forming a base for ecological pyramid. Over the last few decades, plant genomes have been studied extensively bringing about a revolution in this area. With the advent of molecular markers, new generations of markers have been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences.

The discovery of PCR (Polymerase Chain Reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker based gene tags, map-based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping, marker assisted selection of desirable genotypes, etc. This gave new dimensions to concerted efforts of breeding and marker aided selection that can reduce the time span of developing new and better varieties which will make the dream of supper varieties come true (Joshi *et al.*, 1999).

The choice of marker system used for a particular application will depend on its ease of use and on the technical expertise. However, the most important consideration is the type of genomic information required. Each method makes an estimate of genomic polymorphism and identifies polymorphic markers in its own distinct way, and each has different biases (Rafalski *et al.*, 1995).

RFLPs are co-dominant DNA markers that have been applied to a wide range of species, but their effective use has been limited by the large amount of genetic

material required and the labor involved in the analysis. Alternatively methods based on PCR are technically less demanding and offer great experimental simplicity and can be used with the genomes having no prior sequence information. For example, the RAPD assay (Williams *et al*., 1990) can identify extremely large numbers of genomic polymorphisms, require minimal amounts of the input DNA, and is technically simple and straightforward. One of the disadvantages is that they are dominant markers as homozygous dominant genotypes can't be distinguished from heterozygotes. Multiplexed genome assay methods, such as AFLP (Zabeau and Vos, 1993), inter-simple sequence repeat amplification (Zietkiewicz *et al.* 1994), can reveal several polymorphisms simultaneously. These multiplexed methods are becoming increasingly useful for fingerprinting, cultivar's identification, marker discovery and mapping.

As the genome size is very small, very limited information is available on phenotypic variation. Even though, the crop has great medicinal value studies have been conducted on growth and yield attributes of this crop. However, chemical composition of seeds with respect to biochemical parameters and molecular characterization has not been reported. Hence, the present investigation was under taken in the seeds of 12 genotypes of Isabgol with the following objectives:

1. Characterization of different genotypes of Isabgol on the basis of biochemical parameters.
2. Characterization of different genotypes of Isabgol using different molecular markers viz. RAPD, CAPS, ISSR and AFLP.

Review of Literature

CHAPTER – II
REVIEW OF LITERATURE

India, being one of the twelve-mega diversity centers, has immense biotic wealth marked by remarkable ecosystems, species and genetic diversity, is matched equally by rich cultural diversity and health traditions (Groombridge, 1992). Since a vast majority of Indian population is dependent on traditional medicine for primary health care and, as against the recent revival of interest in plant medicines across the globe and consequent pressure on precious herbal resources, it makes sense to rationalize the use of medicinal plants through scientific screening and validation.

Plant resources are widely employed in nature. Over 7000 species out of an estimated 17,000 higher angiosperms recorded from India are reported useful for medicinal purposes. An infinite variety of plants and tree resins exist. Many have been studied scientifically and the pharmacological effects have been proved for their active ingredients. Many others however, are still used empirically (Chirif, 1978).

Psyllium (*Plantago ovata*):

For the most amazingly pleasurable "morning sit-down", try bulk Psyllium: one of the God's greatest creations (Herbtrader.com). Psyllium has traditionally been used internally for constipation and diarrhea. In Ayurvedic medicines it is used with buttermilk for diarrhea, and with warm milk for constipation. Psyllium is a common name used for several members of the plant genus *Plantago*, whose seeds are used commercially for production of mucilage. The genus *Plantago* contains over 200 species. *Plantago ovata* and *Plantago psyllium* are produced commercially in several European countries, the former Soviet Union, Pakistan and India. Plantago seeds commonly known as black, French or Spanish psyllium, is obtained from

P.psyllium and *P.arenaria.* Seeds produced from *P.ovata* are known in trading circle as white or blond psyllium, Indian Plantago or Isabgol/Isabgul. The common name in India for *P.ovata* comes from the Persian words "isap" and "ghol" that means horse ear, which is descriptive of the shape of the seed. India dominates the world market in the production of psyllium for export. Its taxonomic classification is as (Newcropsearch.com) follows-

KINGDOM-	PLANTAE (PLANTS)
SUB-KINGDOM-	TRACHEOBIONTA (VASCULAR PLANTS)
SUPERDIVISON-	SPERMATOPHYTA (SEED PLANTS)
DIVISON-	MAGNOLIOPHYTA (FLOWERING PLANTS)
CLASS-	MAGNOLIOPSIDA (DICOTYLEDONS)
SUBCLASS-	ASTERIDAE
ORDER-	PLANTAGINALES
FAMILY-	PLANTAGINACEAE (PLANTAIN FAMILY)
GENUS-	PLANTAGO L. (PLANTAIN)
SPECIES-	*PLANTAGO OVATA* FORSK (Indian Desert Wheat)

Recent interest in Plantago has come primarily due to its use in high fiber breakfast cereals and these high fiber cereals containing Plantago are effective in reducing cholesterol (Herbmed.com). Several studies point to a cholesterol reduction attributed to a diet that includes dietary fiber such as Plantago. Research reported in The American Journal of Clinical Nutrition concluded, that the use of soluble fiber cereals is an effective and well-tolerated part of a prudent diet for the treatment of mild to moderate hypercholesterolemia. Many research works also indicate that Isabgol incorporated into food products is more effective in reducing blood glucose than use of a soluble fiber supplement that is separate from the food.

SEED PREPARATION AND GERMINATION:

Isabgol has small seeds, 1000 seeds weighing less than 2 grams. Under ideal conditions of adequate moisture and low temperature (50 to 68^{0}F), 30% of the seeds

germinate in 5 to 8 days. The seeds show some innate dormancy (3 months) following harvest. Various treatments including wet and dry heat, cold, scarification, ethylene and CO_2 do not eliminate this dormancy period. Post-dormancy seeds show 90% germination at 84^0F and germination rate decreases as temperature increases.

USES: (HerbMed.com)

Psyllium is produced mainly for its mucilage contents, which is highest in *P.ovata*. Mucilage describes a group of clear, colorless, gelling agents derived from plants. The mucilage obtained from Isabgol comes from the seed coat. The United States is the world's largest importer of its "husk" with over 60% of total imports going to pharmaceutical firms for their use in products such as "**Metamucil**", "**Effersyllium**" and "**Fiberall**". The mucilage is also used as a natural dietary fiber for animals. The dehusked seeds, that remain after the seed coat is milled off, is rich in starch and fatty acids which is used in India as chicken and cattle feed. Isabgol has been used in connection with the following conditions:

RANKING	HEALTH CONCERN
PRIMARY	Artherosclerosis
	Constipation
	Diabetes
	Diverticular Disease
	Hemorrhoids
	High cholesterol
	Irritable Bowel Syndrome
SECONDARY	Diarrhea
	High triglycerides
	Weight loss and obesity
OTHERS	Parkinson's disease
	Psoriasis
	Ulcerative colitis

In addition to its traditional and current use for constipation, Isabgol is also used topically to treat skin irritations, including poison ivy reactions, insect bites and stings. It has also been used in traditional herbal systems of China and India to treat diarrhea, hemorrhoids, bladder problems and high blood pressure. The husk or mucilaginous seed coat is used to cure the inflammation of mucous membrane of gastro-intestinal and genito-urinary tracts, amoebic and bacillary dysentery and diarrhea, duodenal ulcers, gonorrhea and piles (Bhagat, 1980).

HUSK

Plantago seed mucilage is often referred to as husk or psyllium husk. The milled seed mucilage is a white fibrous material that is hydrophilic (water loving). Upon absorbing water, the clear colorless mucilaginous gel that forms, increases in the volume by ten-fold or more. Isabgol is mainly used as a dietary fiber, which is not digested by the action of enzymes in small intestine. Action of Isabgol mucilage which absorbs excess water while stimulating normal bowel elimination is purely a mechanical one. Although, it's main use has been as a laxative, it is more appropriately termed a true dietary fiber. Isabgol seeds contain 10 to 30% mucilage. The resulting bulk stimulates a reflex contraction of the walls of the bowel, followed by emptying (Leung and Foster, 1996).

In one of the studies, Isabgol seeds successfully treated constipation due to poor life style (low fiber, low exercise etc), not when an actual disease was the cause (Voderholzer *et al.*, 1997). Numerous double-blind studies confirm Isabgol can lower total cholesterol and Low Density Lipoprotein (LDL), "the bad" cholesterol (Anderson *et al.*, 1999). However, levels of High Density Lipoprotein (HDL), "the good" cholesterol are not affected (Oson *et al.*, 1997). The cholesterol lowering effect of Isabgol has

been reported in children (Davidson *et al.*, 1996), as well as in adults. Probably due to its soluble fiber content, Isabgol has improved glucose tolerance in some people with diabetes (Florholmen *et al.*, 1996).

The seed husk is also used in printing, agar-agar media preparation, gum and jelly making, as binder in tablets and also to terminate pregnancy. It is also reported to be useful against cancer. The usefulness of Isabgol is entirely due to the large quantity of mucilage having a remarkable property of a thickener and therefore, is used as a fixative in ice-cream industries in the western countries. It is widely used in USA, UK and many other countries as a cattle feed and bird feed (Punia *et al.*, 1985).

CHEMISTRY OF ISABGOL

OIL

Pendse and Dutt (1934) reported a pale yellow oil from the seeds of *Plantago ovata* (11.42%). The oil contains both saturated and unsaturated fatty acids. Saturated fatty acids are composed of 32.77% palmitic acid, 60.37% stearic acid and 6.80% lignoceric acids (Pendse, 1937). Bhunvara and Khurana (1950) estimated 6.23% oil in Indian seeds as extracted with petroleum ether at 40-60°C, and found that this oil did not interfere with the swelling properties of the seeds.

Chakraborty and Patel (1992) reported 8.6% oil from the dehusked seeds of Isabgol as extracted with n-hexane. They have also reported the presence of palmitic acid (10.9%), stearic acid (1.7%), oleic acid (48.0%), linoleic acid (37.7%) and linolenic acid (2.10%) in the oil.

CARBOHYDRATES AND RELATED COMPOUNDS

Plant energy storage components are referred to as carbohydrates. The group, which the term carbohydrates represent includes, di- (sucrose, lactose etc), and poly-

(stach, inulin) saccharides, acids that are produced after cellular carbohydrate respiration, alcohols such as sorbitol, cellulose, gums and mucilage. For the purpose of therapeutics, usually the cellulose and gum/mucilage subgroups are most important.

A study was designed to evaluate the effect of Plantago mucilage and acarbose on glycemic index by Frati *et al.* (1998). They reported that the addition of acarbose or Iasbgol to meal may reduce the glycemic index of carbohydrate foods and may help diabetic control. Pastors *et al.* (1991) have reported that Plantago fiber reduces rise in postprandial glucose and insulin concentrations in patients with non-insulin dependent diabetes. Wolever *et al.* (1991) determined the effect of method of administration of Isabgol on glycemic response and carbohydrate digestibility.

The seeds upon extraction with water yield mucilage which constitute about 30% of the seeds (Chakraborty and Patel, 1992), cosists of D-xylose and L-arabinose (Smith and Montagomery, 1959).

The husk is reported to be a mixture of at least two distinct components-a branched polysaccharide with a polyxylose backbone and pectin like compound containing galactouranate and rhamnose, whose basic composition varies from species to species (Salyers *et al.*, 1978). The exact compositions of the polymers remain to be determined (Tyler *et al.*, 1976).

Naresh *et al.* (1992) estimated the total carbohydrate contents from 43 genotypes of *Plantago ovata* seeds and it ranged from 70.09 to 74.14%.

A glycoside named acubin was isolated from the Isabgol plant by Chopra *et al.*, in 1958 and reported to be pharmacologically inactive.

French *et al.* (1953) isolated a sugar from Plantago named plantiose. Plantiose is a non-reducing trisaccharide and gives glucose, fructose and galactose on hydrolysis. However, Pendse and Dutt (1934), didn't find any glycoside or alkaloid in *Plantago ovata* seeds but reported that they contain large amount of mucilaginous matter, inorganic ash and reducing sugars.

Pendse *et al.* (1976) reported the varied chemical composition of *Plantago ovata* from Poona and Gujarat material. Gujarat material was higher in ash (6.85 vs 2.7%) and crude fiber (23.5 vs 18.9%) whereas, Poona material was higher in protein (9.4 vs 8.7%) and carbohydrates (58.2 vs 50.65%).

PROTEINS AND AMINOACIDS

There are 20 amino acids, derived from the acid hydrolysate of plant proteins. Plant proteins are essential for carrying out specific cellular functions both internally and externally. Plant proteins are seed-based storehouses for nitrogen and guard against would-be predators. Some are toxic to humans but some other are included in the human diet. Furthermore, some proteins have therapeutic use, L-DOPA, from fava bean, is used in the treatment of Parkinson's disease; L-cysteine, found in all the plants, is used in eye drops and topical antibiotics. Plants also contain essential amino acids: those amino acids humans can't synthesize, for that they are dependent on plant sources eg Methionine, Lysine and Tryptophan etc (Harbone, 2000).

Patel *et al.* (1980) screened seed proteins of *Plantago ovata* by chromatographic technique and found amino acid asparagine in free form, glycine and cystine in bound form and other amino acids both in free and bound form.

Chakraborty and Patel (1992), reported that Isabgol seeds have sufficient amount of essential amino acids eg lysine (4.1%) and methionine (1.7%) comparable to cereal and pulses.

Isabgol-gola, the byproduct of Isabgol husk processing industry, was found to have high protein content with good profile of limiting essential amino acids. Although, oil content was much less, its oleic/linoleic ratio (1:27) ensures it to be a good grade of edible oil (Anon., 1989).

ENZYMES OR BIOCHEMICAL MARKERS

Isoenzymes of plant tissues are useful biochemical index reflecting changes in metabolic activity during growth, development and differentiation. The conventional selection process is long, difficult and exposed to the environment, which has a negative effect on the expression of resistance. However, biochemical markers, which may or may not be a part of resistance mechanism, are a practical and reliable tool for predicting resistance to disease.

In lettuce (*Lactuca sativa*), an apparent trend indicated that one of the components of field resistance to downy mildew (*Bremia lactucae*) could be related to a high level of Peroxidase (Pox) prior to infection (Reuveni *et al.*, 1991). The highest activity was detected in the "Iceberg" and "Santa Anna" cultivars (the one with the highest level of field resistance and a source of resistance being used in current breeding programs). An intermediate level of Pox activity was detected in cvs "Grand Rapids" and "Lobjoits Cos", which exhibit moderate to low field resistance. In "Cobham Green" (a susceptible butterhead type) and "Ithaca" (a highly susceptible crisphead type), Pox activity was low. A similar trend was also found between Pox

activity and field resistance of three wild (*L. serriola*) lines. Total activity of Pox might be a useful tool to predict nonspecific resistance.

Esterases are a group of hydrolytic enzymes with broad substrate specificity. Esterases can be classified on the basis of the type of substrate hydrolyzed. Esterases in general exist as isoenzymes, and are useful to study the polymorphism, both within and among the species. Esterases have a definite role to play in plant growth and development, cell wall expansion, somatic embryogenesis (as a cytochemical marker), stomatal movement, resistance against infection and as a bioassay for environmental quality.

Pramanik *et al.* (1996) reported the changes in esterase and superoxide dismutase isoenzymes during *in vitro* morphogenesis in *Plantago ovata* Forsk and postulated that the new enzyme forms in esterase as well as in superoxide dismutase may either arise *de novo* or due to post-transcriptional modification of the genes and are essential for shoot tip multiplication of *Plantago ovata.*

DNA BASED MOLECULAR MARKERS:

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. A wide variety of techniques have, therefore, been developed in the past few years for visualizing DNA sequence polymorphism.

Alec-Jeffery et al., (1985) introduced the term DNA fingerprinting for the first time, to describe a bar code like DNA fragment patterns generated by multilocus probe after electrophoretic separation of genomic DNA fragments. The emerging patterns make up a unique feature of the analyzed individual and are currently

considered to be the ultimate tool for biological individualization. Recently, the term DNA fingerprinting/profiling is used to describe the combined use of several single locus detection systems and is being used as a versatile tool for investigating various aspects of plant genomes. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding and diagnostics (Joshi *et al.*, 1999).

Properties desirable for ideal DNA markers-

- Highly polymorphic nature
- Co dominant inheritance (determination of homozygous and heterozygous state of diploid organisms)
- Frequent occurrence in genome
- Selective natural behavior (the DNA sequence of any organism is neutral to environmental conditions or management practices)
- Easy access (availability)
- Easy and fast assay
- High reproducibility
- Easy exchange of data between laboratories.

RAPD:

A widely applied approach for characterization of DNA from plants and other organisms is to use PCR with short oligonucleotide primers of arbitrary (random) sequences to generate genetic markers. This is the basis of the Random Amplified Polymorphic DNA (RAPD) method (Williams *et al.*, 1990), Arbitrary Primed Polymerase Chain Reaction (AP-PCR) (Welsh and McClelland, 1990) and DNA Amplification Fingerprinting (DAF) (Caetano-Anolles *et al.*, 1991). These random

primers were shown reproducibility to detect polymorphism in the absence of specific nucleotide sequence information in DNA from bacterial, fungal, human and plant origins. The technique has good reproducibility under identical conditions.

RAPD markers are well suited for genetic mapping, for plant and animal breeding applications and for DNA fingerprinting, with particular utility for studies on population genetics. RAPD markers can also provide an efficient assay for polymorphisms, which should allow rapid identification and isolation of chromosome specific DNA fragments. Hybrid cell lines or genetic stocks carrying deletions or additions of large chromosomal segments could be screened relative to appropriate controls, to identify the region of the genome carrying the deletions or additions. Like most molecular markers, the information content of an individual RAPD marker is very low. It is only when many of those anonymous markers are used to define a genome that they begin to have utility. High-density genetic maps comprising molecular markers have lead to the identification of several previously unidentified loci of biological importance (Taksley *et al.*, 1989).

RAPD markers hold promise for the automation of the genome mapping extending the power of genetic analysis to organisms, which lack an ample number of phenotypic markers to completely describe their genome. Genetic mapping using RAPD markers has several advantages over other methods: (i) a universal set of primers can be used for genome analysis in a wide variety of species (ii) no preliminary work, such as isolation of cloned DNA probes, preparation of filters for hybridizations or nucleotide sequencing, is required (iii) each RAPD marker is equivalent of a Sequence Tagged Site (Olson *et al.*, 1989), which can greatly simplify information transfer in collaborative research programs. Perhaps the most significant

advantage of this method is that the determination of genotype can be automated. Genetic maps consisting of RAPD markers can be obtained more efficiently and with greater marker density, than by RFLP or tagged PCR based methods.

RAPDs have been used for a variety of purposes including the construction of genetic linkage maps (Reiter *et al.*, 1992), genetic tagging, identification of cultivars (Nybom, 1994), assessment of genetic variation in populations (Chalmers *et al.*, 1992) and species (Nesbitt *et al.*, 1995), study of phylogenetic relationships among species, subspecies and cultivars (Landry *et al.*, 1994), and for many other purposes in a large number of plant species. These applications have also led to the development of species-specific (Chen *et al.*, 1998), genome-specific and chromosome-specific markers (Wang *et al.*, 1995) and more importantly to the development of molecular markers for identification and selection of the desired genotypes (for a variety of traits of economic importance) in segregating populations during breeding programs.

The sweet chestnut (*Castanea sativa*) is a typical nut crop of the Mediterranean basin, whose importance is related to both fruit and wood production. Chestnut cultivars and clonal variants are often classified according to morphological triats and geographical origin. This approach makes discrimination among cultivars ambiguous and difficult. Galderisi *et al.* (1998) presented a random amplified polymorphic DNA (RAPD) based procedure for molecular typing of chestnut cultivars in fruit production.

Chinese water chestnut is a crop new to Australia. To establish a reputable industry, the influence of both genotype and environment on yield and quality need to be evaluated. To that end, the genetic relationship of cultivated Chinese water chestnut in Australia was investigated by using RAPD analysis by Mei Li and Midmore

(1999). It is therefore, suggested that the observed morphological and physiological variations in Chinese water chestnut produced in Australia are phenotypic and reflects the differences of environment and cultivation rather than genetic diversity.

Nabauer *et al.* (1999) used RAPD markers to assess levels and patterns of genetic diversity in *Digitalis obscura* and out crossing cardanolide producing medicinal plant species. Six arbitrarily chosen decamer primers resulting in highly reproducible polymorphic bands analyzed a total of 50 plants from six Spanish natural populations. The analysis of molecular variance (AMOVA) showed that most of the variation (8.8%) occurred among individuals within the population, which was expected from the out crossing system.

RAPD analysis was done to determine intraspecific variability in *Andrographis paniculata* (Padmesh *et al.*, 1999), a popular antipyretic and hepatoprotective drug used in India. The results indicated that the RAPD could be effectively used for genetic diversity analysis in wild species of prospective value as it is reliable, rapid and superior to those based on pedigree information.

DNA fingerprints were produced for samples of the Chinese herb dngshen, the roots of *Codonopsis pilosula*, from 11 sites in the Chinese provinces (Zhang *et al.*, 1999). Similarity index analysis revealed that samples from the same province generated similar DNA fingerprints, while samples from different provinces displayed different DNA fingerprints. This method may be an useful tool for authentication of the sources of other Chinese herbal medicinal materials.

Darokar et al. (2000) to assess the extent of genetic diversity, carried out RAPD analysis in six accessions each of *Tagetes minuta* and *Tagetes patula* collected from different geographical parts of India. The comparison of phylogenetic trees

based on their genetic diversity (RAPDs), indicated much higher extent of variation detectable through DNA analysis compared to morphotypic variation indicating utility of RAPD technique in analyzing genetic variation in Tagetes species for further exploitation.

Gemas *et al.* (2000) carried out Inter- and Intra- varietal analysis of three *Olea europaea* L. cultivars using the RAPD technique. The method has the potential use in varietal certification and breeding programs that need to analyze a high number of samples.

Persson *et al.* (2000) carried out identification of culninary rhubarb (*Rheum* spp) cultivars using morphological characterization and RAPD markers. When results were compared on morphology and RAPD with pedigree information for four cultivars, a combination of the two types of data sets appeared to give fairly good information about relatedness among cultivars.

Shasany *et al.* (2000) screened twenty-three accessions of *Allium sativum* L. (Garlic) from different parts of India and two accessions from Argentina by RAPD analysis and bioactivity evaluation. They observed that the combination of morphological and RAPD data will be of immense importance for genetic improvement through marker selections. This will also be helpful for effective management of germplasm core collection.

Dioecy is well established in animals, but occurs sporadically in the plant kingdom. The great majority of flowering plants are hermaphrodite, but a large proportion of angiosperm families include dioecious species, with separate male and female individual plants. Kafkas *et al.* (2001) have conducted the study on development of sex-associated RAPD markers in wild Pistacia spp.

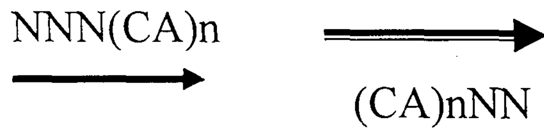
ISSR:

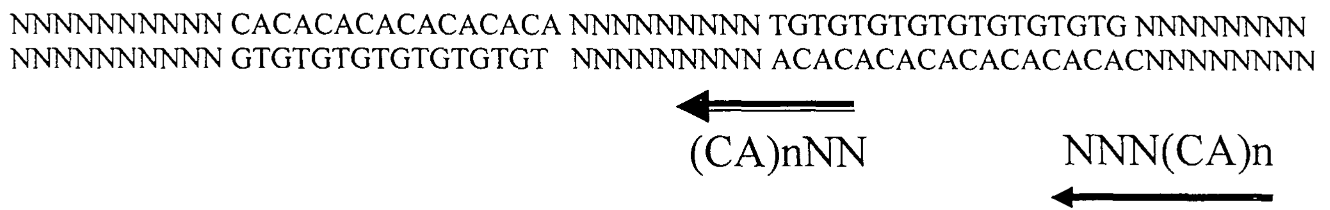
Simple repeat sequences, di- or tri- nucleotide repeats are common in plant genomes. Simple (or short) sequence repeats (SSRs) are also known as STRs (short tandom repeats) or microsatellites. PCR using primers to the sequences flanking these repeats can be used to generate polymorphisms because of the frequent variation in the length of the repeat region. Microsatellites have been established as useful genetic markers in plant species (Wu and Tanksley, 1993; Zhao and Kochert, 1993; Saghai-Maroof *et al.*, 1994; Roder *et al.*, 1995). Another approach is to amplify the regions between SSRs in inter-SSR-PCR (Zietkewicz *et al.*, 1994). In this technique, primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. Various microsatellites anchored at the 3' end are used for amplifying genomic DNA which increases their simplicity. These are mostly dominant markers; though occasionally a few of them exhibit co-dominance. An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and pentanucleotides [(4) 3=64, (4) 4=256] etc with an anchor made up of a few bases and can be exploited for a broad range of applications in plant species (Fig. 2.1).

Inter-SSR PCR provides a novel fingerprinting approach applicable for taxonomic and phylogenetic comparisons and mapping tool in a wide range of organisms.

Wolf and Morgan (1998), surveyed *Plantago major* plants from several Scottish and Dutch locations for their genetic variation using PCR markers, namely RAPD analysis, anchored- inter-SSR PCR and chloroplast PCR followed by RFLP analysis. The RAPD and inter-SSR markers showed a differentiation between the two sub-species of *P. major*.

ISSR





PCR Product 3'-anchored primer

PCR Product 5'-anchored primer

Figure:2.1- Inter-SSR PCR: A single primer targeting a (CA)n repeat, anchored either at the 3' or at 5' end of the repeat, is used to amplify genomic sequence flanked by two inversely oriented (CA)n elements.

AFLP:

Amplified Fragment Length Polymorphism is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Zabeau and Vos, 1993; Vos *et al.*, 1995). DNA from the plant to be analyzed (Fig. 2.2) is digested with a restriction enzyme. Short oligonucleotides are then ligated to the ends of all fragments. A subset of the restriction fragments is then amplified by PCR with primers complementary to the added oligonucleotides and restriction site. Additional specificity is provided by a small number of specific nucleotides added at the 3' end of the PCR primer. Each base added at the 3' end of the primer, reduces the number of fragments amplified by a factor of 16, on an average because of the four fold selection at each end (Henry, 1997).

AFLP can be used for DNAs of any origin or complexity. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques (Saiki *et al.*, 1988; Ehrlich et al., 1991) and is extremely useful in detecting polymorphisms between closely related genotypes.

This approach is very useful in saturation mapping and for discrimination between varieties. High reproducibility, rapid generation and high frequency of identifiable AFLP polymorphisms make AFLP DNA analysis an attractive technique for identifying polymorphisms and for determining linkages by analyzing individuals

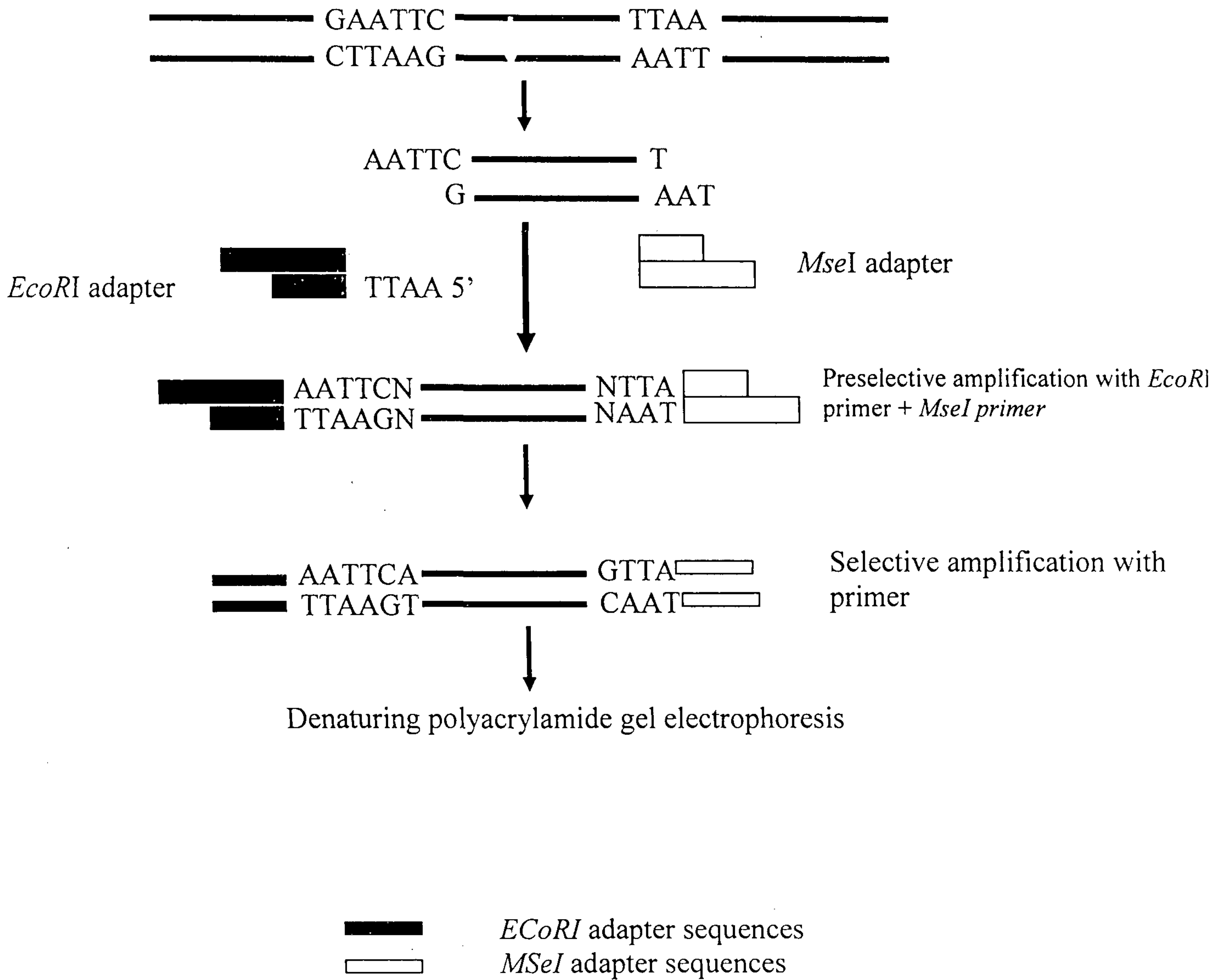


Figure 2.2 : Flow diagram for AFLP

rom a segregating population. However, AFLPs are still expensive to generate as the ›ands are detected by silver staining, fluorescent dye or radioactivity (Mohan *et al.*, 997).

Ronald *et al.* (2000) performed an evaluation, to reveal polymorphisms among *olium perenne* with different degree of Kinship by AFLP markers. They applied adioactive and fluorescent detection techniques. The use of a fluorescent detection pproach contributed greatly to the speed and ease of conducting and interpreting the AFLP pattern. The great discriminative power of AFLP markers and their capacity to represent genetic relationship among rye grass plants was shown.

Classical methods of identification and characterization of sweet cherry (*Prunus avium* L.) or relatedness among groups of cherry plants have relied upon morphological characters, which can be influenced by environmental or agronomic factors. Struss *et al.*, (2000) have made an attempt, for the detection of genetic diversity among populations of sweet cherry by AFLP.

Powell *et al.* (1996) compared the efficiency of RFLP, RAPD, AFLP and SSR (microsatellites) markers for germplasm analysis in soybean. The utility of these DNA markers in germplasm analysis was determined by evaluating information content (expected heterozygosity), number of loci analyzed (multiplex ratio) and effectiveness relationships between accessions. A comparison of genetic similarity matrices revealed that, estimates based on RFLPs, AFLPs and SSRs are highly correlated, indicating congruence between these assays. However, correlations of RAPD marker data with those obtained using other marker systems were lower, because of higher estimates of inter-specific similarities produced by RAPDs. The similarity estimates

obtained by RAPD and AFLP were more closely related than those involving other marker systems.

RAPD, ISSR and AFLP markers were used to generate fingerprints and to examine the genetic diversity in gooseberry (Ribes grossularia subgenus Grossularia) germplasm by Lanham and Brennan, (1999). AFLP generated unique profiles for each genotype. Whereas, RAPD and ISSR could not distinguish all genotypes separately. AFLP alone were sufficient to achieve this but RAPD and ISSR required complementation by AFLP data. The broader implications of these results suggest that success of molecular markers in fingerprinting for some plant species may depend on which marker type is being used and that a combination of marker types may be the best option.

Germplasms from the genus Ribes were assayed for molecular polymorphism using RAPD and ISSR markers by Lanham *et al.* (2000). The levels of genetic diversity were found high in Ribes.

Materials and Methods

CHAPTER – III
MATERIALS AND METHODS

The present study was conducted at the Department of Biochemistry, B.A.College of Agriculture, Gujarat Agricultural University, Anand in collaboration with Department of Animal Genetics and Breeding, College of Veterinary Sciences and Animal Husbandry, Gujarat Agricultural University, Anand.

3.1 EXPERIMENTAL MATERIAL

For the present study, 12 genotypes of Isabgol seeds (*Plantago ovata* Forsk) were received from Medicinal and Aromatic plants Unit, Gujarat Agricultural University, Anand.

3.2 LABORATORY WARE

All glassware used were obtained from Corning/Borosil and disposable plasticware from Axygen Ltd.

All glassware and plasticware were washed thoroughly and dried in oven. Glassware was sterilized in oven and plasticware was autoclaved before use.

3.3 CHEMICALS

All the biochemicals used were of analytical reagent grade and were obtained from Sisco Research Lab. (SRL), E-merck and Sigma.

The chemicals used for the molecular study were of molecular reagent grade.

3.4 EQUIPMENTS

Centrifuge	:	Universal 30 RF, Hettich Zentrifuge, Germany.
Variable Volume Micropipettes	:	Finnpipette, Labsystems, Finland, Nichipette, Nichriyo, Japan and Eppendorf, Eppendorf Research, Germany.
Waterbath		Yorco YSI-413, York Scientific Industries Ltd., India.
Spectrophotometer	:	Unicam UV/Vis Spectrophotometer, U.K.
DNA Thermal cycler	:	Mastercycler Gradient, Eppendorf, Germany.

Gel Electrophoretic Unit (Sub-Marine) (Vertical Slab gel)	:	Bangalore Genei Ltd., India Atto, Japan
Power Pac	:	Power Pac-1000, Bio-Rad, USA AE-8750, Atto, Japan
Weighing Balance	:	BP 210 D, Sartorius, Germany
Microwave Oven	:	LG India Ltd., India
Bio Imaging System	:	Gene Genius Syngene, U.K.

3.5 PREPARATION OF CHEMICALS AND REAGENTS

3.5.1 Total oil :- Hexane

3.5.2 Husk % :- 0.1 N HCl

3.5.3 Total carbohydrate

(a) 5% Phenol :- 50 g re-distilled phenol was dissolved in water and diluted to 1 litre.

(b) Sulphuric Acid 96% analytical reagent grade.

(c) 2.5 N HCl.

(d) Solid sodium carbonate.

3.5.4 Total soluble sugars

(a) 80% Ethanol : 80 ml Absolute alcohol (98%) made to 98 ml with distilled water.

(b) 5% Phenol.

(c) Concentrated sulphuric acid.

3.5.5 Reducing sugars

(a) Anhydrous sodium carbonate (2.5 g), Sodium bicarbonate (2g), potassium sodium tartrate (92.5 g) and anhydrous sodium sulphate (20 g) were dissolved in 80 ml of distilled water and volume made up to 100 ml.

(b) Copper sulphate (15 g) was dissolved in a small volume of distilled water. One drop of sulphuric acid was added and volume was made up to 100 ml.

4 ml of (b) and 96 ml of (a) were mixed before use.

(c) Arsenomolybdate Reagent: Ammonium molybdate (2.5 g) was dissolved in 45 ml of distilled water, then 25 ml of sulphuric acid was added to it and mixed properly. Then 0.3 g di sodium hydrogen arsenate dissolved in 25 ml of distilled water was added. Mixed well and was incubated at 37°C for 48 hrs in an oven.

3.5.6 Cellulose

(a) Acetic/Nitric acid reagent : Acetic acid (150 ml of 80%) and 15 ml of concentrated nitric acid were mixed.

(b) Anthrone reagent : Anthrone (200 mg) was dissolved in 100 ml of concentrated sulphuric acid. (Prepared fresh and chilled for 2 hrs before use).

(c) 67% sulphuric acid.

3.5.7 Ascorbic acid

(a) 9 N H_2SO_4 : Concentrated sulphuric acid (250 ml, sp. gr. 1.84) was added to 700 ml of distilled water and kept on ice. After cooling diluted to one litre.

(b) 2,4-Dinitrophenyl hydrazine (DNPH) : DNPH (2.0 g) was dissolved in 100 ml of 9 N H_2SO_4 and filtered through filter paper.

(c) 10% Thiourea : Thiourea (10 g) was dissolved in 50% alcohol and volume was made up to 100 ml with 50% alcohol.

(d) 4% Trichloroacetic acid (TCA) : TCA (40g) was dissolved in 800 ml of distilled water and volume was made up to 1000 ml.

3.5.8 Total free amino acid

(a) Ninhydrin reagent : 1% ninhydrin was prepared in 0.5M citrate buffer (pH 5.5).

(b) Pure Glycerol.

(c) 0.5 M citrate buffer (pH 5.5)

(a), (b) and (c) were mixed in the ratio of 5:12:2.

(d) 80% Ethanol :

3.5.9 Total phenol

(a) Folin ciocalteau Reagent : Folin reagent was diluted 1:2 with distilled water and prepared fresh.

(b) 35% sodium carbonate : Na_2CO_3 (35g) was dissolved in 100 ml of hot distilled water and kept overnight for saturation. Filtered through glass wool before use. Prepared fresh.

(c) 0.3 N HCl in methanol.

3.5.10 Total protein (Microkjeldahl Method)

(a) Sulphuric acid (Sp. gr. 1.84) :- N-free.

(b) Catalyst Mixture : 99.0 g of K_2SO_4, 4.1 g of HgO and 0.8 g of $CuSO_4$ were ground together in a mortar and pestle.

(c) Sodium hydroxide-sodium thiosulphate solution :- NaOH (50 g) and 5 g $Na_2S_2O_3.5H_2O$ were dissolved in distilled water and made up to 100 ml (Prepared under cold conditions).

(d) Boric Acid solution : Boric acid (4 g) was dissolved in warm water and diluted to 100 ml.

(e) 0.3 N sulphuric acid.

(f) Methyl red Bromocresol green indicator solution : One part 0.2% methyl red in ethanol with 5 parts of 0.2% bromo cresol green in ethanol were mixed.

3.5.11 Proline

(a) 1% sulphosalicylic acid : Sulphosalicylic acid (1 g) was dissolved in 100 ml distilled water.

(b) Ninhydrin solution : Ninhydrin (1.25 g) was dissolved in 30 ml of glacial acetic acid, 8 m. of orthophosphoric acid and 12 ml of distilled water was added and mixed properly.

(c) Toluene.

3.5.12 Lysine

(a) Papain solution : Papain (4 mg) was dissolved in 1 ml of 0.03M phosphate buffer (pH 7.4) and filtered.

(b) 0.6 M carbonate buffer (pH 9.0).

(c) 0.05 M borate buffer (pH 9.0).

(d) Copper phosphate suspension :

Solution A : $CuCl_2.12H_2O$ (2.8 g) was dissolved in 100 ml of distilled water.

Solution B : $Na_3PO_4.12H_2O$ (13.6 g) was dissolved in 200 ml of distilled water.

Solution A and B were mixed with swirling, then centrifuged at 3000 g for 10 min. Precipitate so obtained was washed with 0.05 M borate buffer four times and was suspended in 80 ml of borate buffer (can be stored in refrigerator and used up to 2 weeks).

(e) 2-Chloro-3,5-dinitropyridine solution : 2-chloro-3,5-dinitropyridine (30mg) was dissolved in one ml of methanol (ethanol can be used). Prepared fresh before use.

(f) 1.2 N HCl.

(g) Ethyl acetate.

(h) Mixture of amino acids : Cystine (20 mg), Methionine (20 mg), Histidine (30 mg), Alanine (30 mg), Isoleucine (30 mg), Threonine (30 mg), Tyrosine (30 mg), Glycine (40 mg), Phenylalanine (40 mg), Valine (40 mg), Arginine (50 mg), Serine (50 mg), Aspartic acid (60 mg), Glutamic acid (300 mg), Leucine (80 mg) and Proline (80 mg) were mixed well. 200 mg of amino acid mixture was dissolved in 20 ml of carbonate buffer (0.6 M, pH 9.0).

3.5.13 Methionine

(a) 2 N HCl.

(b) 10 N NaOH (40%) : NaOH (40 g) was dissolved in 100 ml distilled water under cold conditions.

(c) 10% NaOH – NaOH (10 g) was dissolved in 100 ml of distilled water.

(d) 10% sodium nitroprusside : Sodium nitroprusside (10 g) was dissolved in 100 ml of distilled water.

(e) 3% Glycine : Glycine (3g) was dissolved in 100 ml of distilled water.

(f) Orthophosphoric acid.

(g) 20% HCl.

3.5.14 Peroxidase

(a) 0.1 M phosphate buffer (pH 7.2) containing 1 mM polyvinyl polypyrolidone (PVPP) and 10% Glycerol was used for extraction.

(b) 0.03% H_2O_2 was prepared in 0.01 M phosphate buffer (pH 6.0).

(c) 1% Orthodianisidine : Orthodianisidine dye (1g) was dissolved in 100 ml of Methanol.

3.5.15 Poly phenol oxidase

(a) Extraction buffer : 0.1 M phosphate buffer (pH 7.2) containing 1 mM polyvinyl polypyrolidone (PVPP) and 10% Glycerol was prepared.

(b) 0.1 M catechol was prepared in 0.1 M phosphate buffer (pH 6.0).

3.5.16 Esterase

0.1 M phosphate buffer (pH 7.2) containing :

0.25 M Sucrose
1% PVPP
1 mM DTT
0.1% Ascorbic acid
0.1% Cysteine HCl
1.0 mM EDTA
1.0 mM $MgCl_2$

3.5.17 Phenyl Alanine Ammonia Lyase (PAL)

(a) Extraction buffer : Borate-HCl (pH 8.8).

(b) Reagent buffer : Borate-Borax (pH 8.8).

(c) 0.1 M Phenylalanine : Phenylalanine (0.1652g) was dissolved in 10 ml of reagent buffer (Prepared fresh).

3.5.18 Tyrosine ammonia Lyase (TAL)

(a) Extraction buffer : Borate-HCl (pH 8.8).

(b) Reagent buffer : Borate–Borax (pH 8.8).

(c) Tyrosine : 0.1 M Tyrosine was prepared in Borate-Borax buffer and used as substrate.

3.5.19 β-Glucosidase

(a) Citrate buffer (0.1 M, pH 4.8).

(b) Substrate was prepared by dissolving 15 mg of P-nitrophenyl β-D-glucopyranoside in 2 ml of distilled water.

(c) 1M sodium carbonate.

3.5.20 Alkaline Phosphatase

Glycine-NaOH buffer (50 mM, pH 10.4) was prepared for enzyme extraction.

3.5.21 Polyacrylamide Gel Electrophoresis (PAGE)

(a) Stock Acrylamide solution (30%)

29.2 g Acrylamide

0.8 g N,N'-Methylene bis acrylamide

Final volume made upto 100 ml with distilled water.

(b) Stock 1.5 M Tris-HCl :

Tris-buffer (18.16g) was dissolved in 60 ml distilled water. pH was then adjusted to 8.8 with HCl and final volume was made up to 100 ml with distilled water.

(c) Stock 0.5 M Tris-HCl :

Tris buffer (3 g,0.025 M) was dissolved in 35 ml of distilled water. pH was then adjusted to 6.8 and final volume was made up to 50 ml with distilled water.

(d) Electrode buffer (pH 8.3) :

Tris buffer (3 g, 0.025 M) and 14.4 g Glycine (0.192 M) were dissolved in distilled water and finally adjusted to 1000 ml. For SDS-PAGE

10 ml of 10% SDS was added and then finally volume was made up to 1000 ml.

(e) 10% APS (Ammonium per sulfate) : APS (100mg) was dissolved in 1 ml of distilled water. Prepared fresh at the time of gel casting.

(f) TEMED (N,N,N'N'-Tetra methylethelendiamine).

(g) 10% SDS (Sodium Dodecyl sulfate) : SDS (10g) was dissolved in 100 ml of distilled water.

(h) Gel loading dye :

50 mM Tris-HCl (pH 6.8)
100 mM DTT (Dithiothreitol)
2% SDS
0.1% BPB (Bromophenol blue)
10% Glycerol

(i) Staining dye :

Coomassie blue R-250 (0.1g) was dissolved in 100 ml solution of Methanol (40) : Acetic acid (10) : Distilled water (50).

(j) Destaining solution was prepared by mixing Methanol, Acetic acid and distilled water in the ratio of 40:10:50.

(k) Protein Extraction buffer :

Tris HCl (0.2 M, pH 7.2)
7 M Urea
2 M Thiourea
2% Triton X-100
1mM EDTA (pH 8.0)
1% PVPP
1% β-mercaptoethanol

(l) Preparation of 7.5% running gel (For isoenzymes)

10 ml stock solution of acrylamide
7.5 ml 1.5 M Tris-HCl (pH 8.8)
22.5 ml distilled water were mixed and degassed
250 µl APS
25 µl TEMED

(m) Preparation of 10% running gel (For protein)

10 ml stock solution of acrylamide
6.5 ml stock 1.5 M Tris-HCl (pH 8.8)
13.2 ml distilled water were mixed and degassed
250 µl APS   300 µl SDS (10%)
25 µl TEMED

(n) Preparation of 4% stacking gel

3.1 ml stock solution of acrylamide
2.5 ml stock 0.5 M Tris-HCl (pH 6.8)
14.1 ml distilled water were mixed and degassed
300 µl APS
50 µl TEMED

*For SDS PAGE ~100 µl SDS (10%) was added.

3.5.21 DNA Extraction

(a) Homogenizing buffer :

0.4 M NaCl
10 m M Tris-HCl (pH 8.0)
2mM EDTA (pH 8.0) (Ethylene di amine tetra acetic acid)

(b) 20% SDS : Sodium dodecyl sulphate (Sodium lauryl sulphate, SLS) (20g) was dissolved in 100 ml of distilled water.

(c) Proteinase K : Proteinase K (20mg) was dissolved in 1 ml of distilled water.

(d) 6 M NaCl.

(e) 2 X Cetyl di methyl ethyl ammonium bromide (CTAB) (100 ml)

2% CTAB (w/v)	=	2 g CTAB
100 mM Tris (pH 8.0)	=	1.2114 g
20 mM EDTA (pH 8.0)	=	0.5845 g
1.4 M NaCl	=	8.816 g

Dissolved in 50 ml of distilled water and pH adjusted to 8.0 then volume was made up to 100 ml.

(f) 5 X CTAB (100 ml)

5% CTAB (w/v)	=	5 g
0.7 M NaCl	=	4.09 g

Dissolved in 100 ml of distilled water.

(g) High Salt TE : (pH 8.0)

10 mM Tris	=	0.1211 g
1mM EDTA	=	0.02923 g
1M NaCl	=	5.844 g

Dissolved in 50 ml of distilled water, pH was adjusted to 8.0 and then volume was made up to 100 ml.

(h) Phenol (Saturated, pH 8.0)

The commercially available phenol was distilled. The hydroxyquinoline was added as an antioxidant to a final concentration of 0.1%. Then equal volume of Tris-HCl buffer (0.5 M, pH 8.0) was added at room temperature and stirred on magnetic stirrer for 15 minutes and allowed to separate in two phases. The upper aqueous phase was removed. Equal volume of 0.1 M Tris HCl (pH 8.0) was added to lower phenolic phase and was stirred for 15 minutes. The upper aqueous phase was removed and the

process was repeated with 0.1 M Tris-HCl until the pH of the phenolic phase had risen to more than 7.8. This phenol was stored in 0.1 volume of 0.1 M Tris-HCl (pH 8.0) containing 0.2% β-mercaptoethanol and stored at 4°C.

(i) Chloroform

(j) Isoamyl alcohol

(k) Ethanol (Absolute)

3.5.22 Polymerase chain reaction (PCR)

(i) dNTPs : Pharmecia (100 mM each), which was diluted to 10 mM each.

(ii) Taq DNA polymerase : Bangalore Genei (3 U/μl).

(iii) Primers : Primers were custom synthesized at Labware scientific Inc.

List of Primers :

		Primer (Local code)	Sequence
(iii a)	RAPD		
		Plantago 01	5'CAG GCC CTT C 3'
		Plantago 02	5'TGC CGA GCT G 3'
		Plantago 03	5'GTG ATC GCA G 3'
		Plantago 04	5'AGG TGA CCG T 3'
		Plantago 05	5'CAA ACG TCG G 3'
		Plantago 06	5'GTT TCG CTC C 3'
		Plantago 07	5'GGA CTG GAG T 3'
		Plantago 08	5'CCG CAT CTA C 3'
		Plantago 09	5'TGG ACC GGT C 3'
		Plantago 10	5'TGT CTG GGT G 3'
		Primer BG 50	5'GGG ACG TCT C 3'
		Primer BG 51	5'TTA GCG CCC C 3'
(iii b)	ISSR		
		UBC 887	5'AGT ACG AGT TCT CTC TCT CTC TC 3'
		UBC 889	5'ACT CGT AGT ACA CAC ACA CAC AC 3'
		UBC 891	5'ACT ACG ACT TGT GTG TGT GTG TG 3'

All the primers were diluted to 10 pmols/μl with autoclaved HPLC water.

3.5.23 Restriction enzymes

*EcoR*I : 10 U/μl

*Rsa*I : 10 U/μl

Supplied by MBI fermentas, USA.

3.5.24 Electrophoresis

(a) Agarose (Low EEO type) Bangalore Genei

(b) 5X Tris Borate EDTA (TBE), pH 8.3

0.9 M Tris HCl, 0.9 M Boric acid, 20 mM EDTA

pH adjusted to 8.3

(c) Gel loading dye (6X)

0.25% Bromophenol blue

0.25% xylene cyanol

40% Sucrose

Prepared in distilled water and stored at 4°C.

(d) Ethidium Bromide (1 mg/ml).

(e) 100 bp and 1 Kb plus DNA ladder (Gibco BRL)

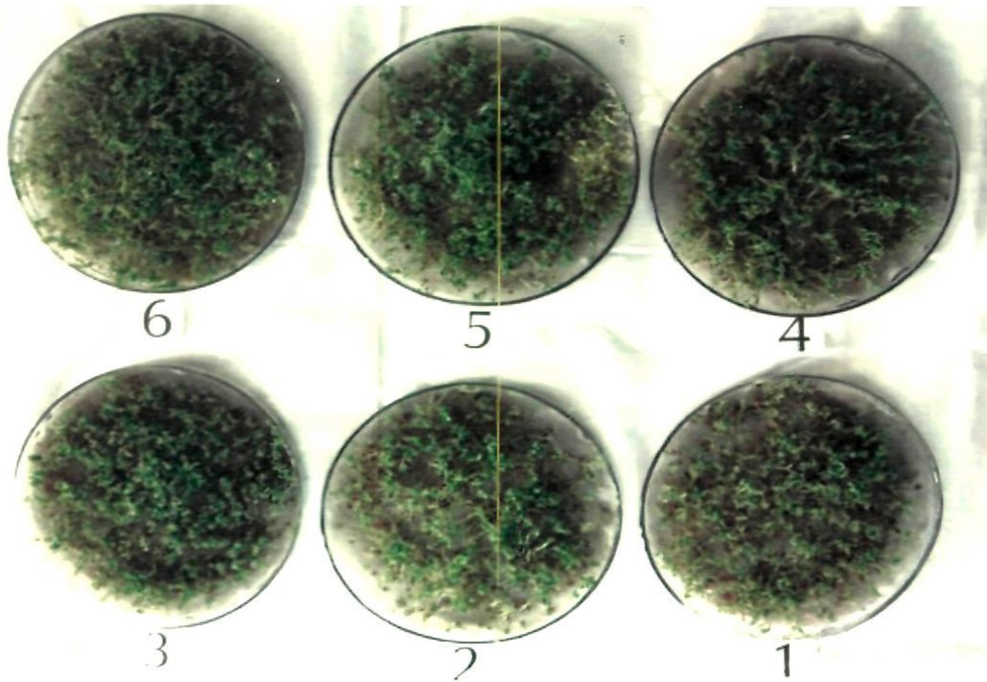
3.5.25 AFLP (Amplified Fragment Length Polymorphism)

The kit for AFLP was supplied by GIBCO BRL India Pvt. Ltd. (AFLP Analysis system II).

3.6 METHODS

3.6.1 Germination of seeds

The seeds of 12 different genotypes of Isabgol were germinated in petriplates at 4°C (Plate 1). After germination the seedlings were grown at room temperature. The seedlings at two-week stage were analysed for:



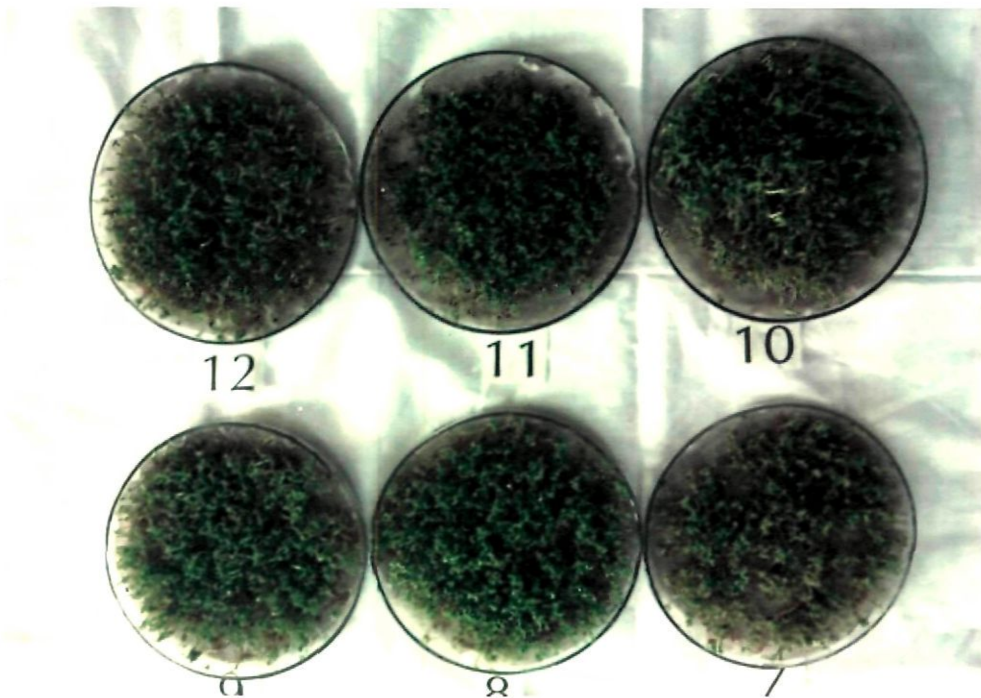


PLATE-1:GERMINATING SEEDLINGS OF ISABGOL

(1=GI2;2=DM1;3=JI10;4=JI56;5=JI53;6=JI42;

7=DM3;8=DRP46;9=DM2;10=JI65;11=DM4;12=EC124345)

1. Total soluble sugars
2. Total free amino acids
3. Total phenols
4. Enzymes : a) Peroxidase
   b) Polyphenol oxidase (PPO)
   c) Esterase
   d) ß-Glucosidase
   e) Alkaline phosphatase

The whole seeds were powdered in mixer and sieved through 60-mesh sieve.

The 60-mesh seed powder was analyzed for:

1. Total lipid
2. Husk %
3. Total carbohydrate
4. Total soluble sugars
5. Reducing sugars
6. Cellulose
7. Ascorbic acid
8. Total free amino acids
9. Total phenols
10. Total protein
11. Proline
12. Lysine
13. Methionine
14. Enzymes : a) Peroxidase
    b) Polyphenol oxidase (PPO)
    c) Phenyl alanine ammonialyase (PAL)
    d) Tyrosine ammonialyase (TAL)
    e) ß-Glucosidase
15. DNA Fingerprinting
    - DNA Extraction
    - RAPD Profile
    - ISSR
    - CAPS
    - AFLP

3.6.2 Extraction of total oil

The total oil from the seeds was extracted by soxhlet extraction method using hexane. 20 g of powdered seeds were extracted in hexane for 8 hrs (Mehta and Lodha, 1979). The solvent was distilled out and the flasks were then transferred to vacuum oven maintained at 55°C for 24 hrs. The flasks were removed from oven and put in the desiccator until it comes to room temperature. The flasks were weighed and per cent oil was calculated as :

$$\% \text{ oil} = \frac{(\text{Weight of flasks} + \text{Oil}) - \text{Weight of flask}}{\text{Weight of sample (20g)}} \text{ x } 100$$

3.6.3 Estimation of husk

The husk % was determined by the chemical method (Thanki and Talati, 1983). 20 ml of 0.1N HCl was taken in 150 ml beaker and heated to boiling. Beaker was removed from the flame and 1 g seeds were transferred to 0.1 N HCl without being deposited on the sides of beaker to the possible extent. The heating was continued until the husk from the seeds dissolved and all seeds turned to pale yellow color from the original reddish brown color and were visibly free from husk. The beaker was taken off from the flame and the seeds were allowed to settle. The hot supernatant acidic solution was decanted off through a previously weighed porcelain gooch crucible. The seeds were washed thoroughly with hot water by decanting water. The crucible was dried at 90°C for 3 hrs and the weight of dried dehusked seeds were calculated as :

$$\% \text{ Husk} = \frac{(\text{Weight of crucible} + \text{Seeds}) - \text{Weight of crucible}}{\text{Weight of sample}} \text{ x } 100$$

3.6.4 Estimation of total carbohydrate

Seed sample (100 mg) was crushed into fine powder and transferred to boiling tube and hydrolyzed by 5 ml of 2.5 N HCl. Tubes were then placed in boiling water bath for three hrs. The tubes were cooled to room temperature. The acidic solution was neutralized with solid sodium carbonate until the effervescence ceases. Volume was made up to 100 ml and centrifuged. Supernatant (0.1 ml) was taken for carbohydrate estimation. 0.1 ml of supernatant was transferred to the test tube and volume was made up to 1 ml. To this 1 ml of 5% phenol and 5 ml of 96% sulphuric acid were added. Contents were mixed well and tubes were placed in water bath at 25-30°C for 30 minutes and the color was read at 490 nm.

For the standard graph standard stock solution of glucose was prepared by dissolving 100 mg / 100 ml in distilled water. 10 ml of this stock was then diluted to 100 ml. From this working, stock standard graph was drawn ranging from 0-100 µg concentration. The amount of carbohydrate present in the samples was calculated as :

Total carbohydrate (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.5 Estimation of total soluble sugars

Total soluble sugars from Isabgol seeds and seedlings were determined by phenol-sulphuric acid method described by Dubois *et al.* (1956).

Two hundred mg of the powdered seed sample and 500 mg of seedlings were weighed and extracted for sugars in 80% ethanol. 5 ml of the extract was evaporated to dryness and dissolved in 25 ml of hot water. One ml of the sample was pipetted in 30 ml test tube. In a similar way 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard glucose solution (0-100 µg) was pipetted into a series of tubes. The volume in each tube was made up to 1 ml with distilled water. A blank was set simultaneously by

taking 1 ml of distilled water in a tube. One ml of 5% phenol solution and 5 ml of 96% sulphuric acid were added to each tube and shaken well. Again after 10 minutes the contents in the tubes were shaken and placed in a ice-bath for 20 minutes. The absorbance was read at 490 nm. The amount of total soluble sugars was calculated by using the standard graph as :

Total soluble sugars (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.6 Estimation of reducing sugars

The reducing sugar content from the seeds of Isabgol was analyzed by the method described by Somogyi (1952).

Two hundred mg of powdered seed samples were crushed and was extracted with 80% ethanol (25 ml) for reducing sugars. The supernatant was collected and 5 ml of this supernatant was evaporated to dryness. Ten ml of distilled water was added to dissolve the sugars. 0.5 ml of solution was pipetted out in a test tube. In a similar way 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard of glucose (0-100 μg) solution was pipetted into a series of test tubes. The volume was made up to 2 ml with distilled water in each test tube. Two ml-distilled water in a separate tube was used as a blank. One ml of copper tartrate reagent was added to each tube. The tubes were placed in boiling water bath for 10 minutes and then cooled. One ml of arsenomolybdic acid reagent was added and the volume was made up to 10 ml with distilled water. The absorbance of blue color was read at 620 nm after 10 minutes. From the standard graph drawn, the amount of reducing sugars present in the sample was calculated as :

Amount of reducing sugars (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.7 Estimation of cellulose

The amount of cellulose in the Isabgol seed samples was estimated by the method described by Sadasivam and Manickam (1992).

Five hundred mg of seed samples were ground into fine powder and transferred to the test tubes. The tubes were placed in water bath at 100°C for 30 minutes. The tubes were cooled and centrifuged at 10,000 g for 15-20 minutes. Residue was washed thrice with distilled water. To the residue, 67% H_2SO_4 was added and kept for 1 hr. One ml of this solution was diluted to 100 ml. Pipetted 1 ml aliquot of the diluted solution to 30 ml test tube and 10 ml of anthrone reagent was added and mixed well. Placed the tubes in boiling water bath for 10 minutes. After cooling the color was read at 630 nm. A blank with distilled water and anthrone reagent was also run simultaneously.

For standard graph 100 ml of cellulose in a test tube was taken and digested with 10 ml of 67% H_2SO_4 for 1 hr. Pipetted 0.4 ml to 2 ml of aliquots in a series of test tubes corresponding to 40-200 μg of cellulose and developed the color.

The amount of cellulose present in the sample was calculated as :

Cellulose (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.9 Estimation of ascorbic acid

Ascorbic acid content was estimated by 2,4-dinitrophenyl hydrazine method by ISI (1971).

Extraction :- One gram powdered Isabgol seeds were extracted with 25 ml of 4% TCA.

Activated animal charcoal treatment :- Approximately 0.5 g of activated animal charcoal was added to 25 ml of the extract, shaken well and allowed to stand for 10 minutes. Filtered through Whatman No. 42 filter paper.

Treatment to charcoal filtrate :

One ml aliquot of the filterate was taken into test tubes and the volume was made up to 4 ml with 4% TCA. 0.05 ml of Thiourea and 1 ml of 2% DNPH were added serially to each test tube and incubated at 37°C for 3 hrs. Similarly, a tube containing 4 ml of extracting medium was also incubated and used as the reagent blank. At the end of incubation period, the tubes were placed in crushed ice and 5 ml of 85% H_2SO_4 was added drop by drop from a burette. The tubes were allowed to stand for 30 minutes at room temperature, before reading the color at 540 nm.

A standard solution of ascorbic acid was prepared by dissolving 50 mg of ascorbic acid in 50 ml of 4% TCA. One ml of this stock standard solution was diluted to 100 ml. From this working standard, a standard graph was prepared ranging from 5 to 50 μg concentration.

The amount of ascorbic acid present in the sample was calculated as :

Ascorbic acid (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.9 Estimation of total free amino acids

The amino acids in the free pool of plant cells or in protein hydrolysate can be determined using ninhydrin (Triketohydrindene hydrate).

A reference graph was prepared by using Glycine (0 to 30 μg) as a standard amino acid and the amount of amino acids was calculated in the Isabgol seed samples.

Powdered seeds (200 mg) and seedlings (500 mg) were ground in a homogenizer with 25 ml (10+5+5+5) of 80% ethyl alcohol. The contents were

centrifuge and pooled all the supernatants and volume was made up to 25 ml with 80% ethanol.

0.1 ml of supernatant was taken and 5 ml of ninhydrin reagent was added followed by vigorous shaking. The tubes were placed in boiling water bath for 20 minutes and brought to room temperature under running tape water. Absorbance was read at 570 nm against blank prepared by adding 0.1 ml of 80% ethanol in place of extract. The amount of free amino acids present in the samples was calculated as :

Amount of free amino acids = Sample O.D. x Standard O.D. x Dilution factor
(g/100 g)

3.6.10 Estimation of total phenols

For phenol estimation, standard stock solution of pyrocatechol was prepared in distilled water containing 1 mg/ml of pyrocatechol. This stock solution was diluted to 10 μg/ml concentration, from this diluted working solution standard graph was drawn ranging between 2 to 10 μg concentration.

Powdered seeds and seedlings (500 mg) of Isabgol were crushed and ground in 0.3 N HCl and kept for shaking for about 1 hr. After that crude extract was centrifuged at 8000 rpm for 10 minutes. Supernatant so obtained was evaporated to dryness. To the residue hot water was added and final volume was adjusted to 25 ml with distilled water in volumetric flask. One ml of the above aliquot was taken in the test tube. To this 1 ml each of FCR (1:2) and 35% Na_2CO_3 was added. After 1 hr 2 ml distilled water was added to adjust the final volume to 5 ml and the absorbance was read at 620 nm. Blank was prepared by taking 1 ml of distilled water instead of the sample. The amount of phenol present in the sample was calculated as :

Amount of phenol (g/100 g) = Sample O.D. x Standard O.D. x Dilution factor

3.6.11 Estimation of total protein

The protein content in seeds was analyzed by Microkjeldahl's method described by AOAC (1985).

Weighed seed sample (50 mg) were transferred to the digestion flask. One-gram catalyst mixture and 2 ml of concentrated H_2SO_4 were added to it. The contents were digested until the solution became colorless. After cooling the contents were dissolved in minimum amount of distilled water. 10 ml of boric acid solution was pipetted out into a 100 ml erlenmeyer flask, 2-3 drops of indicator solution was added and placed under condenser. Digested mixture then transferred to distillation apparatus and flask was rinsed with 2-3 ml of distilled water. Ten ml of sodium hydroxide-sodium thiosulphate solution was added to distillation apparatus and about 20 ml of distillate was collected (20 minutes).

The receiving flask was removed and titrated with 0.02 N H_2SO_4 till the first appearance of violet color. Blank was also analyzed in a similar manner without sample. The protein content was calculated from the samples as :

$$\% \text{ Nitrogen} = \frac{(\text{ml } H_2SO_4 \text{ in determination} - \text{ml blank}) \times \text{Normality} \times 1000 \times 14.007)}{\text{mg sample}}$$

% Protein = % Nitrogen x 6.25

3.6.12 Estimation of proline

The proline content in the seed samples was analyzed by the method suggested by Malik and Singh (1980).

Powdered seed samples (500 mg) were homogenized in a mortar with 5 ml of 1% sulfosalicylic acid. The contents were centrifuged at 2000 g for 10-15 minutes and the volume of clear supernatant was adjusted to 5 ml with distilled water. Glacial acetic acid (5 ml) and 1 ml of acid ninhydrin was added to it and mixed well. The

tubes were placed in boiling water bath for one hour and then cooled. The mixture was then extracted with the addition of 10 ml of toluene and allowed to stand for 2-3 minutes. The absorbance of toluene layer was recorded at 520 nm. Blank was also run simultaneously with 5 ml of 1% sulfosalicylic acid without sample.

Standard stock solution of proline was prepared by dissolving 5 mg of standard proline in 50 ml of distilled water. 0.5 ml of stock solution was diluted to 25 ml of working standard solution with distilled water and pipetted out 0, 1, 2, 3 and 4 ml of working standard in 30 ml test tubes. Volume was adjusted to 5 ml. Glacial acetic acid (5 ml) and 1 ml of acid ninhydrin was added to the tubes. The tubes were placed in boiling water bath for one hour and then cooled. Ten ml of toluene was added to extract the color into the toluene layer. The tubes were allowed to stand for 2-3 minutes and read the absorbance of toluene layer at 520 nm.

The amount of proline in the sample was calculated as :

% Proline = Sample O.D. x Standard O.D. x Dilution factor

3.6.13 Estimation of lysine

The lysine content in the seed samples was analyzed by the method suggested by Tsai *et al.* (1972).

The fine powder of defatted sample of Isabgol seeds (100 mg) was transferred to a glass vial and 5 ml of papain solution was added. All the vials were sealed and shaken well so as to make the sample totally wet. A blank with papain solution without sample was also run simultaneously. All the vials were incubated overnight at 65°C. Samples were shaken well, one hour after putting and one hour before taking out from the incubator.

Hydrolyzed samples were removed, mixed well and allowed to cool to room temperature. One ml of clear supernatant was transferred to centrifuge tube containing 0.5 ml of carbonate buffer. Copper phosphate suspension (0.5 ml) was added to each

tube. The mixture was mixed well and centrifuged to precipitate the excess copper phosphate. One ml aliquot of the supernatant was pipetted out into a test tube and 2-chloro-3,5-dinitropyridine (0.1 ml) solution was added and mixed well. The reaction was allowed to proceed for 2 hours at room temperature with shaking at every 30 minutes.

At the end of the reaction, mixture was acidified by addition of 5 ml of 1.2 N HCl and vortexed well. Five ml of ethyl acetate was added to each tube and mixed well by inversion of tubes. Then the upper phase was extracted out by using the syringe adapted with a polyethylene tube. This step was repeated thrice. The intensity of aqueous layer was read at 390 nm against a sample free blank.

The standard graph was prepared by dissolving 62.5 mg of standard lysine in 100 ml of distilled water (1 mg/ml). Pipetted 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard stock solution was pipetted into a series of test tubes and volume was made up to 1 ml with distilled water. Four ml of papain solution was added and mixed well. 1 ml of this and 0.5 ml of amino acid mixture was added and then 0.5 ml of copper phosphate suspension. The components were mixed well and centrifuged at 8000 g for 10 minutes. One ml of this clear supernatant was drawn into a fresh tube. 2,chloro-3,5-dinitropyridine (0.1 ml) was added and mixed well. The tubes were incubated at room temperature for two hours with shaking at every 30 minutes. After two hours 5 ml of 1.2 N HCl was added followed by the addition of 5 ml of ethyl acetate, mixed well and kept for 10 minutes. The upper layer was removed and the step was repeated thrice. Absorbance of aqueous layer was read at 390 nm.

The amount of lysine present in the samples was calculated from the standard graph as :

$$\% \text{ lysine} = \frac{\text{Sample O.D.} \times 780}{\% \text{ protein present in the sample}}$$

3.6.14 Estimation of methionine

The methionine content in the seed samples was analyzed by the method suggested by Sadasivum and Manickam (1992).

The defatted seed samples (500 mg) were weighed into a 50 ml conical flask and 6 ml of 2 N HCl was added. The mixture was autoclaved at 15 lb pressure for one hour. Small amount of activated charcoal was added to the autoclaved sample (hydrolysate) and was boiled on hot plate. The contents were filtered when hot and washed with hot water.

The filtrate was neutralized with 10 N NaOH and pH was adjusted to 6.5. Volume was then made up to 50 ml with distilled water after cooling. From this 25 ml solution was transferred into a 100 ml conical flask. Three ml of 10% NaOH was added followed by 0.15 ml of sodium nitroprusside. After 10 minutes, 1 ml of glycine solution was added. 10 minutes later, 2 ml of orthophosphoric acid was added with vigorous shaking. The intensity of the color was read at 520 nm after 10 minutes against a blank prepared in the same way without sodium nitroprusside.

Standard stock solution of methionine was prepared by dissolving 100 ml of DL-methionine in 4 ml of 20% HCl and diluted to 100 ml with distilled water. 0, 1, 2, 3, 4 and 5 ml of standard solution of methionine was pipetted and the volume was made up to 25 ml with distilled water and color was developed. The methionine content present in the samples was calculated as :

$$\% \text{ Methionine} = \frac{\text{Sample O.D.} \times 437.52}{\% \text{ protein present in the sample}}$$

3.6.15 Extraction of enzymes

3.6.15.1 Peroxidase and Poly phenol Oxidase

Isabgol seeds (1 g) and seedlings (1 g) were ground with glass powder in cold 0.1 M phosphate buffer, pH 7.2 containing 1 m M PVPP and 10% glycerol. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant containing 50 µg of protein was used for enzyme assay and electrophoresis.

Assay :

Peroxidase: - The reaction mixture contained 2.99 ml of 0.03% H_2O_2 (substrate) in 0.01 M phosphate buffer (pH 6.0), 25 µl of 1% orthodianisidine in methanol and 100 µl of enzyme extract. The reaction was initiated by the addition of enzyme. The change in the color of oxidized dye was read at 460 nm up to 1 minute, at the interval of 15 seconds. Blank was run without the addition of substrate. The enzyme activity was expressed as change in OD/minute/g fresh tissue.

$$\text{Enzyme activity} = \frac{\text{Change in O.D./min}}{\text{Weight of sample present in 0.1 ml of extract}}$$

Poly phenol oxidase (PPO): - The reaction mixture contained 3.0 ml of 0.01 M catechol in 0.1 M phosphate buffer (pH 6.0) (substrate) and 100 µl of enzyme extract. The reaction was initiated in the blank with enzyme without the substrate. The color change in oxidized catechol (light green color) was read at 490 nm up to 3 minutes, at the interval of 15 seconds. The enzyme activity was expressed as change in OD/min/g fresh tissue.

$$\text{Enzyme activity} = \frac{\text{Change in O.D./min}}{\text{Weight of sample present in 0.1 ml extract}}$$

3.6.15.2 Enzyme extraction for esterase

Isabgol seedlings (1 g) were ground in cold extraction buffer and homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C. The clear supernatant was used for electrophoresis.

3.6.15.3 Enzyme extraction for alkaline phosphatase

One gram of Isabgol seedlings were crushed in cold extraction buffer and homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The clear supernatant was used for electrophoresis.

3.6.15.4 Electrophoresis

Electrophoresis was conducted on vertical slab gel PAGE (7.5%). Electrophoresis was carried out at 25 mA for 3-4 hours at 4°. The samples were loaded on the gel, mixed with tracking dye in the ratio of 2:1. After electrophoretic run, the gels were washed and stained for peroxidase, PPO, esterase and alkaline phosphatease.

Gel was stained for peroxidase activity in 100 ml solution containing 1 g benzidine, 9 ml acetic acid and 3% Hydrogen peroxide (Sadasivam and Manickam, 1992). For poly phenol oxidase, gel was incubated in 0.01M DL-dihydroxy phenylalanine (DOPA) in dark at 30°C for 1 hour (Mahadevan and Sridhar, 1996). The gel was stained for esterase activity at room temperature using sodium dihydrogen phosphate (2.8 g), disodium hydrogen phosphate (1.1 g), Fast blue RR salt (0.2 g), 2-napthyl acetate (0.03 g) and water to 200 ml. The gel was incubated at 37°C in dark. The reaction was stopped by the addition of methanol (10) : water (10) : acetic acid (2) : ethyl alcohol (1) (Sadasivam and Manickam, 1992). For Alkaline phosphatase isozymes, the gel was incubated in 100 ml solution containing 50mg Fast

blue, RR Salt 50mg Sodium-naphthyl phosphate, 123mg Magnesium sulphate, 374mg Boric acid and 5ml of 1N NaOH. The gel was incubated in dark for 30min at 37^{0}C (Harris and Hopkins, 1977).

3.6.15.5 Enzyme extraction for PAL and TAL

The seedlings were homogenized in borate-HCl buffer containing 0.04% ß-mercaptoethanol. The homogenates were centrifuged at 12,000 rpm for 20 minutes. The clear yellow green supernatant was used as the enzyme source for the assay of PAL and TAL.

Enzyme assay: -

The reaction mixture containing 3.0 ml of 0.1 M sodium borate buffer (pH 8.8), 0.5 ml of substrate (0.1 M phenylalanine in 0.1 M sodium borate (pH 8.8) for PAL and 0.1 M Tyrosine in 0.1 M sodium borate (pH 8.8) for TAL and 100 µl of enzyme extract. The tubes were incubated at 37°C for 2 hours. The blank was also set without enzyme and without substrate. After two hours, the absorbance was read at 290 nm for PAL and at 335 nm for TAL and the enzyme activity was expressed as change in O.D./h/g of tissue (Mahadevan and Sridhar, 1980).

3.6.15.6 Extraction for ß-Glucosidase enzyme

The seeds of Isabgol and seedlings were crushed into chilled mortar and pestle with (5 ml/500 mg) citrate phosphate buffer (0.1 M, pH 4.8). Crude homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C. Clear supernatant so obtained was used for enzyme assay (Malik and Singh, 1980).

The reaction mixture was prepared by mixing 0.1 ml of substrate and 0.8 ml of 0.1 M citrate buffer in test tubes. The tubes were incubated in water bath at 30°C. The enzyme (0.1 ml) was added to initiate the reaction and incubated for 30 minutes at

37°C. The reaction was terminated with 2 ml of 1 M Na_2CO_3, and the intensity of the color developed was recorded at 410 nm. Blank was prepared by adding 2 ml of sodium carbonate to substrate followed by the addition of enzyme extract. The activity of the enzyme was expressed in terms of change in O.D./h/g tissue.

3.6.16 Extraction for total protein

The seedlings (1 g) were homogenized in 1 ml of protein extraction buffer. The homogenates were centrifuged at 15,000 rpm for 15 minutes at 4°C. The clear supernatant containing 50μg protein mixed with gel loading dye (with SDS) was loaded on to the gel.

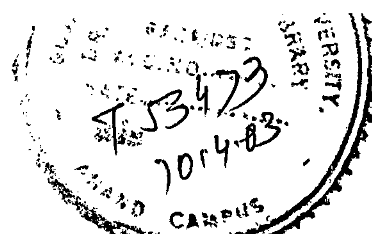
Electrophoresis :

Electrophoresis was conducted on vertical SDS-PAGE (10.5%). Electrophoresis was carried out at 25 mA for 3-4 hours. The gel was washed to remove excess of SDS and stained for proteins with Coomassie blue stain. After staining and destain the gel was fixed in 7% acetic acid.

3.6.17 DNA extraction

The Isabgol seeds were used for DNA extraction. The DNA was extracted by two methods viz., Universal and rapid salt-extraction method (Salah and Martinez, 1997) and CTAB method (Doyle and Doyle, 1990) with some modifications.

In Universal DNA extraction method, the seeds (500 mg) were homogenized in 2 ml of sterile salt homogenizing buffer in mortar and pestle on ice. Then 20% SDS was added to the final concentration of 2% and proteinase k (20 mg/ml) to the final concentration of 400 μg/ml and mixed well. The samples were incubated overnight at 65°C and then 1 ml of 6 M NaCl was added. Samples were vortexed for 30 seconds at maximum speed. The tubes were spun down for 30 minutes at 10,000 g. The



supernatant was transferred to fresh tubes. An equal volume of isopropanol was added and mixed well by the inversion of the tubes and then incubated at -20°C for 1 hour. Samples were then centrifuged at 4°C for 20 minutes (10,000 g). The pellet was washed with 70% ethanol, dried and dissolved in 200 µl of sterile distilled water.

In CTAB method, 500 mg of seeds were powdered in pre-chilled mortar and pestle and transferred to the centrifuge tubes. 5 ml of 2x CTAB buffer was added which was pre-heated to 65°C. The tubes were rotated slowly for homogenization. Five ml of phenol: chloroform: Isoamyl alcohol (25:24:1) was added and incubated at 65°C for 30 min. Centrifuged at 10,000 rpm for 10 minutes and aqueous phase was taken out. One ml of 5X CTAB was added to it and rotated slowly. Five ml chloroform: Isoamyl alcohol (24:1) was added and rotated upside down and incubated for 20 minutes on a slow moving shaker, centrifuged at 11,000 rpm for 10 minutes. The aqueous phase was transferred to new tube and 2 volumes of pre-chilled absolute alcohol was added. It was Spun at 11,000 rpm for 10 minutes and pelleted DNA was air dried and dissolved in 2 ml TE buffer (at 65°C for 30 min). 500 µl of dissolved DNA in TE was transferred to 1.5 ml tubes and 1 ml of pre-chilled absolute alcohol was added and incubated at -20°C overnight for effective precipitation. The DNA pellet was washed with 70% ethanol and air-dried. DNA was dissolved in 200 µl of sterile distilled water.

3.6.18 Quality check and quantitation of DNA

The quality of DNA was checked by agarose gel electrophoresis and quantitation was carried out by UV-spectrophotometer. For the quantitation the stock of genomic DNA was diluted to 1:50 in distilled water and OD was measured at 260 nm. The concentration of DNA was calculated as :

$$DNA\ (\mu g/ml) = O.D._{260} \times 50 \times 50$$

The quality of DNA was checked on 0.8% agarose containing 10 µl of Ethedium bromide (1 mg/ml) per 100 ml of gel. Genomic DNA (5 µl) from stock mixed with 2 µl of BPB tracking dye and loaded on to the gel. The gel was run at 80 V for one hour. The bands were visualized under UV and scanned by gel documentation system.

The DNA samples with the ratio of 1.7-1.8 at $OD_{260/280}$ were retained for DNA fingerprinting. The stock solution was diluted to the concentration of 30 ng/µl and used for further analysis.

3.6.19 Randomly Amplified Polymorphic DNA (RAPD)

The genomic DNA was amplified using random Primers, Plantago 1 to 10, BG-50 and BG-51. PCR was carried out in a reaction volume of 25 µl (Lanham *et al.*, 2000).

3.6.20 Inter-Simple Sequence Repeat (ISSR)

The genomic DNA fingerprinting by Inter Simple Sequence Repeat (ISSR) was subjected to Anchored polymerase chain reaction amplification with three ISSR primers: UBC 887, UBC 889 and UBC 891 (Zietkiewicz *et al.*, 1994; Wolf and Morgan, 1998).

3.6.21 Cleaved Amplified Polymorphic Sequences (CAPS)

The polymorphic patterns were generated by restriction enzyme digestion of genomic DNA followed by PCR amplification with RAPD Primers.

Restriction digestion :-

Genomic DNA was digested for 24 h with *EcoR*I and *Rsa*I restriction enzymes. The samples were kept for digestion in 200 µl thin walled PCR tubes and incubated at 37°C. The digestion mixture (25µl) consisted of:

Restriction buffer (10 x) = 2.5 µl

Enzyme (10 U/µl) = 0.2 µl

DNA (30 ng/µl) = 15 µl (450 ng)

Autoclaved HPLC water = 7.3 µl

The digested genomic DNA was subjected to the amplification using Plantago 1 to 3, BG-50 and BG-51 random (decamer) primers.

PCR reactions for RAPD, ISSR and CAPS were carried out in a reaction volume of 25 µl. The PCR mix consisted of :

Components	RAPD	ISSR	CAPS
PCR buffer (10x) with 15mM $MgCl_2$	2.5 µl	2.5 µl	2.5 µl
Primer (10 pmole/µl)	1.0 µl	1.0 µl	1.0 µl
dNTPs mix (10 mM each)	0.5 µl	0.5 µl	0.5 µl
Taq DNA polymerase (3 U/µl)	0.3 µl	0.3 µl	0.3 µl
Template DNA (30 ng/µl)	4.0 µl	2.0 µl	5.0 µl (Digested)

All the PCR reactions were carried out in 200 µl thin walled PCR tubes. PCR tubes containing reaction mixture were tapped gently and spun briefly at 10,000 rpm. The PCR amplification was carried out in eppendorf master cycler and subjected to following PCR protocol.

Steps	RAPD		ISSR		CAPS	
	Temperature (°C)	Duration (min)	Temperature (°C)	Duration (min)	Temperature (°C)	Duration (min)
Initial denaturation	94	2	94	7	94	2
Denaturation	94	30 sec.	94	30 sec.	94	30 sec.
Annealing	37	1	52	45 sec.	37	1
Extension	72	1	72	2	72	1
	40 times to step 2		30 times to step 2		40 times to step 2	
Final extension	72	10	72	7	72	10
Hold	10	-	10	-	10	-

3.6.22 Electrophoresis

All the PCR products were run on 1.5% agarose containing 10 µl of ethidium bromide (1 mg/ml). Ten µl of PCR product was mixed with 2 µl of 6X tracking dye and loaded on to the well. The gel was run at 80 V (constant) to separate the amplified bands. The standard DNA marker was also run along with the samples. The separated bands were seen under UV and photographed by Gel documentation system and analyzed by Gene tool.

3.6.23 Amplified Fragment Length Polymorphism (AFLP)

AFLP was carried out using standard AFLP kit (Gibco BRL Life technologies Inc.).

(i) Digestion :- To produce DNA fingerprints by AFLP, the genomic DNA was digested in a reaction volume of 12.5 µl.

DNA (30 ng/µl)	=	4.2 µl (≅ 125 ng)
Enzyme Mix (*Eco*RI + *Mse*I)	=	1.0 µl
Buffer (10 x)	=	1.2 µl
Autoclaved HPLC water	=	6.05 µl

The tubes containing digestion mixture were incubated at 37°C for 3 hours. At the end of this incubation the tubes were transferred to water bath, set at 70°C for 10 minutes.

(ii) Ligation :-

After restriction digestion the restricted product was ligated with the enzyme adaptors at both the ends in reaction volume of 25 μl. The reaction mixture contained

Digested product	=	12.5 μl
Adapter (*Eco*RI + *Mse*I)	=	9.5 μl
Buffer (10 X)	=	2.5 μl
Ligase enzyme	=	0.5 μl

The reaction mixture was incubated in a water bath set at 20°C for 3 hrs. The resultant product of ligation was diluted 10 times (1:10 – Dilution I).

(iii) Pre-amplification/Ist PCR :

The mixture for Ist PCR consisted of

Product of Ist dilution	=	2.5 μl
Pre-amplification mix (Primers and dNTPs)	=	20.0 μl
PCR buffer (10 X)	=	2.5 μl
Taq polymerase	=	0.5 μl
		25.5 μl

The reaction mixture was subjected to the Perkin-Elmer PCR thermal cycler with the following PCR protocol.

	Step	Temperature (°C)	Duration (Sec.)
1.	Denaturation	94	30
2.	Annealing	56	60
3.	Extension	72	60

20 times to step 1.

At the end of PCR the product was diluted 50 times (1:50 – Dilution 2nd) in TE buffer.

(iv) Second PCR :-

Before second PCR mix I and mix II were prepared and *Eco*RI primers were radiolabelled.

Kinetion :- Radiolabelling of forward (*EcoR*I Primers) primers :

*EcoR*I Primer	=	1.8 µl
Buffer (10 x)	=	0.5 µl
dATP	=	1.2 µl
T_4 PNR (Kinase enzyme)	=	0.5 µl
Autoclaved HPLC water	=	1.0 µl
		5.0 µl

The reaction mixture was incubated at 37°C for 1 hour.

Mix I was prepared by mixing 1 µl of radiolabelled *EcoR*I primer (forward primer) and 9 µl of *Mse*I primer (Reverse primer).

Three primer set combinations were used :

1.	G_1	E-AGG + M-CAC
2.	G_2	E-ACA + M-CAT
3.	G_3	E-ACC + M-CTG

Mix II was prepared in a reaction volume of 100 µl by mixing.

Autoclaved HPLC water	=	78 µl
Taq	=	2 µl
Buffer (10 x)	=	20 µl
		100 µl

The reaction mixture for IInd PCR was prepared in a reaction volume of 20 µl. The reaction mixture consisted of :

Product of IInd dilution	=	5 µl
Mix I	=	5 µl
Mix II	=	10 µl

The tubes were transferred to the Perkin-Elmer Thermal cycler for PCR and subjected to the following PCR protocol.

Step	Temperature (°C)	Duration (Sec.)	Cycles
1	94	30	
2	68	30	5
3	72	60	
4	94	30	
5	64	30	5
6	72	60	
7	94	30	
8	60	30	5
9	72	60	
10	94	30	
11	56	30	5
12	72	60	

The samples (end product of IInd PCR) were run on 6% PAGE (Acrylamide : Bis – 19:1) containing 7 M urea. The gel was prepared in 0.5 x TBE. The gel was prepared as :

Acrylamide = 5.7 g
Bis-acrylamide = 0.3 g
TBE (10 x) = 5 ml
Urea = 42 g

Dissolved in 50 ml of distilled water and volume was made upto 100 ml.

APS (10%) = 910 µl
TEMED = 91 µl

Running buffer = 0.5 x TBE

Before gel casting, plates were washed thoroughly and dried. The samples (20 µl) were mixed with 4 µl of tracking dye and 10 µl of mixture per well was loaded on to the gel. The gel was pre-run at 60 W for 1 hr. The gel was run after sample loading at 60 W for 3 hrs or till the tracking dye reached the end. At the end of the

run, the gel was taken out and fixed in fixative containing 15% methanol and 5% acetic acid for 15 minutes.

The gel was kept for band transferring on to the X-ray film in the cassettes for two days and autoradiogram was developed in developing solution and stopped using fixing solution.

STATISTICAL ANALYSIS

1. **Statistical analysis of Biochemical results**: Observations for all the biochemical parameters were taken in four replications, which were analyzed by Completely Randomized Design (CRD). Pearson Correlation Coefficient and Squared Euclidean Distances were calculated and dendogram was constructed using Hirarchial Linkage method released by SPSS (Norusis, 1990)

2. **Statistical analysis of PCR results: -**

Band Sharing (Bs) :

The presence or absence of band within RAPD, ISSR, CAPS and AFLP patterns were scored as one or zero, respectively were used to estimate BS between the genotypes. The similarity between the genotypes based upon BS for each genotype population (Bab) was estimated using the formula (Lynch, 1990).

$$Bs = 2\,Nab / (Na + Nb)$$

Where Nab is number of common fragments observed in individual a and b, and Na and Nb are the total number of fragments scored in individual a and b, respectively.

Genetic Distance :

Between population BS estimates were used to determine genetic distance (Dxy) according to Lynch (1991) as :

$$Dxy = -\ln [Bxy / \sqrt{Bx\,By}\,]$$

Where Bxy is number of common fragments observed in individual x and y, and Bx and By are the total number of fragments scored in individual a and b, respectively.

Percentage Difference (PD) :

The PD was calculated by making a series of pair-wise comparisons using following formula :

$$PD = [1\text{-}Bs] \times 100$$

Average percentage difference (APD) :

$$APD = 1/C\ (\Sigma PDi)$$

Where C is the number of pair-wise comparisons between genotypes (Thangrajan, 2000).

Mean Average Percentage Difference (MAPD):

$$MAPD = 1R/(\Sigma\ APD)$$

Where, R is the no of Primer used.

Polymorphic Information Content:

$$PICi = 2\ fi\ (1\text{-}\ fi)$$

Where, PICi is polymorphic information content for each marker 'i', fi is the frequency of the polymorphic amplified allele and (1-fi) is the frequency of the null allele (Ronald-Ruiz *et al.*, 2000).

Average Heterozygosity: (Powell *et al.*, 1996)

$Hn = 1 - \Sigma Pi^2$	Where, Hn is the expected heterozygosity
	Pi is the frequency of polymorphic allele
$Hav = \Sigma Hn/N$	Hav is the average heterozygosity
	N is the no. of total polymorphic alleles

Phylogenetic analysis :

Dendrograms from presence or absence of bands from RAPD, ISSR, CAPS and AFLP primers were constructed by the Neighbor joining, Phylip 3.6a version (Felsenstein, 1993), based on Nei's formula (Nei and Li, 1979). Squared Euclidean Distances were calculated and dendogram was constructed using Hirarchial Linkage method released by SPSS.

Phylogenetic tree pooled over all genetic markers :

The data (presence or absence of allele) from all 12 RAPD, 3 ISSR, 10 CAPS and 3 AFLP markers were pooled together for phylogenetic analysis (Felsenstein, 1993). The data were subjected to bootstrapping to create 100 multiple data set. The genetic distances obtained were subjected to neighbor joining analysis. A consensus tree was constructed.

Network analysis :

The data obtained from presence or absence of bands from RAPD, ISSR, CAPS and AFLP primers were analyzed by the Median Joining Network 200L (Cheng *et al.*, 1995) version 3.1.0.1.

Results and Discussion

CHAPTER – IV
RESULTS AND DISCUSSION

India holds a near monopoly in the production and export of Isabgol seed husk in the world market. It is mainly cultivated in Gujarat and Rajasthan. The dehusked seed, which constitutes about 70% by weight of annual seed crop, does not find an effective use other than animal feed. Lack of scientific information about seed composition precludes its proper exploitation. Recent interest in the Western countries for Isabgol as cholesterol reducing agent has led to renewed interest in its detailed composition.

BIOCHEMICAL CHARACTERIZATION

The variability in quality parameters and proximate principles in 12 Isabgol genotypes is presented in Table 4.1. Food in seeds is generally stored either in the form of oil, carbohydrates, proteins or more than one form. The seed of *Plantago ovata* contains protein, a fixed oil, mucilage, cellulose and traces of starch (Anon., 1968).

4.1 TOTAL OIL:

The total oil percent in the whole seeds (husk + gola) of Isabgol varied from 3.25% to 5.06% in different genotypes (Figure 4.1.1). The highest percentage of oil was found in DM3 genotype (5.06%), while the lowest percent of oil was found in JI10 (3.25%). Chakraborty and Patel (1992) estimated oil (8.6%) from dehusked seeds collected from the market and Naresh *et al.* (1992) estimated oil (8.05 to 10.45%) from dehusked seeds from 43 different genotypes of Isabgol. Pendse *et al.* (1976) also reported 5% pale yellow oil from Isabgol seeds from Gujarat region.

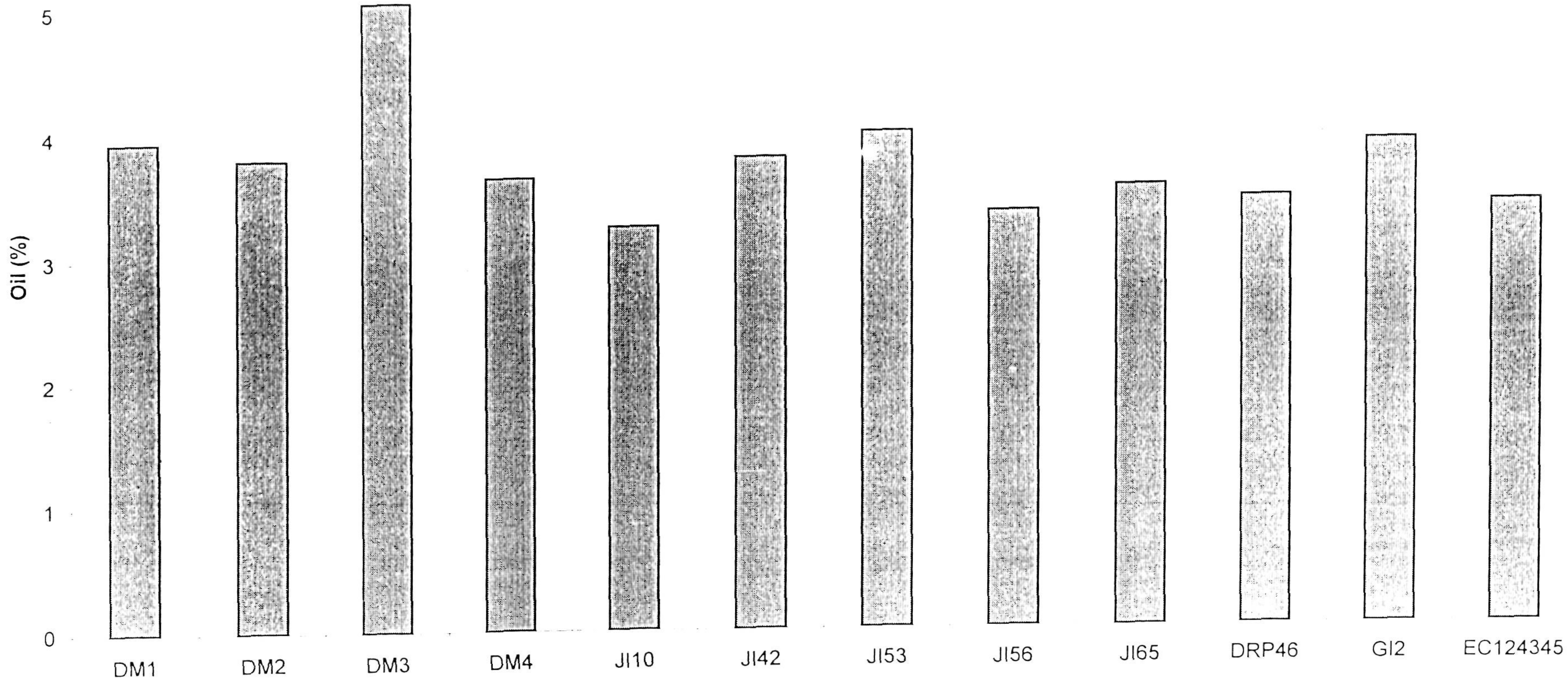


Figure 4.1.1 : Oil percentage in different genotypes of Isabgol seeds

4.2 CABOHYDRATES:

4.2.1 HUSK

Husk, the main constituent of Isabgol, is mainly composed of carbohydrates and primarily used as a laxative. The husk content (Figure 4.2.1.1) varied from 31% to 36% in the examined genotypes (Fig. 4.2.1.1). The data (Table 4.1) revealed that the husk content of genotypes EC124345 and JI65 was significantly higher (36%) as compared to rest of the genotypes. The lowest husk content was found in DM3 genotype (31%). Naresh *et al.* (1992) also reported husk content from 43 genotypes of Isabgol, which ranged from 28.45 to 35.65%.

4.2.2 TOTAL CARBOHYDRATES

Carbohydrates are the main stored products in the seeds, which may be in the form of starch, hemicelluloses or glycans. In Isabgol seeds, total carbohydrates varied from 51% to 63% (Figure 4.2.2.1). A perusal of the data, on total carbohydrates indicated that the genotypes EC124345 and JI65 contained the highest total carbohydrate content (63%). While, the lowest carbohydrate content was found in DM3 and DM1 (51%) genotype.

4.2.3 TOTAL SOLUBLE SUGARS (TSS)

Soluble sugars are the products of photosynthesis. Plants always maintain equilibrium of soluble sugars in the source and whenever the concentration exceeds, it is either converted to the polysaccharides or inter-converted to other primary products or translocated to other organs for the synthesis of secondary compounds. Once it is in the form of polysaccharides, further transformations are slow or limited. The seeds of genotype DM4 contained the highest TSS content (2.9%), which was found to be at par

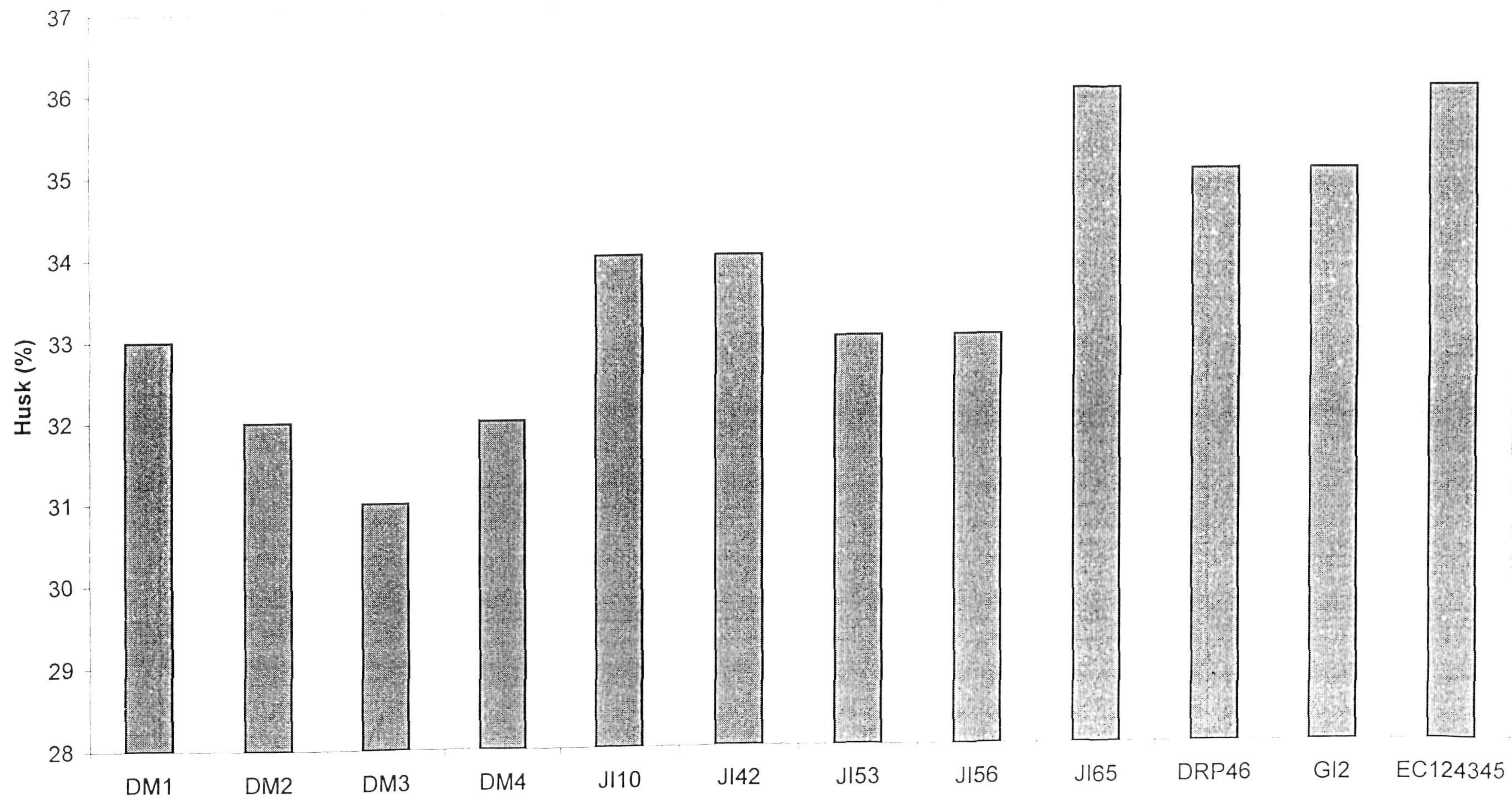


Figure 4.2.1.1 : Husk percentage in different genotypes of Isabgol seeds

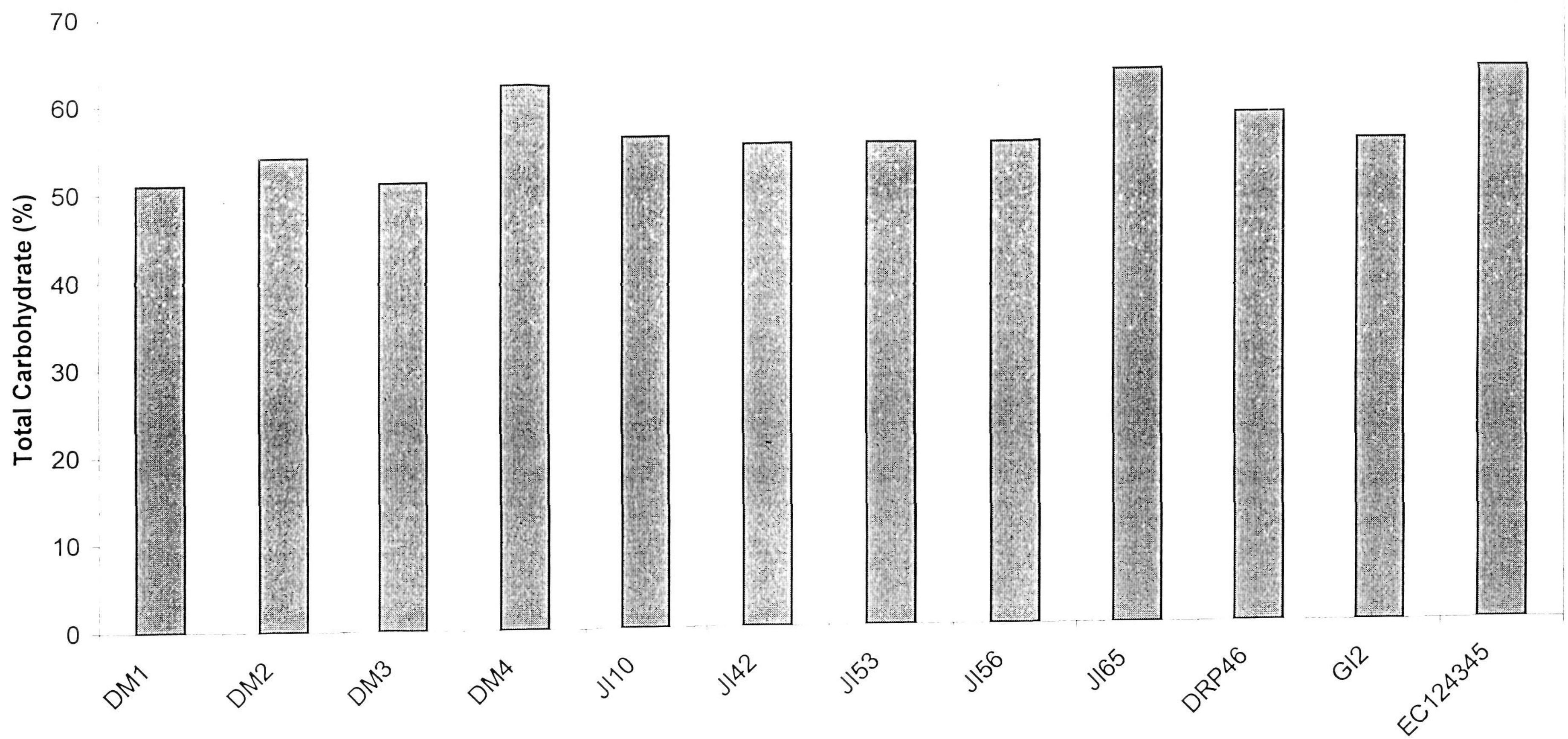


Figure 4.2.2.1 : Total carbohydrate percentage in different genotypes of Isabgol seeds

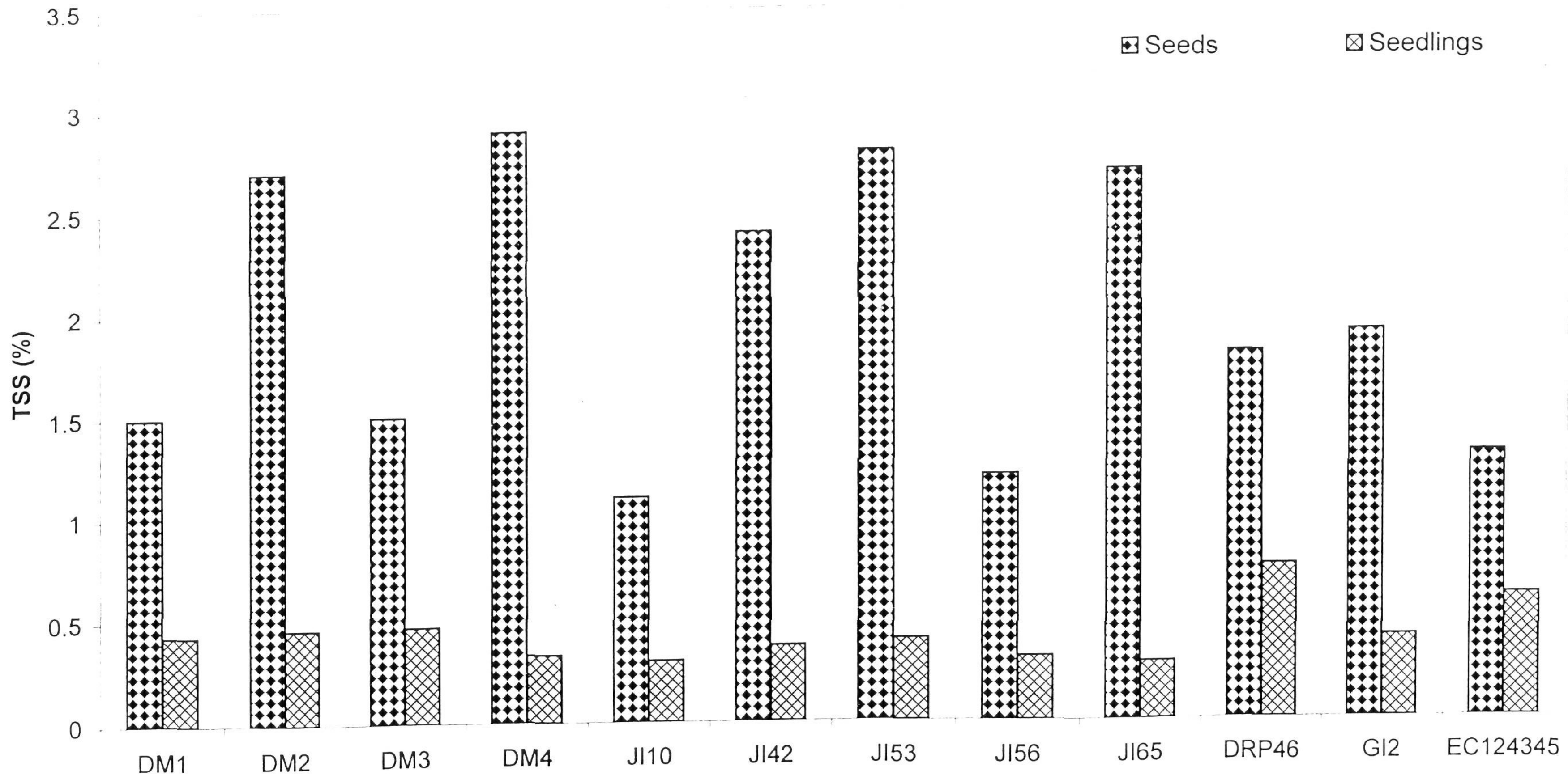


Figure 4.2.3.1 : Total soluble sugar percentage in different genotypes of Isabgol seeds and seedlings

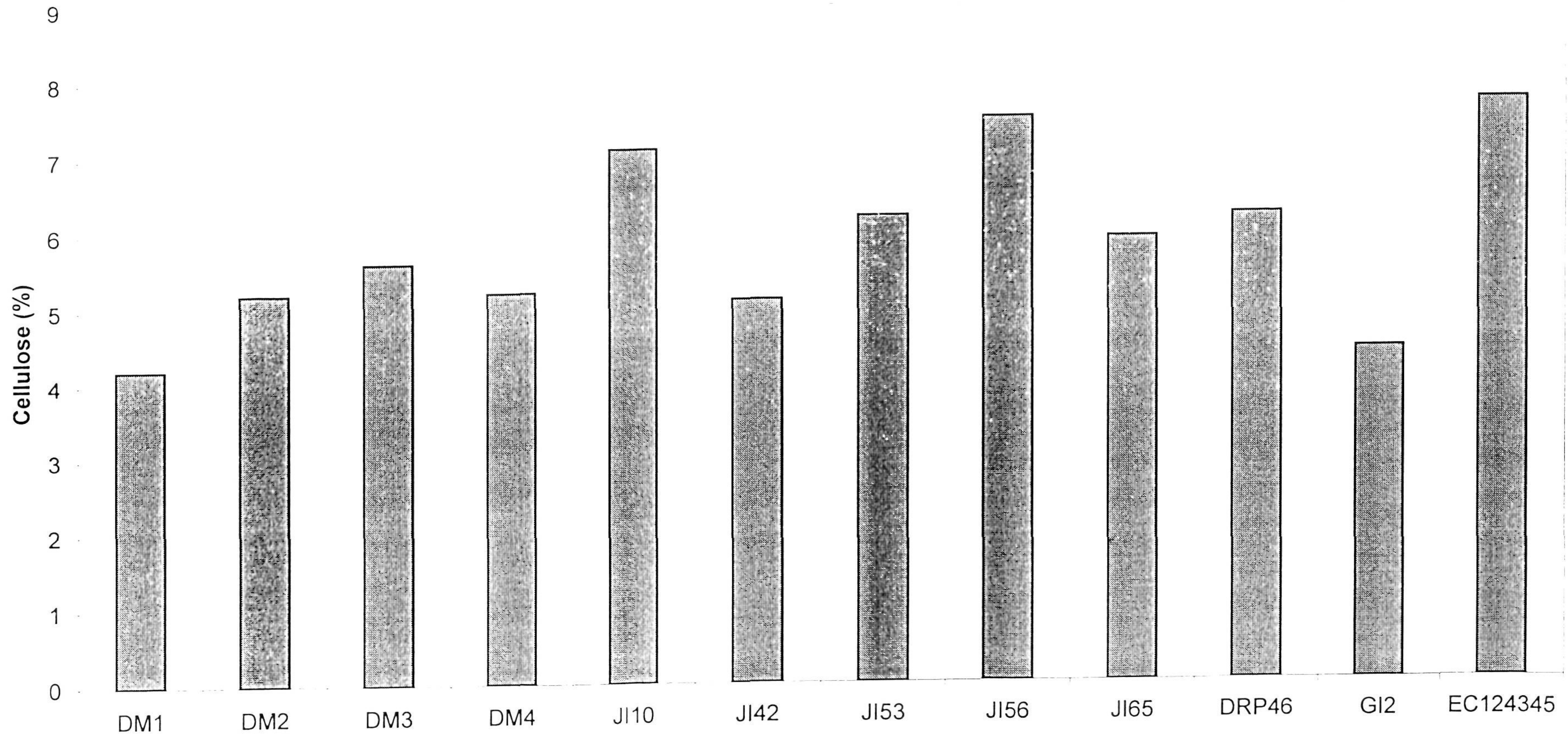


Figure 4.2.5.1 : Cellulose percentage of different genotypes of Isabgol seeds

with genotype JI53 (2.8%). Whereas, genotype JI10 was found to have the lowest (1.1%) TSS content (Figure 4.2.3.1).

The TSS content in Isabgol seeds is somewhat comparable to the cereals (Sorghum-1.9%TSS), which adds sweet taste to it.

At seedling stage the highest TSS content was found in genotype DRP46 (0.75%) and the lowest content was found in the seedlings of genotype JI65 (0.28%).

4.2.4 REDUCING SUGARS

The mean data indicated in Table 4.1, revealed that genotype GI2 showed minimum amount of reducing sugars (0.15%), whereas, DM2 showed maximum reducing sugar content (0.45%).

4.2.5 CELLULOSE

From the structural point of view cellulose is probably the best understood of all the carbohydrates of the plant cell wall (Nevell and Zeronian, 1995). A perusal of the data on cellulose (Table 4.1, Figure 4.2.5.1) showed that the cellulose content was found significantly higher in genotype EC124345 (7.7%), which was found to be at par with JI10 (7.1%). Genotype DM1 contained the lowest amount of cellulose (4.2%), which was found to be at par with GI2 (4.4%).

4.3 ASCORBIC ACID:

The mean values pertaining to ascorbic acid of different Isabgol genotypes listed in Table 4.1, which explained that the genotype JI42 (0.089%) contained significantly high ascorbic acid, which was found to be at par with genotypes DM1, DM2, DM4, JI10 and EC124345 (0.081% to 0.086%).While, the lowest ascorbic acid content was found in genotype JI53 (0.066%), which was found to be at par with DRP46 and JI56 (0.076%).

Shah and Gandhi, (1976), has also reported the presence of ascorbic acid in the cells of developing seedlings of Isabgol.

4.4 TOTAL FREE AMINO ACIDS (TFA):

Amino acids are known precursors of majority of secondary products. The precursors for the biosynthesis of alkaloids reported to be amino acids such as ornithine, lysine, phenylalanine, tyrosine and tryptophan (Kudesia and Jetely, 1995). Hence, high content of TFA is highly advantageous to medicinal plants, if they are utilized in the production of valuable secondary products.

The data on TFA (Table 4.1) revealed that, the seeds of genotype DM2 contained significantly higher (0.061%) TFA and was found to be at par with JI65 (0.058%). The lowest TFA content was observed in the seeds of DM4 (0.017%), which was found to be at par with JI42 (0.021%) and JI53 (0.020%). At seedling stage the genotype GI2 showed the highest amount of TFA (0.22%) and it was found to be at par with EC124345 (0.21%) and DRP46 (0.20%), while the lowest amount of TFA was found in the seedlings of JI42 and JI56 (0.09%), which were found to be at par with JI10 (0.11%) and JI53 (0.10%).

4.5 TOTAL PHENOLS:

A Wide range of phenolic compounds are synthesized in plant tissues during normal growth and development via the phenyl propanoid biosynthetic pathway. The compounds are building blocks for cell wall structure (Hahlbrock and Grisbact, 1979) and plant pigment production (Ebel and Hohlbrock, 1982) and serve as a defense against pathogens (Dixon *et al.*, 1983).

The total phenols in Isabgol seeds ranged from 0.08% to 0.14% (Figure 4.5.1). The maximum total phenols were found in DM1 and JI42 (0.14%), which was found to

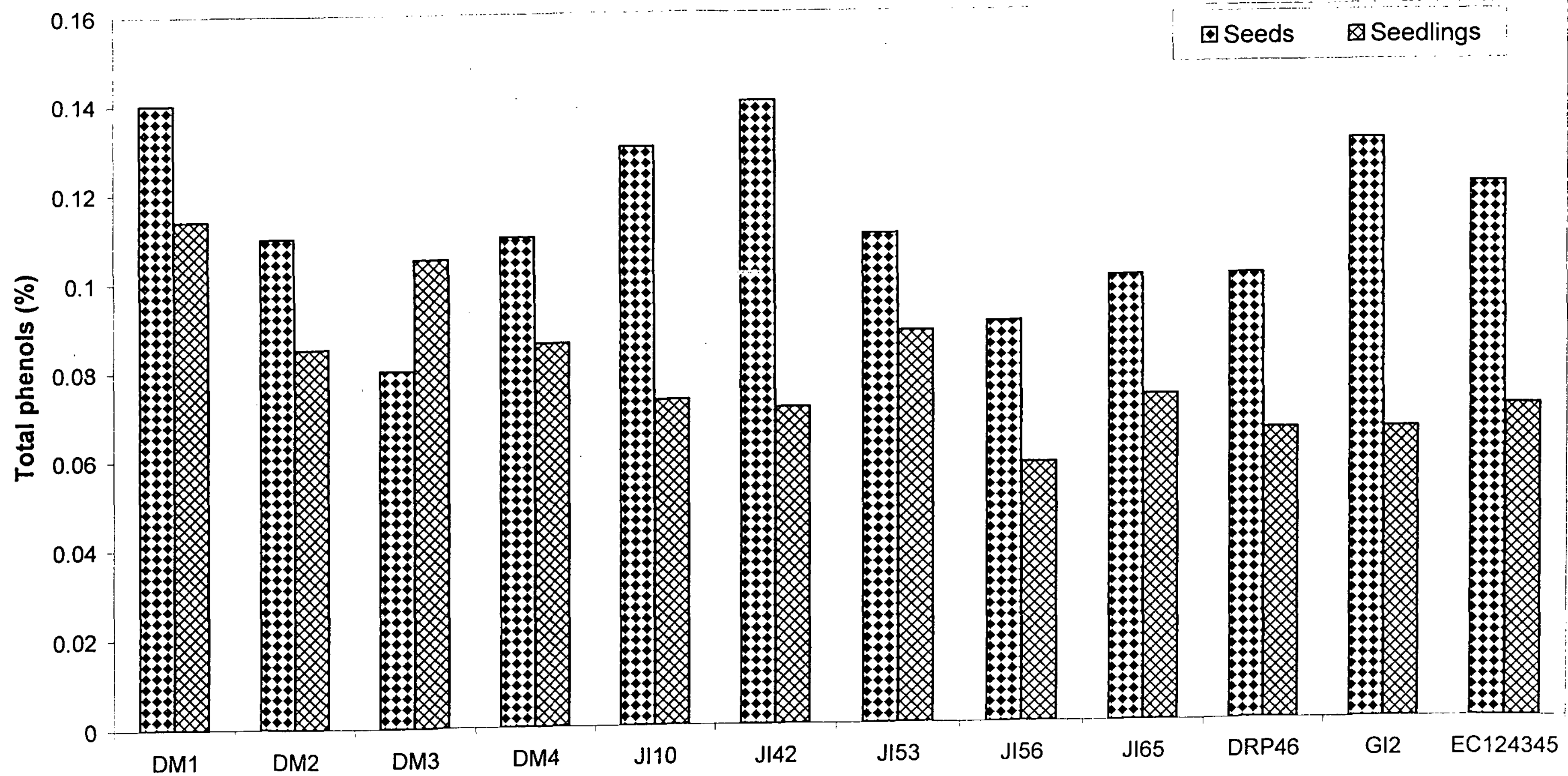


Figure 4.5.1 : Total phenols percentage in different genotypes of Isabgol seeds and seedlings

be at par with JI10 and GI2 (0.13%). The lowest content of phenols were found in genotype DM3 (0.08%), which was found to be at par with JI56 (0.09%). At seedling stage, the genotype DM1 showed maximum phenol contents (0.114%), while, the genotype JI56 showed minimum phenol content (0.058%).

4.6 TOTAL PROTEIN:

The genotype DM3 (Table 4.1, Figure 4.6.1) showed significantly higher (18%) amount of protein content. While, the lowest protein content was found in genotype JI65 and EC124345 (15%). Chakraborty and Patel (1992) reported crude protein in seed (20%) and crude protein in defatted meal (29.3%) from dehusked seeds. Naresh *et al.* (1992), also have been reported 13 to 16.81% protein content in 43 genotypes of Isabgol.

4.7 PROLINE:

Accumulation of proline is common in plants exposed to a variety of stresses including infection. The accumulation of proline is an adaptive mechanism to overcome the stress conditions. Enhanced synthesis of abscisic acid, which is related to drought resistance, is also characteristic of diseased plants, which exhibit severe stunting (Mohanty and Sridhar, 1982).

The mean data of proline content on percent protein basis presented in Table 4.1, revealed that the proline content in Isabgol seeds varied from 0.15% to 0.43%. The proline content of genotype JI53 was found significantly high (0.43%) as compared with rest of the genotypes. The lowest proline content was found in genotype DM2 (0.15%).

4.8 ESSENTIAL AMINO ACIDS:

Essential amino acids generally regarded as limiting factors in cereals and pulses are Lysine, Methionine and Tryptophan.

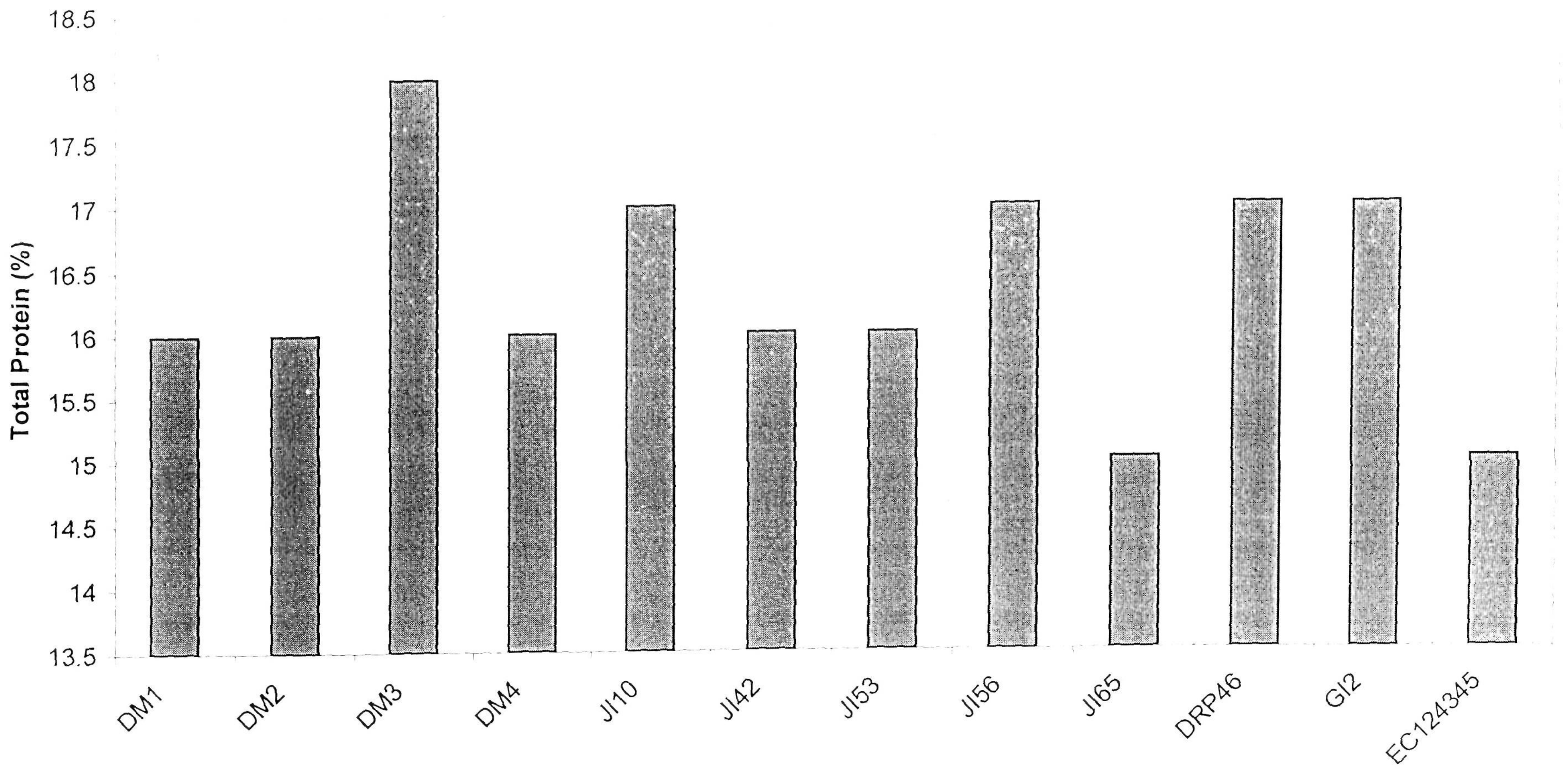


Figure 4.6.1 : Total protein percentage in different genotypes of Isabgol seeds

4.8.1 LYSINE

The lysine content of Isabgol seeds varied from 3.3% to 4.6% (Table 4.1). The genotype JI65 (4.6%) showed the highest amount of lysine, whereas, the lowest lysine content was found in EC124345, DRP46 and JI56 (3.3%), which were found to be at par with DM2 (3.6%) and GI2 (3.7%).

4.8.2 METHIONINE

The maximum methionine content was found in genotype JI53 (1.9%), which was found to be at par with DM3 and EC124345 (1.8%). The lowest methionine content was found in DM1 (1.2%) and it was found to be at par with JI10, JI56, JI65 and GI2 (1.2% to 1.3%).

Lysine content of Isabgol seed protein was found to be relatively higher than cereal protein like wheat, maize, sorghum and pearl millet, somewhat comparable to rice, but less than pulse protein (Gopalan *et al.*, 1994). Methionine content was much higher than pulse protein like pigeon pea and chickpea and comparable to cereals, but less than the FAO provisional pattern (which is for total sulphur amino acids). Chakraborty and Patel (1992) evaluated Isabgol dehusked seeds for their lysine (4.1%) and methionine content (1.7%)

The perusal of the data (Table 4.1) indicated less variability in quality parameters among the genotypes studied. The variability in husk and cellulose (quality parameter) was found to vary from 31 to 36%and 4.2 to7.7%, respectively (Naresh *et al.*, 1992). The variability in proximate principles viz oil, total carbohydrate, protein, ascorbic acid, TFA (seeds/seedlings) and total phenols (seeds/seedlings) were found to be 3.25 to 5.06, 51 to 63, 15 to 18, 0.066 to 0.089, 0.017

to 0.061/0.09 to 0.21 and 0.08 to 0.14/0.058 to 0.114%, respectively. All other proximate principles also exhibited poor variability among these genotypes.

4.9 ENZYME ASSAYS:

4.9.1 PEROXIDASE (Pox)

Enzyme Pox plays an important role in the oxidation of Indole-3-acetic acid (Stonier *et al.*, 1979; Siegel and Galston, 1995), ethylene biosynthesis, hydroxylation of proline (Ridge and Osborne, 1970), in lignification and in disease resistance (Reuveni and Karchi, 1987).

The Isabgol seeds of different genotypes showed no peroxidase enzyme activity. The activity was not found in seedlings up to 7 days after germination while two-weeks-old seedlings showed high Pox activity (Table 4.1, Figure 4.9.1.1). The maximum Pox activity was shown by EC124345 (18.20 ΔO.D./min/g fresh wt.), while the genotype DM3 showed the lowest Pox activity at this stage (1.68 ΔO.D./min/g fresh wt.).

Pox activity is frequently increased in plants infected by pathogens, and the level of its activity is often closely correlated with disease resistance (Kusuge, 1969). Enhanced Pox activity is also very often associated with resistance phenomena such as lignin production (Vance *et al.*, 1980), phenylalanine ammonia lyase activity and phenol accumulation (Rahtmell, 1973; Tena and Valbeuna, 1983).

4.9.2 POLY PHENOL OXIDASE (PPO)

The presence of phenolic compounds in plants, their oxidation following injury, either mechanical or due to infection and the relatively high toxicity of the oxidation products have since long drawn attention. The possible relationships of these properties to plant disease resistance has prompted many research workers to ascribe a role to PPO

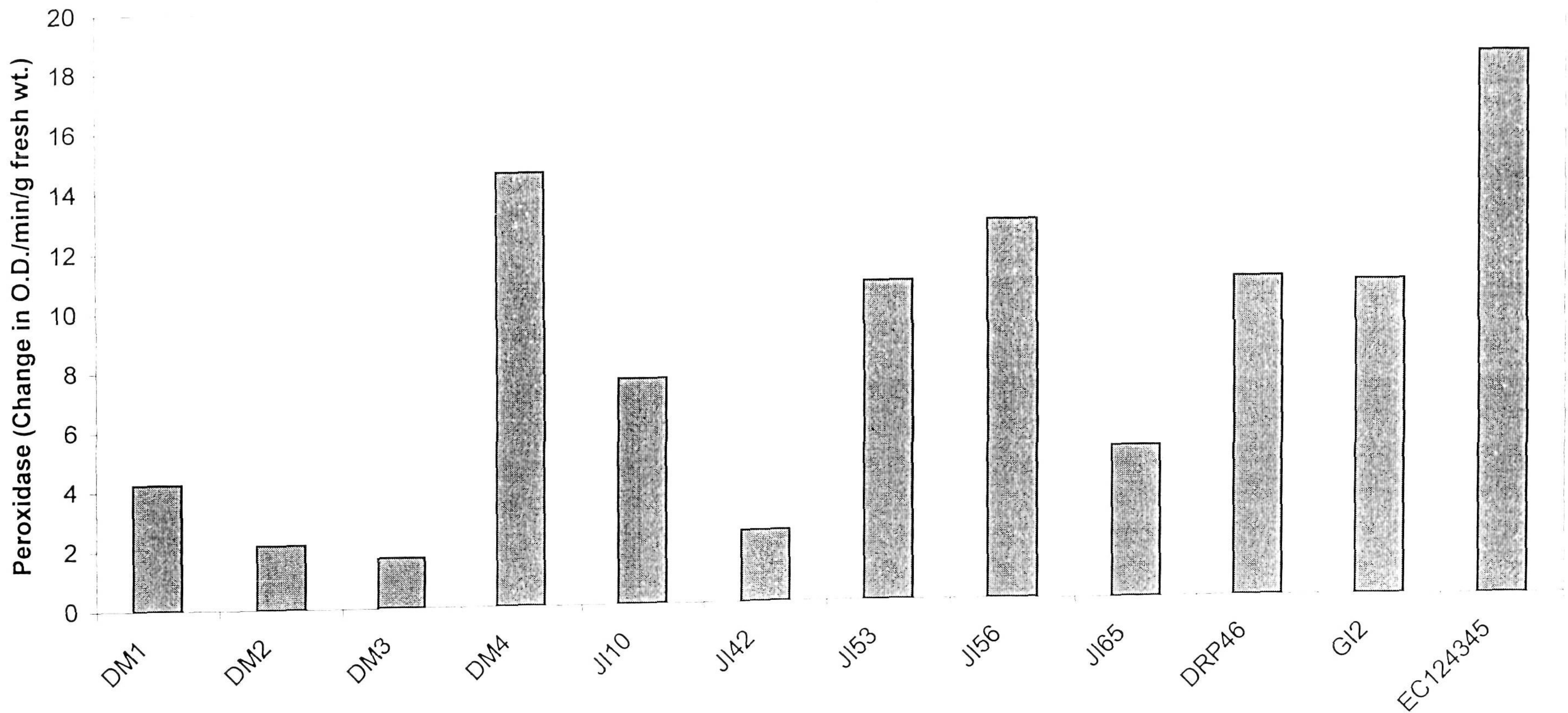


Figure 4.9.1.1 : Peroxidase activity in different genotypes of Isabgol seedlings

in disease resistance. PPO has been found to increase following infection by virus, bacteria, fungi and nematodes or mechanical injury (Brueske and Dropkin, 1973).

Seeds and seven days old seedlings of Isabgol showed no marked PPO activity. The enzyme activity at two-week-old seedlings elucidated in Table 4.1 (Figure 4.9.2.1), revealed that the genotype DM3 showed the highest PPO activity (31.76 ΔO.D./min/g fresh wt.), while genotype EC124345 showed the least PPO activity at this stage (13.32 ΔO.D./min/g fresh wt.).

4.9.3 PAL AND TAL:

In disease resistance mechanism these enzymes play an important role in the conversion of phenylalanine and tyrosine to coumaric acids. These provide the phenyl propane carbon skeleton for the synthesis of flavanoids, phenolic phenylpropanes and lignin (Mahadeven, 1979).

The enzyme activity of PAL (Table 4.1) was found to be the highest in genotype DM2 (0.50 ΔO.D./h/g), while the lowest activity was found in DM1 (0.36 ΔO.D./h/g) genotype. The TAL activity of different genotypes as shown in Table 4.1, revealed that the genotype JI10 (2.20 ΔO.D./h/g) showed the maximum activity, while the genotype DM2 (0.85 ΔO.D./h/g) showed the minimum activity.

4.9.4 β-GLUCOSIDASE:

Glucosidases play a direct role in cell wall modification and cell expansion. An increase in β-Glucosidase enzyme activity was shown to be roughly correlated in time with the decrease in cell wall glucan and an increase in growth rate of hypocotyls of seedlings (Malik and Singh, 1980).

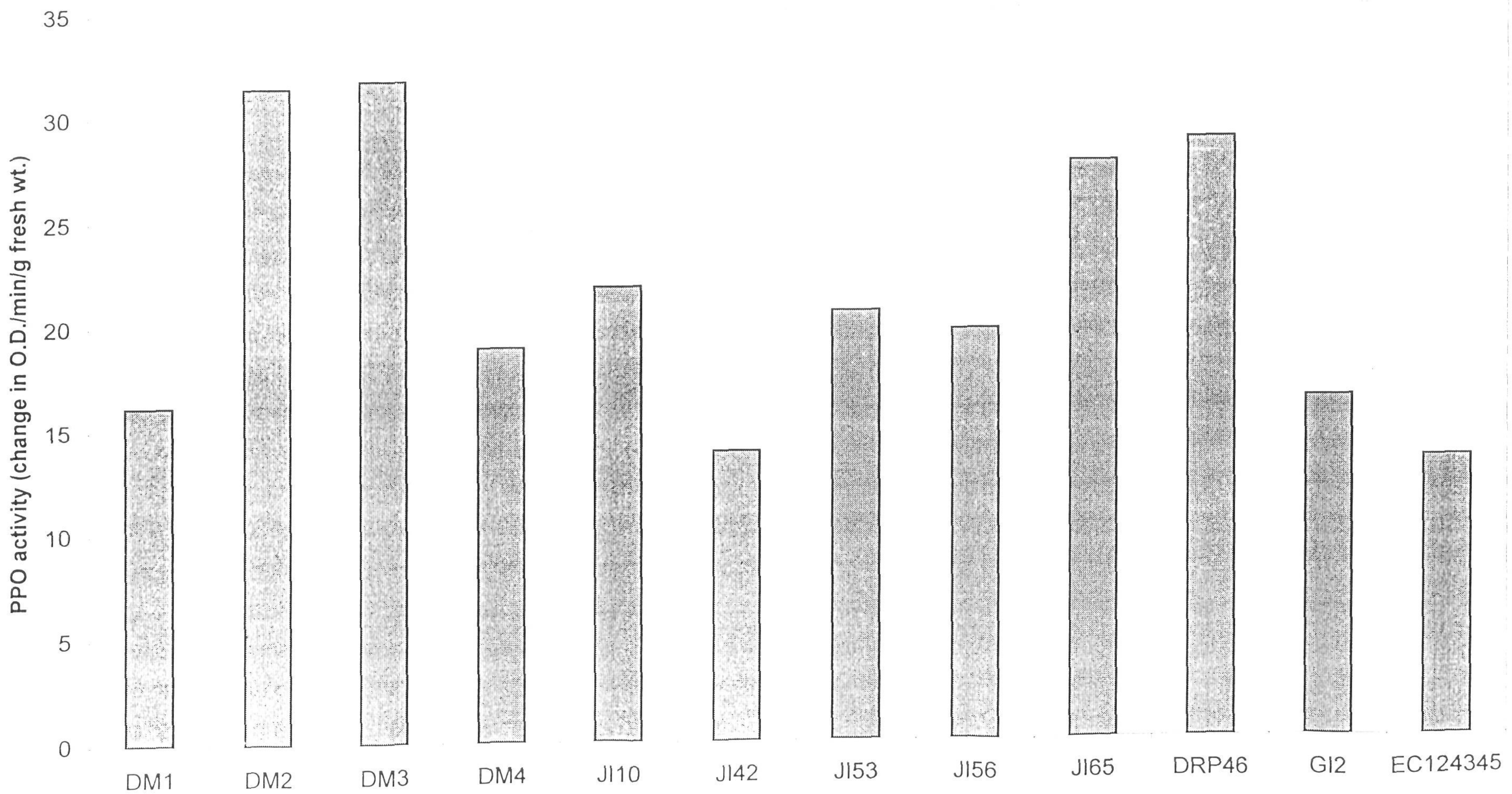


Figure 4.9.2.1 : Activity of polyphenol oxidase in different genotypes of Isabgol seedlings

The β-Glucosidase enzyme activity from seeds and seedlings of different genotypes is presented in Table 4.1. The enzyme activity was the highest in the seeds of EC124345 (0.32 ΔOD/h/g), while the lowest was found in GI2 (0.10 ΔOD/h/g). In seedlings, the enzyme activity was found to be maximum in genotypes JI10 (0.10 ΔOD/h/g fresh wt.), while the least activity was showed by the genotypes DM3 and DM4 (~0.063 ΔOD/h/g fresh wt.).

β-Glucosidase releases reducing sugars from glucosides or oligosaccharides. This enzyme is particularly important in disease resistance mechanisms where phenols are released from phenolic glucosides (Mahadeven and Sridhar, 1996).

4.10 PROTEIN AND ISOENZYMES:

Seed protein and isoenzyme banding pattern is a rapid technique useful to differentiate between genotypes. Variation in protein composition can be taken which are most commonly used in cultivar identification through proteins/enzymes which are encoded at several loci within the genome and exhibit extensive polymorphism both in relation to size and charge (Larkins, 1982). The term isoenzyme proposed as multiple molecular forms of an enzyme, sharing a catalytic activity derived from the tissues (Markert and Holler, 1959) but differ in their physical properties. The isoenzymes technique has been applied extensively to plant cultivars.

The electrophoregrams of Pox, PPO, Esterase, Alkaline phosphatase and protein are shown in Plates-2, 3, 4, 5 and 6, respectively. The isoenzyme pattern of Pox and Alkaline phosphatase indicated no variation between the genotypes. But the bands were found to be differing in their intensity for Pox, which showed different intensity of

Table 4.1 : Variability in quality parameters and proximate principles in *Plantago ovata* Forsk

Characters	Material	DM1	DM2	DM3	DM4	JI10	JI42	JI53	JI56	JI65	DRP46	GI2	EC124345	MEAN	S.Em.	CD at 5%
Oil (%)	Seeds	3.95	3.81	5.06	3.65	3.25	3.80	4.00	3.35	3.55	3.45	3.90	3.40	3.76	-	-
Husk (%)	Seeds	33	32	31	32	34	34	33	33	36	35	35	36	33.67	0.331	0.95
Total Carbohydrate (%)	Seeds	51	54	51	62	56	55	55	55	63	58	55	63	56.50	0.29	0.9
Total Soluble Sugars (%)	Seeds	1.5	2.7	1.5	2.9	1.1	2.4	2.8	1.2	2.7	1.8	1.9	1.3	1.98	0.037	0.110
	Seedlings	0.43	0.46	0.47	0.33	0.30	0.37	0.40	0.31	0.28	0.75	0.40	0.60	0.425	0.002	0.01
Reducing Sugars (%)	Seeds	0.18	0.45	0.22	0.41	0.18	0.31	0.39	0.20	0.32	0.23	0.15	0.26	0.275	0.004	0.01
Cellulose (%)	Seeds	4.2	5.2	5.6	5.2	7.1	5.1	6.2	7.5	5.9	6.2	4.4	7.7	5.83	0.211	0.61
Ascorbic Acid (%)	Seeds	0.082	0.085	0.081	0.086	0.085	0.089	0.066	0.077	0.076	0.076	0.072	0.086	0.080	0.003	0.01
TFA (%)	Seeds	0.034	0.061	0.055	0.017	0.026	0.021	0.020	0.027	0.058	0.041	0.025	0.040	0.035	0.001	0.004
	Seedlings	0.13	0.13	0.12	0.17	0.11	0.09	0.10	0.09	0.12	0.20	0.22	0.21	0.141	0.005	0.02
Total Phenols (%)	Seeds	0.14	0.11	0.08	0.11	0.13	0.14	0.11	0.09	0.10	0.10	0.13	0.12	0.113	0.004	0.01
	Seedlings	0.114	0.085	0.105	0.086	0.073	0.071	0.088	0.058	0.073	0.065	0.065	0.070	0.079	0.001	0.002
Total Protein (%)	Seeds	16	16	18	16	17	16	16	17	15	17	17	15	16.33	0.141	0.41
Proline (% Protein basis)	Seeds	0.39	0.15	0.29	0.31	0.22	0.36	0.43	0.41	0.33	0.22	0.39	0.37	0.322	0.004	0.01
Lysine (% Protein basis)	Seeds	3.9	3.6	4.0	4.1	4.2	4.1	3.9	3.3	4.6	3.3	3.7	3.3	3.83	0.145	0.42
Methionine (% Protein basis)	Seeds	1.2	1.7	1.8	1.7	1.3	1.7	1.9	1.3	1.2	1.4	1.3	1.8	1.525	0.045	0.13
Peroxidase (ΔO.D./min/g tissue)	Seedlings	4.24	2.16	1.68	14.56	7.56	2.40	10.72	12.72	5.08	10.72	10.56	18.20	-	-	-
PPO (ΔO.D./min/g tissue)	Seedlings	16.16	31.44	31.76	18.92	21.80	13.84	20.56	19.64	27.64	28.72	16.24	13.32	-	-	-
PAL (ΔO.D./h/g tissue)	Seeds	0.36	0.50	0.39	0.41	0.39	0.40	0.38	0.41	0.46	0.39	0.46	0.40	-	-	-
TAL (ΔO.D./h/g tissue)	Seeds	1.78	0.85	1.16	1.56	2.20	1.56	1.95	2.15	1.60	0.92	1.76	1.97	-	-	-
β-Glucosidase (ΔO.D./h/g tissue)	Seeds	0.23	0.30	0.22	0.22	0.26	0.31	0.23	0.31	0.17	0.14	0.10	0.32	-	-	-
	Seedlings	0.074	0.076	0.063	0.064	0.104	0.066	0.070	0.050	0.068	0.079	0.072	0.085	-	-	-

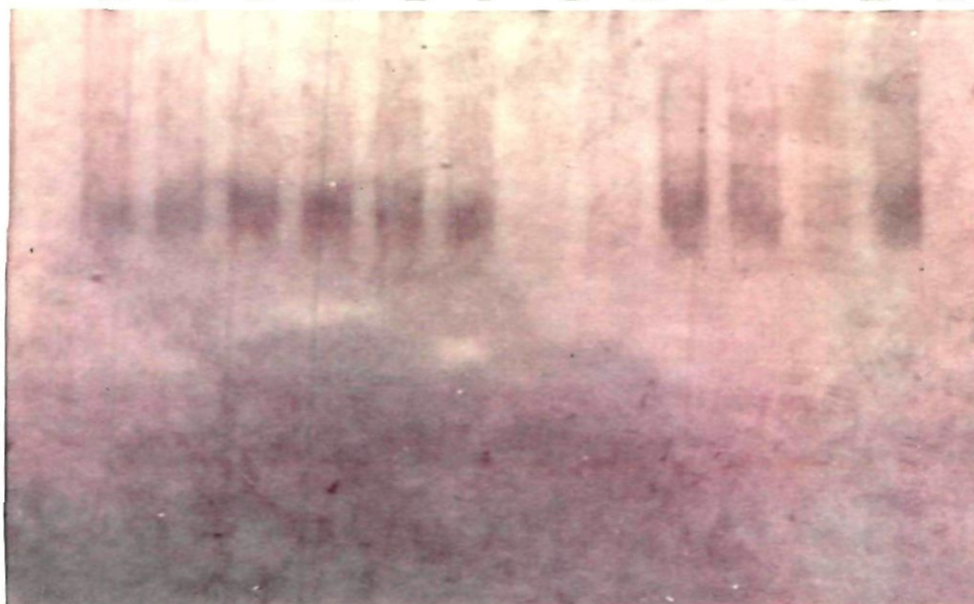


PLATE-2: ISOENZYME PATTERN FOR POX

(A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

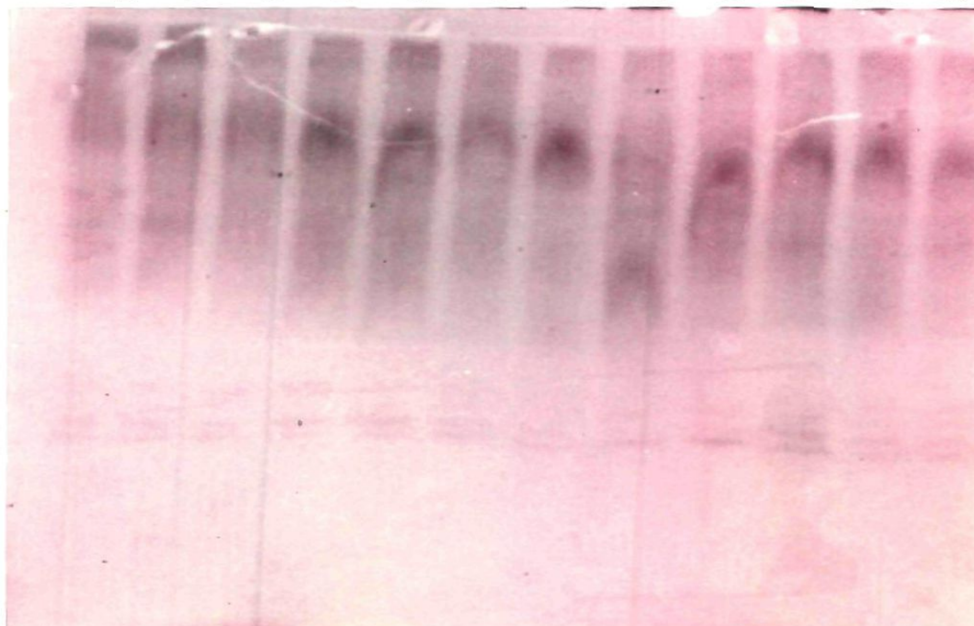


PLATE-3: ISOENZYME PATTERN FOR PPO

(A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

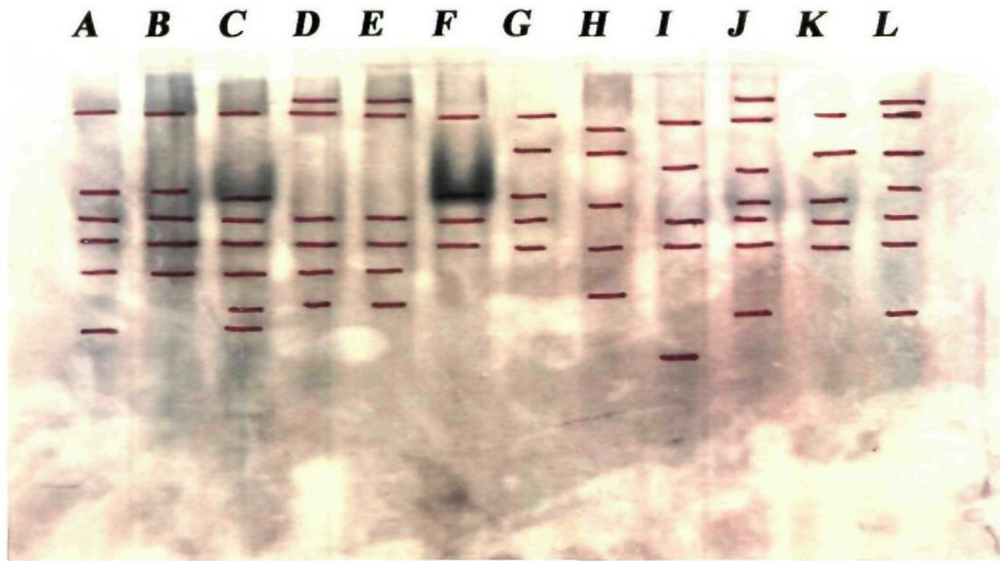


PLATE-4: ISOENZYME PATTERN FOR ESTERASE

(A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

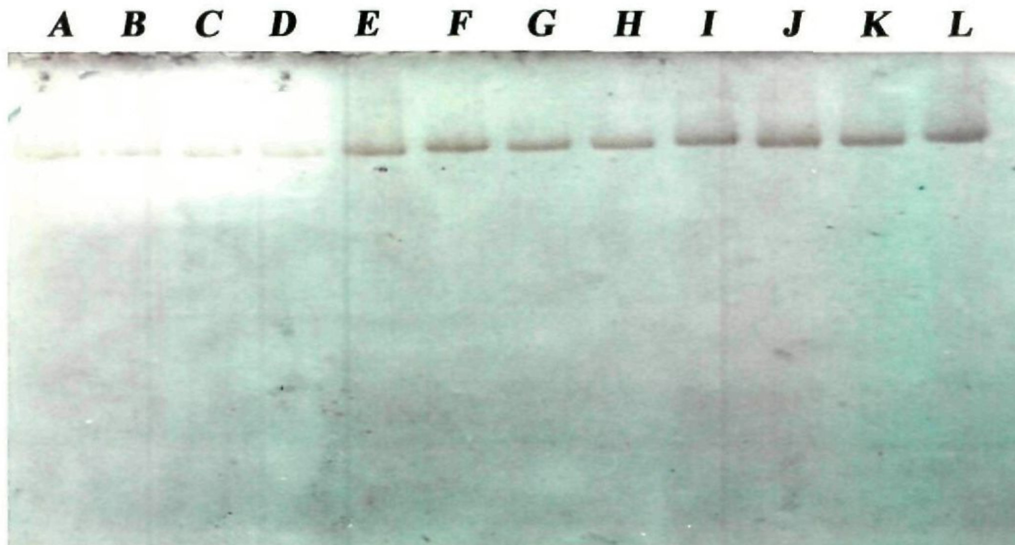


PLATE-5: ISOENZYME PATTERN FOR ALKALINE PHOSPHATASE

(A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

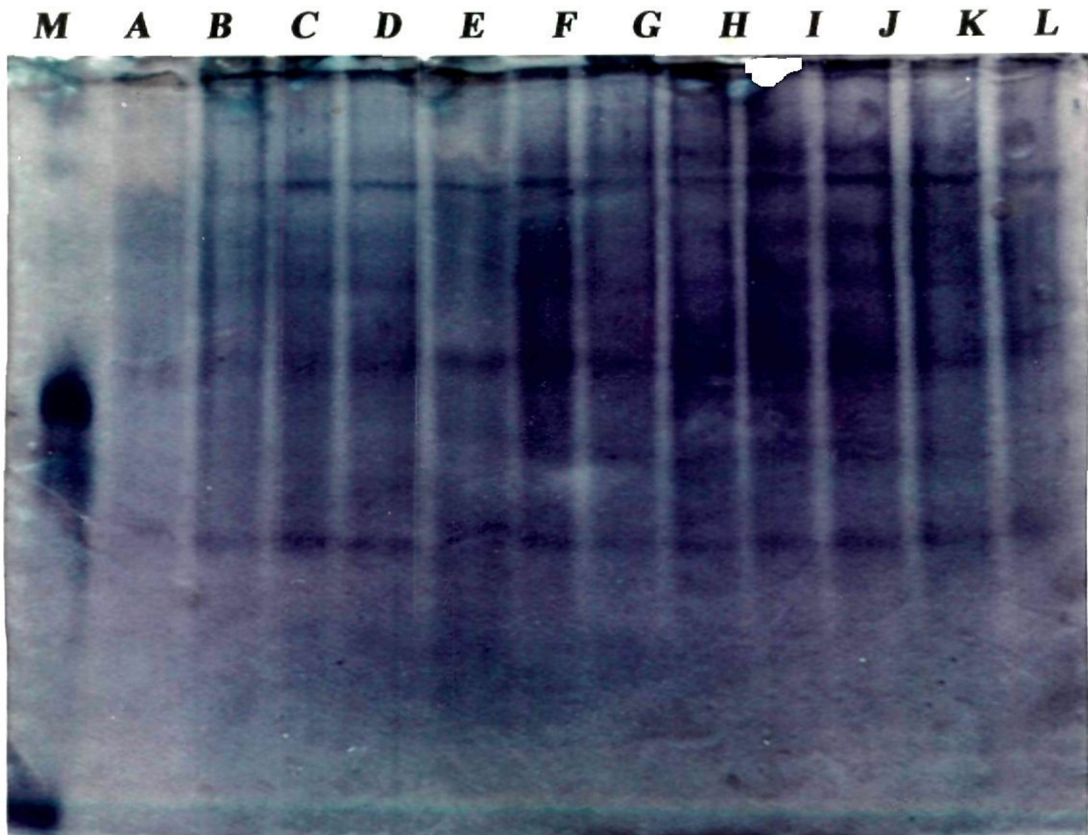


PLATE-6:ELECTROHOROGRAM FOR PROTEIN

(M=MARKER;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

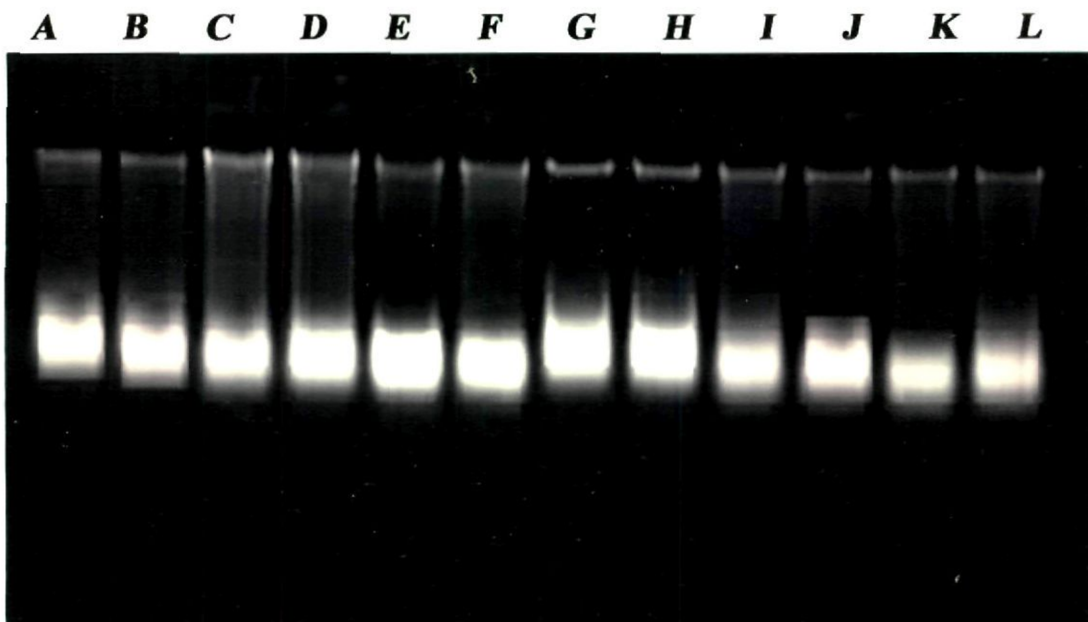


PLATE-7: GENOMIC DNA

(A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

activity of Pox in different genotypes. The Rm values for isozymic bands for PPO and Esterase are elucidated in Table-4.10.1 and 4.10.2, which showed that the PPO isoenzyme bands varied from 5 (DM4) to 8 (GI2, DM1, DRP46, DM2 and JI65), while 4 (JI42) to 7 (JI10, JI65 and EC124345) esterase isozyme bands in different genotypes. The Rm values for PPO varied from 0.09 to 0.70. The band of 0.09 Rm was found to be absent in genotypes JI42, DM3, JI65, DM4 and EC124345. The band of 0.14 Rm value was present only in JI65. The Rm values for Esterase varied from 0.05 to 0.50. The differences in isoenzyme pattern of PPO and Esterase showed that these could be used as a marker in the identification of varieties.

TABLE 4.10.1:Rm Values of PPO Isoenzyme of Isabgol Seedlings

Sr. No.	Genotype	**Relative Mobility (Rm Values)**							
		1	2	3	4	5	6	7	8
1	GI2	0.09**L**	0.19**D**	0.23**D**	0.33**L**	0.39**L**	0.63**D**	0.67**SB**	0.70**SB**
2	DM1	0.09**L**	0.18**D**	0.34**D**	0.42**D**	0.60**D**	0.62**D**	0.66**SB**	0.69**SB**
3	JI10	0.09**L**	0.18**D**	0.23**D**	0.60**D**	0.62**L**	0.66**SB**	0.69**SB**	-
4	JI56	0.09**L**	0.19**L**	0.23**D**	0.62**D**	0.67**SB**	0.68**SB**	-	-
5	JI53	0.09**L**	0.21**D**	0.24**SB**	0.37**D**	0.61**D**	0.66**SB**	0.68**SB**	-
6	JI42	0.19**D**	0.32**D**	0.37**D**	0.40**D**	0.63**L**	0.66**SB**	0.68**SB**	-
7	DM3	0.19**D**	0.31**D**	0.36**L**	0.51**D**	0.56**L**	0.67**L**	0.68**L**	-
8	DRP46	0.09**D**	0.24**L**	0.27**L**	0.32**L**	0.40**D**	0.56**L**	0.63**SB**	0.68**SB**
9	DM2	0.08**L**	0.11**L**	0.24**D**	0.32**L**	0.37**L**	0.56**L**	0.65**SB**	0.67**SB**
10	JI65	0.14**D**	0.24**D**	0.38**D**	0.48**L**	0.56**SB**	0.62**L**	0.65**SB**	0.67**SB**
11	DM4	0.23**D**	0.30**L**	0.36**L**	0.66**SB**	0.69**SB**	-	-	-
12	EC124345	0.23**D**	0.30**L**	0.36**L**	0.64**L**	0.66**SB**	0.69**SB**	-	-

D=Diffused band **L**=Light band **SB**=Sharp bright band

TABLE 4.10.2: Rm Values of Esterase Isoenzyme of Isabgol Seedlings

SR. No.	Genotype	Relative Mobility (Rm Values)						
		1	2	3	4	5	6	7
1	GI2	0.08**L**	0.21**D**	0.28**D**	0.31**D**	0.38**L**	0.50**L**	-
2	DM1	0.08**D**	0.21**D**	0.28**D**	0.33**D**	0.38**L**	-	-
3	JI10	0.09**L**	0.25**VD**	0.29**D**	0.33**L**	0.39**L**	0.45**L**	0.50**L**
4	JI56	0.05**L**	0.08**L**	0.28**D**	0.33**D**	0.38**L**	0.44**L**	-
5	JI53	0.05**L**	0.08**L**	0.28**D**	0.33**D**	0.38**L**	0.44**L**	-
6	JI42	0.15**L**	0.28**VD**	0.33**L**	0.38**L**	-	-	-
7	DM3	0.10**L**	0.18**D**	0.25**D**	0.31**D**	0.36**L**	-	-
8	DRP46	0.13**D**	0.16**D**	0.26**D**	0.34**L**	0.38**L**	-	-
9	DM2	0.10**D**	0.19**L**	0.29**D**	0.33**D**	0.54**L**	-	-
10	JI65	0.06**L**	0.10**L**	0.19**D**	0.25**D**	0.29**D**	0.34**L**	0.46**D**
11	DM4	0.08**L**	0.15**L**	0.24**D**	0.28**D**	0.31**L**	-	-
12	EC124345	0.05**L**	0.08**D**	0.15**L**	0.21**D**	0.26**D**	0.31**L**	0.45**L**

D=Dark band L=Light band VD=Very Dark band

For all the 22 characters Karl Pearson correlation was worked out using SPSS software (Table 4.2). The correlations, which were found significant, are shown in Table 4.3.

TABLE 4.3 :

S4. No.	CHARACTER 1	CHARACTER 2	TYPE OF CORRELATION
1	Oil	Husk	NAGATIVE
2		Total Carbohydrate	NEGATIVE
3		Phenol (Seedling)	POSITIVE
4		Pox (Seedling)	NEGATIVE
5	Husk	Total Carbohydrate	POSITIVE
6		Phenol (Seedling)	NEGATIVE
7	Total Carbohydrate	Protein	NEGATIVE
8		Pox (Seedling)	POSITIVE
9	TSS (Seedling)	TFA (Seedling)	POSITIVE
10		Lysine	NEGATIVE
11		TAL	NEGATIVE
12	Reducing Sugars	Methionine	POSITIVE
13	Cellulose	Phenol (Seedling)	NEGATIVE
14	Ascorbic acid	β-Glucosidase (Seed)	POSITIVE
15	TFA (Seeds)	PPO (Seedlings)	POSITIVE
16		TAL	POSITIVE
17	Phenol (Seeds)	PPO	NEGATIVE
18	Proline	PPO	NEGATIVE
19		TAL	POSITIVE
20	PPO	TAL	NEGATIVE

Table 4.2 Correlations

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22
V1																						
V2	-.600																					
	.039																					
V3	-.583	.605																				
	.047	.037																				
V4	.060	-.205	.287																			
	.853	.523	.366																			
V5	.053	.199	.030	-.175																		
	.871	.535	.925	.587																		
V6	.009	-.287	.300	.920	-.098																	
	.977	.365	.344	.000	.762																	
V7	-.476	.284	.401	-.281	.102	-.095																
	.118	.372	.196	.376	.754	.768																
V8	-.119	-.146	.104	-.021	-.023	.108	.000															
	.713	.650	.748	.949	.943	.739	.999															
V9	.280	.006	-.003	.083	.278	.134	-.004	.083														
	.378	.985	.991	.797	.382	.677	.990	.797														
V10	-.145	.435	.404	-.258	.595	-.195	-.093	-.051	.028													
	.652	.157	.193	.418	.041	.545	.774	.874	.931													
V11	-.295	.303	-.079	-.109	-.125	-.151	-.363	.304	-.465	.107												
	.352	.339	.807	.735	.698	.639	.246	.338	.128	.740												
V12	.682	-.614	-.486	.180	-.025	.129	-.509	.112	.203	-.221	.040											
	.015	.034	.110	.575	.938	.690	.091	.730	.526	.490	.901											
V13	.442	-.486	-.636	-.489	.091	-.492	-.057	-.156	-.070	-.073	-.337	.063										
	.151	.109	.026	.106	.780	.104	.860	.629	.828	.822	.284	.846										
V14	.064	.180	-.006	-.136	-.274	-.248	-.003	-.406	-.520	-.082	.168	-.009	-.199									
	.843	.575	.985	.673	.389	.437	.994	.191	.083	.799	.602	.978	.535									
V15	.198	-.051	.096	.416	-.659	.190	-.336	.084	.025	-.424	.147	.320	-.184	-.013								
	.538	.876	.767	.179	.020	.555	.286	.795	.939	.169	.649	.311	.566	.969								
V16	.408	-.385	.021	.412	.243	.596	.110	.136	-.036	-.031	-.180	.215	-.078	-.019	-.136							
	.188	.217	.949	.183	.447	.041	.734	.674	.911	.923	.577	.502	.810	.954	.674							
V17	-.523	.369	.611	-.192	.206	-.060	.530	-.168	-.453	.542	-.048	-.465	-.227	.344	-.484	.098						
	.081	.237	.035	.549	.521	.853	.076	.602	.139	.069	.882	.128	.478	.274	.111	.762						
V18	.339	-.329	-.147	.247	.182	.246	-.006	-.152	.743	-.148	-.698	.179	.343	-.688	.110	.028	-.469					
	.281	.297	.648	.440	.572	.440	.985	.638	.006	.646	.012	.578	.275	.013	.735	.931	.124					
V19	-.140	.407	.458	.375	-.337	.248	-.069	-.158	.542	-.056	-.218	-.213	-.446	-.117	.501	-.342	-.234	.359				
	.665	.190	.134	.230	.284	.438	.832	.624	.069	.862	.495	.506	.146	.718	.097	.277	.464	.251				
V20	-.389	.266	.127	-.391	-.522	-.377	.417	-.128	-.606	-.202	.330	-.214	-.160	.648	.095	-.235	.452	-.690	-.151			
	.211	.403	.694	.208	.082	.227	.178	.692	.037	.530	.295	.504	.620	.023	.770	.463	.140	.013	.639			
V21	-.151	-.269	-.044	.080	-.151	.306	.462	.570	.003	-.537	.038	.056	-.258	-.032	-.140	.407	-.028	-.172	-.254	.217		
	.641	.397	.893	.804	.639	.334	.130	.053	.994	.072	.907	.862	.418	.921	.664	.190	.931	.593	.426	.497		
V22	-.248	.444	.054	-.423	.164	-.362	-.036	-.002	-.083	.508	.542	-.176	.046	-.265	-.004	-.230	.117	-.161	-.064	.156	-.363	
	.437	.148	.867	.171	.611	.247	.912	.996	.797	.092	.069	.585	.887	.405	.989	.472	.718	.618	.844	.628	.246	

V1=OIL%, V2=HUSK%, V3=TOTAL CARBOHYDRATE, V4=TSS (SEEDS), V5=TSS (SEEDLINGS), V6=REDUCING SUGARS, V7=CELLULOSE, V8=ASCORBIC ACID, V9=TFA (SEEDS), V10=TFA (SEEDLINGS), V11=PHENOL (SEEDS), V12=PHENOL (SEEDLINGS), V13=PROTEIN, V14=PROLINE, V15=LYSINE, V16=METHIONINE, V17=POX, V18=PPO, V19=PAL, V20=TAL, V21=GLUCOSIDASE (SEEDS), V22=GLUCOSIDASE (SEEDLINGS)

The correlation between different characters is shown in Table 4.3. It was observed that the genotypes EC124345 and JI65 were found to have the highest levels of husk, total carbohydrates and the lowest level of proteins. Whereas, the genotype DM3 had lowest level of husk, total carbohydrate and the highest level of protein. It may be concluded that the higher levels of carbohydrate and lower level of protein can be one of the possible reason for high husk content in Isabgol (Table 4.2 and 4.3). Naresh *et al.* (1992) also reported the strong correlation between husk, total carbohydrate and protein. They found positive correlation between husk and total carbohydrate and negative correlation with protein. With these correlations they concluded that the low levels of protein and high levels of carbohydrates are desirable for high husk content.

From the mean data dissimilarity matrix was prepared on the basis of "Squared Euclidean Distances" (Table 4.4) and a dendogram (Fig 4.4) was constructed by Hirarchial linkage clustering using SPSS. The maximum dissimilarities were observed between EC124345 and DM3, while the least distances was shown by JI56 and JI53. Genotypes JI10, GI2, DM1, JI56 and JI53 formed one cluster, while, DM4 and EC124345 formed another cluster. DM3 and DM2; DRP46 and JI65 were found to be closely related.

<u>Table 4.4 : Euclidean Distances for Biochemical characters</u>

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.000	61.257	50.157	30.552	30.403	77.025	356.443	169.779	317.067	234.960	86.629	147.648
DM1	61.257	0.000	79.216	112.901	83.609	27.781	261.876	259.384	251.638	291.720	239.583	370.348
JI10	50.157	79.216	0.000	34.327	19.401	98.597	175.864	66.903	140.040	102.050	105.946	243.788
JI56	30.552	112.901	34.327	0.000	11.335	150.714	298.172	103.319	263.632	206.391	64.392	147.416
JI53	30.403	83.609	19.401	11.335	0.000	117.036	235.022	83.768	196.138	157.514	68.819	187.680
JI42	77.025	27.781	98.597	150.714	117.036	0.000	353.509	304.691	315.768	268.073	226.984	327.925
DM3	356.443	261.876	175.864	298.172	235.022	353.509	0.000	160.816	17.857	211.440	461.110	799.331
DRP46	169.779	259.384	66.903	103.319	83.768	304.691	160.816	0.000	108.977	66.469	140.410	326.956
DM2	317.067	251.638	140.040	263.632	196.138	315.768	17.857	108.977	0.000	123.509	375.404	693.475
JI65	234.960	291.720	102.050	206.391	157.514	268.073	211.440	66.469	123.509	0.000	185.091	384.871
DM4	86.629	239.583	105.946	64.392	68.819	226.984	461.110	140.410	375.404	185.091	0.000	72.407
EC124345	147.648	370.348	243.788	147.416	187.680	327.925	799.331	326.956	693.475	384.871	72.407	0.000

This is a dissimilarity matrix

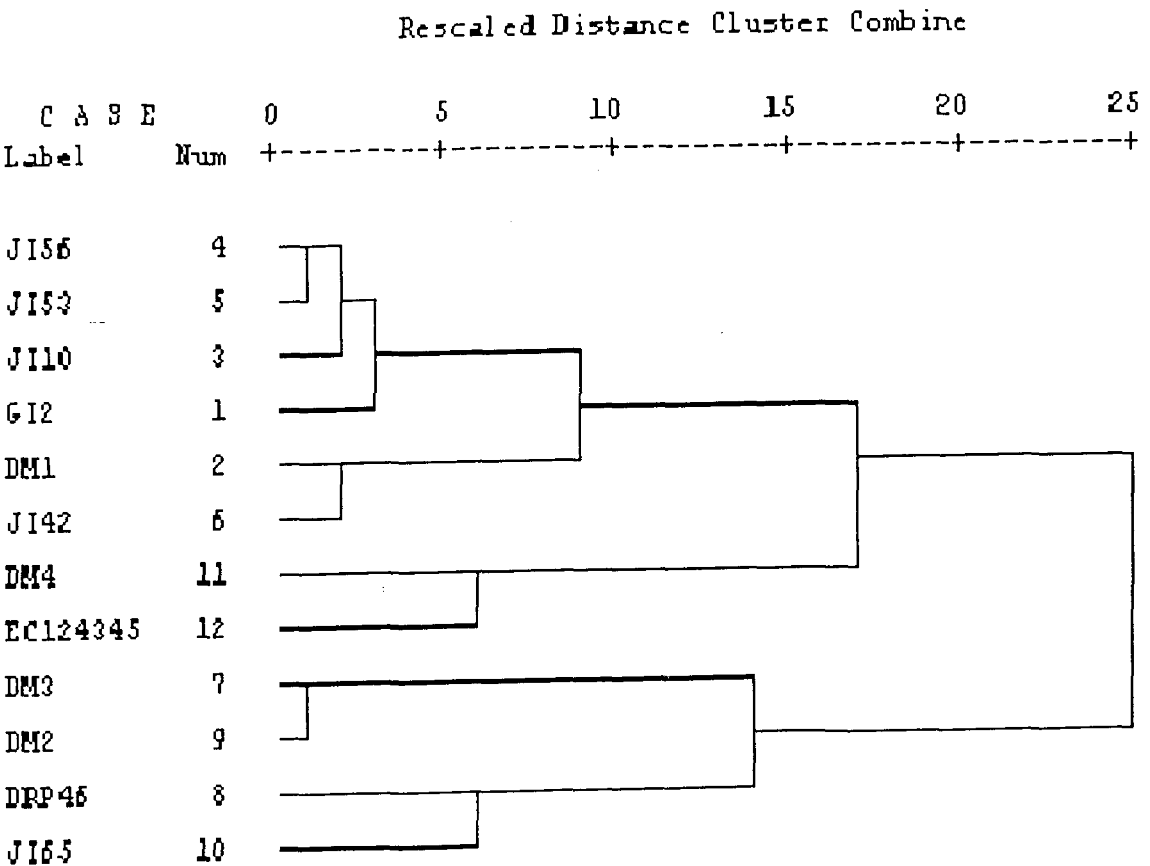


FIGURE 4.4: DENDROGRAM CONSTRUCTED USING BIOCHEMICAL CHARACTERS USING AVERAGE LINKAGE (BETWEEN GROUPS)

MOLECULAR CHARACTERIZATION

4.11 DNA EXTRACTION:

Two methods were used to isolate the DNA from Isabgol seeds. The DNA extracted with universal method gave good results in terms of quality and quantity. The quality and quantity of DNA was not good when isolated by CTAB method. So the DNA isolated by universal method was used for further work (Plate 7).

In *Plantago ovata* all DNA marker types viz. RAPD, CAPS, ISSR and AFLP were able to detect polymorphism. For the analysis only reproducible and polymorphic amplified fragments/bands were considered. For quantification of diversity/similarity, pair wise comparison of banding pattern was evaluated by calculating an index of similarity (Bs) using matching coefficient method of Lynch (1990). The data was entered in Dbase and Bs and Dxy were calculated using the program prepared in foxpro. The dissimilarity (PD%) between A & B was [(1-Bs) X100] (with an expected range of 0-100) also calculated.

4.12 RAPD:

The fingerprints generated by 12 random decamer primers are presented in Plates 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19. Among these primers Plantago 06 didn't show any polymorphism, hence failed to detect any variation among the genotypes. The combined total number of bands scored was 891, of which 444 (50%) were polymorphic. Three to 13 scorable bands ranged from 150-2000 base pairs. The pooled matrix for all RAPD primers of Bs (upper diagonal) and Dxy (lower diagonal) is presented in Table 4.12.1. The overall average values of PIC and Hav were 0.491 and 0.652. The pair wise results for marker size, scorable bands, total alleles, total bands are represented in Table 4.5. Whereas, the primer wise data on Bs, Dxy, PD,

PLATE-8: RAPD PROFILE OF PLANTAGO 01

(M=1Kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

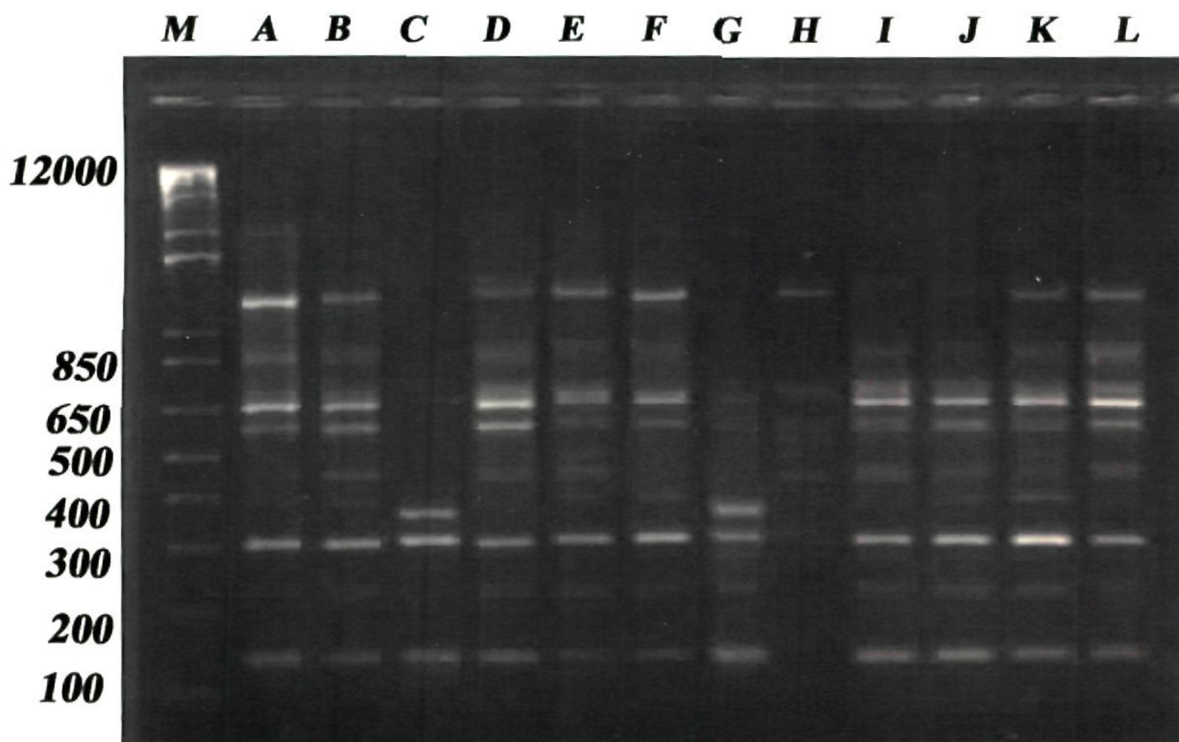


PLATE-9: RAPD PROFILE OF PLANTAGO 02

(M=1Kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

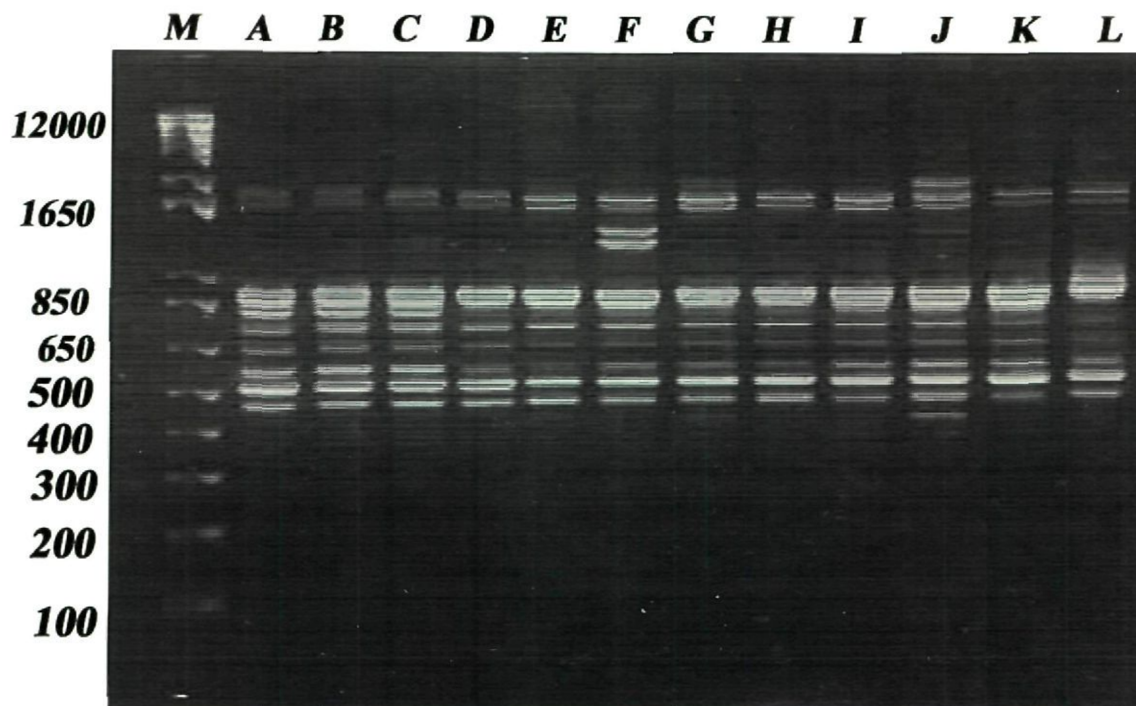


PLATE-10: RAPD PROFILE OF PLANTAGO 03

(M=1Kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

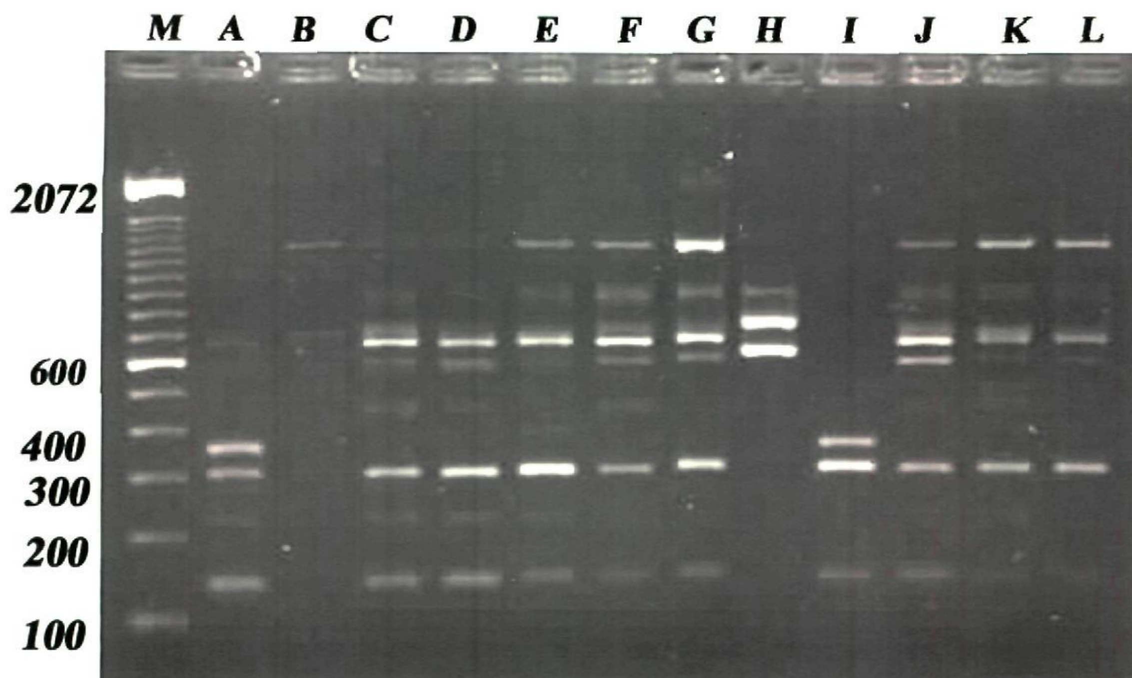


PLATE-11: RAPD PROFILE OF PLANTAGO 04

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

PLATE-12: RAPD PROFILE OF PLANTAGO 05

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

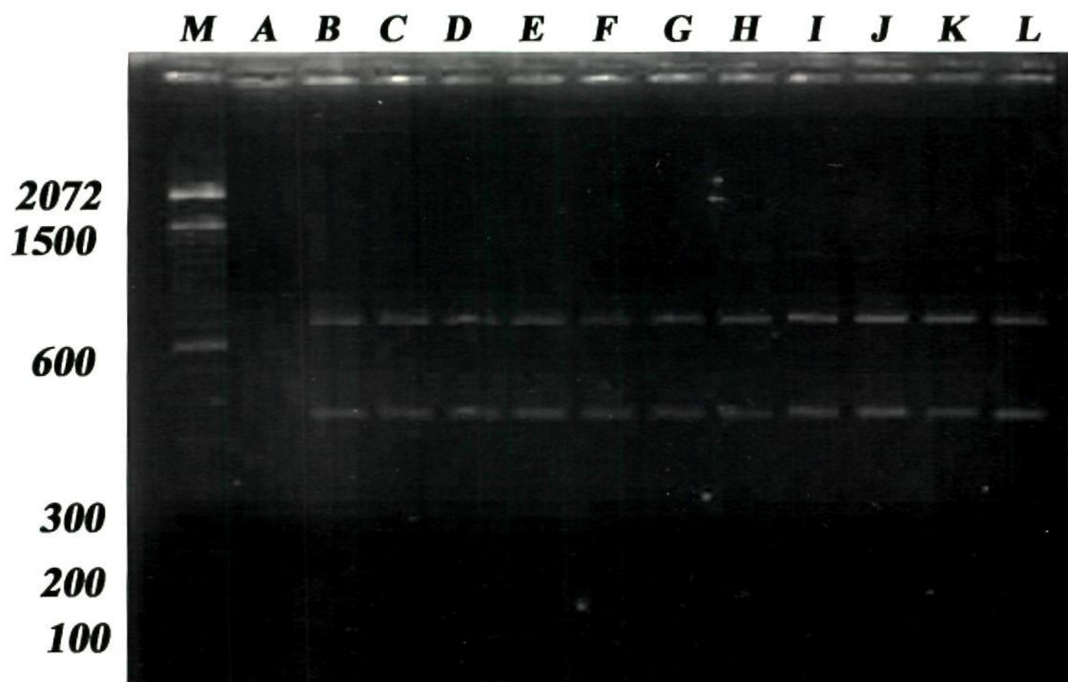


PLATE-13: RAPD PROFILE OF PLANTAGO 06

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

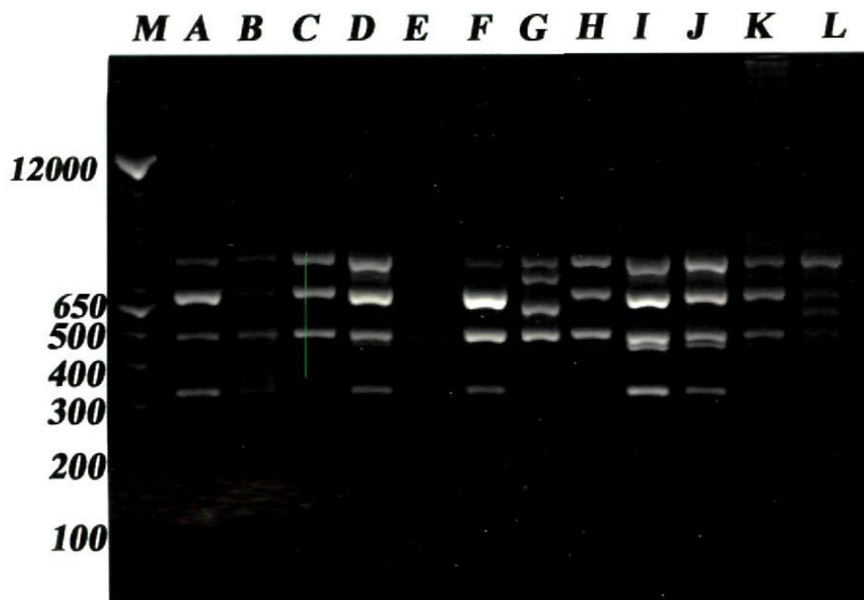


PLATE-14: RAPD PROFILE OF PLANTAGO 07

*(M=1Kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)*

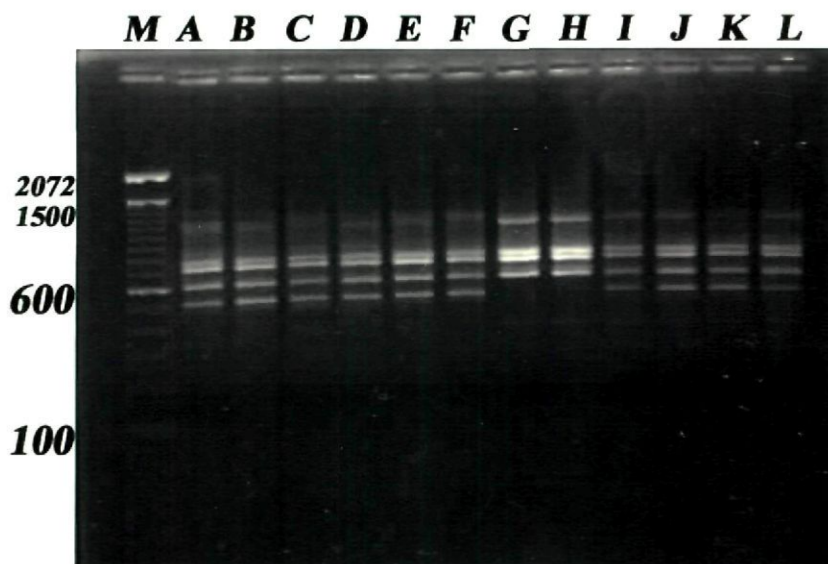


PLATE-15: RAPD PROFILE OF PLANTAGO 08

*(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)*

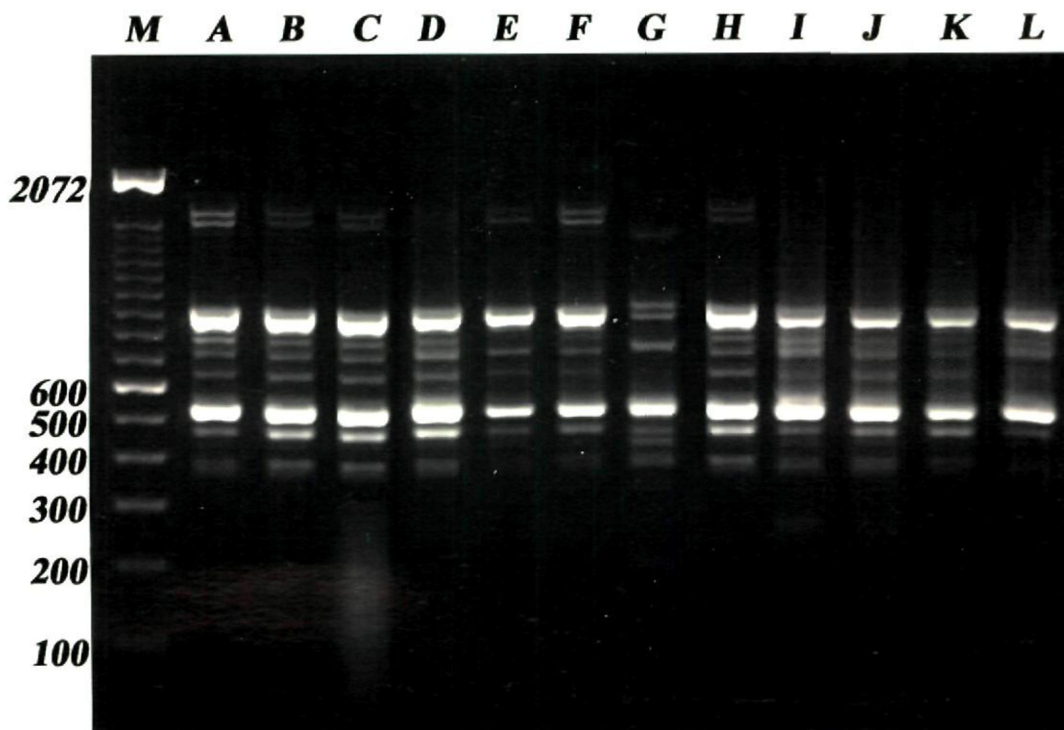


PLATE-16: RAPD PROFILE OF PLANTAGO 09

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

PLATE-17: RAPD PROFILE OF PLANTAGO 10

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

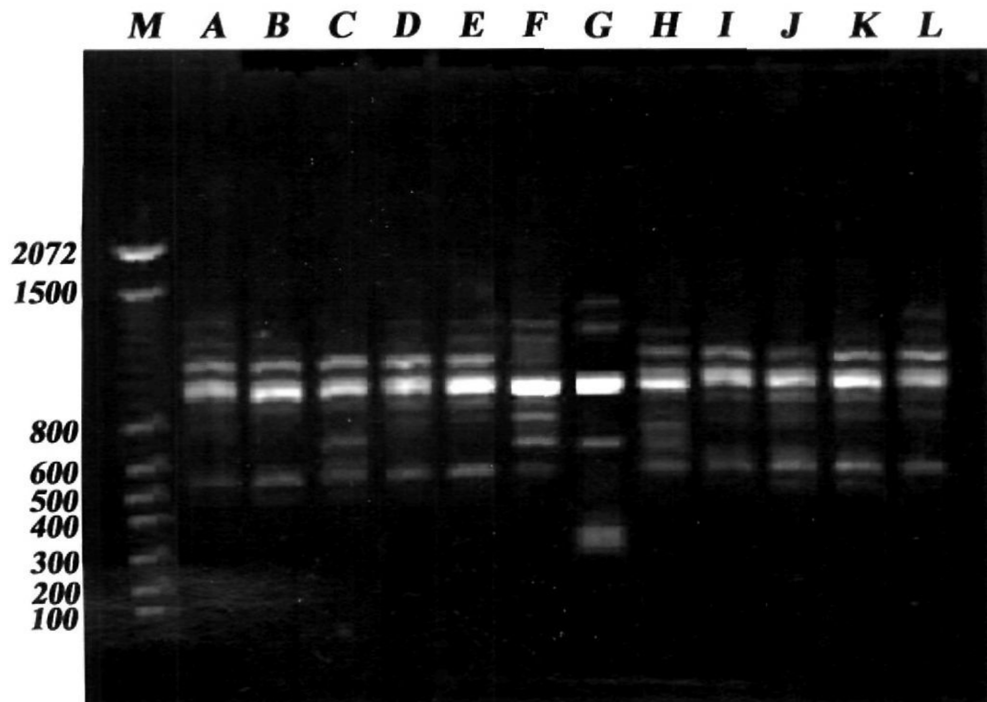


PLATE-18: RAPD PROFILE OF BG50

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

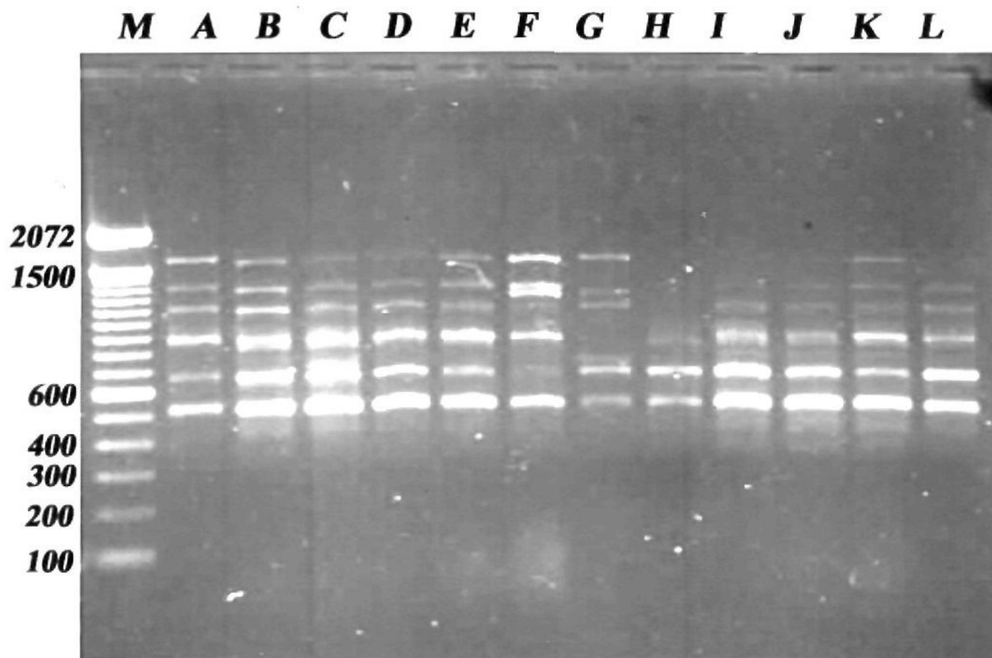


PLATE-19: RAPD PROFILE OF BG51

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

APD, PIC and Hav are shown in Table 4.6. The MAPD was 21% for 12 RAPD primers. The overall lowest genetic distance was found between genotypes DM1 and JI56 (0.099), while, the highest genetic distance was observed for genotypes DM1 and DM3 (0.472) (Table 4.12.1).

Table 4.12.1 : Combined matrix for Bs (Upper Diagonal) and Dxy (Lower Diagonal)

RAPD:(12 Primers)

	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	-	0.915	0.895	0.893	0.782	0.833	0.716	0.665	0.821	0.808	0.869	0.836
DM1	0.129	-	0.826	0.824	0.757	0.814	0.675	0.697	0.776	0.813	0.868	0.819
JI10	0.117	0.100	-	0.831	0.748	0.808	0.740	0.638	0.763	0.767	0.852	0.804
JI56	0.213	0.099	0.200	-	0.840	0.823	0.747	0.694	0.833	0.833	0.846	0.843
JI53	0.153	0.350	0.228	0.215	-	0.856	0.737	0.674	0.769	0.768	0.772	0.792
JI42	0.196	0.248	0.238	0.211	0.158	-	0.723	0.713	0.770	0.796	0.834	0.838
DM3	0.370	0.472	0.325	0.321	0.360	0.325	-	0.639	0.736	0.738	0.743	0.690
DRP46	0.198	0.258	0.244	0.162	0.337	0.258	0.397	-	0.729	0.751	0.693	0.706
DM2	0.214	0.302	0.266	0.244	0.310	0.291	0.372	0.219	-	0.913	0.814	0.805
JI65	0.243	0.238	0.284	0.243	0.303	0.266	0.365	0.275	0.100	-	0.882	0.845
DM4	0.106	0.181	0.178	0.184	0.313	0.178	0.358	0.298	0.238	0.125	-	0.889
EC124345	0.204	0.243	0.240	0.188	0.264	0.181	0.403	0.225	0.264	0.181	0.138	-

4.13 CAPS:

The profiles generated by two restriction enzymes in combination with 5 RAPD primers each are shown in the plates 20 to 29. Genomic DNA, which was amplified using random primer Plantago 01 generated the DNA fragments with in the range 300 to 1000 base pair. In *EcoR*I and *Rsa*I CAPS the profile generated ranged from 450 to 850 base pairs. The fragment of 500 base pair, present in RAPD, disappeared in *EcoR*I and *Rsa*I CAPS. While bands of 550 and 650 base pairs in DRP46, present in RAPD, disappeared in CAPS. Band of 650 base pair in genotype

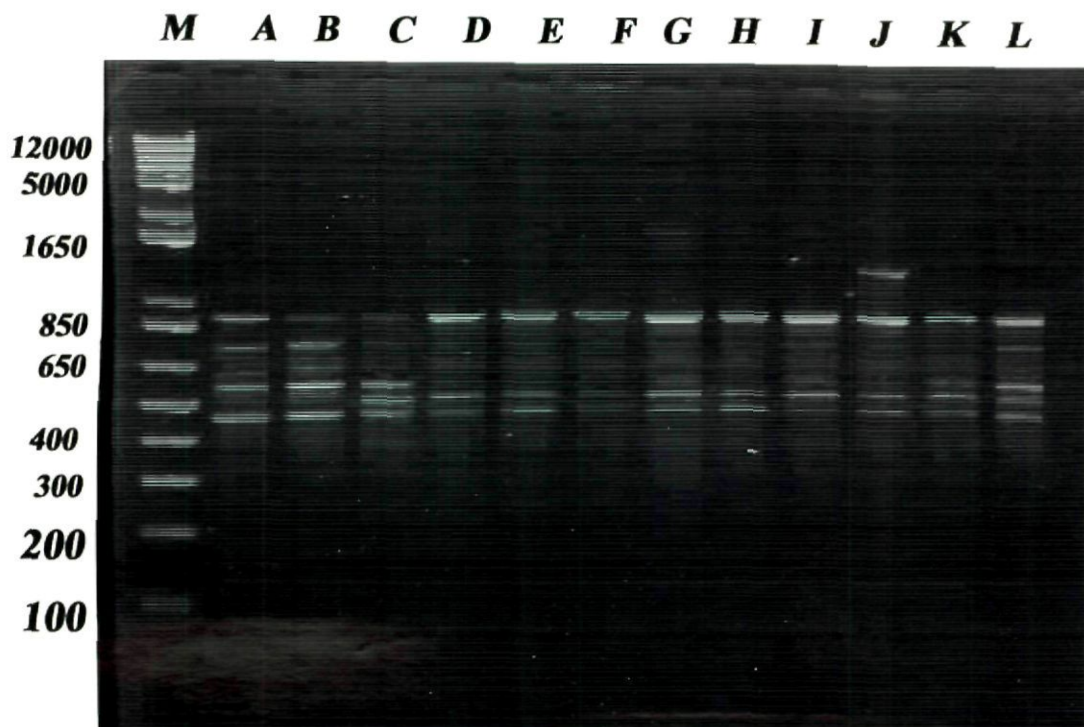


PLATE-20: EcoRI CAPS GENERATED FROM PLANTAGO 01

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

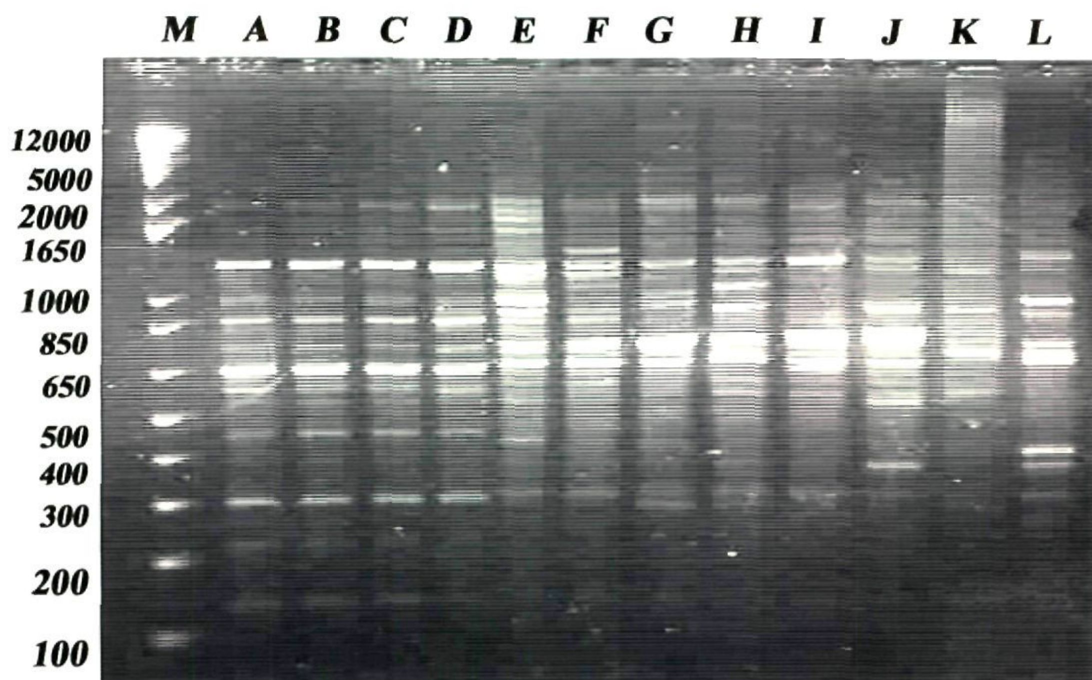


PLATE-21:EcoRI CAPS GENERATED FROM PLANTAGO 02

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

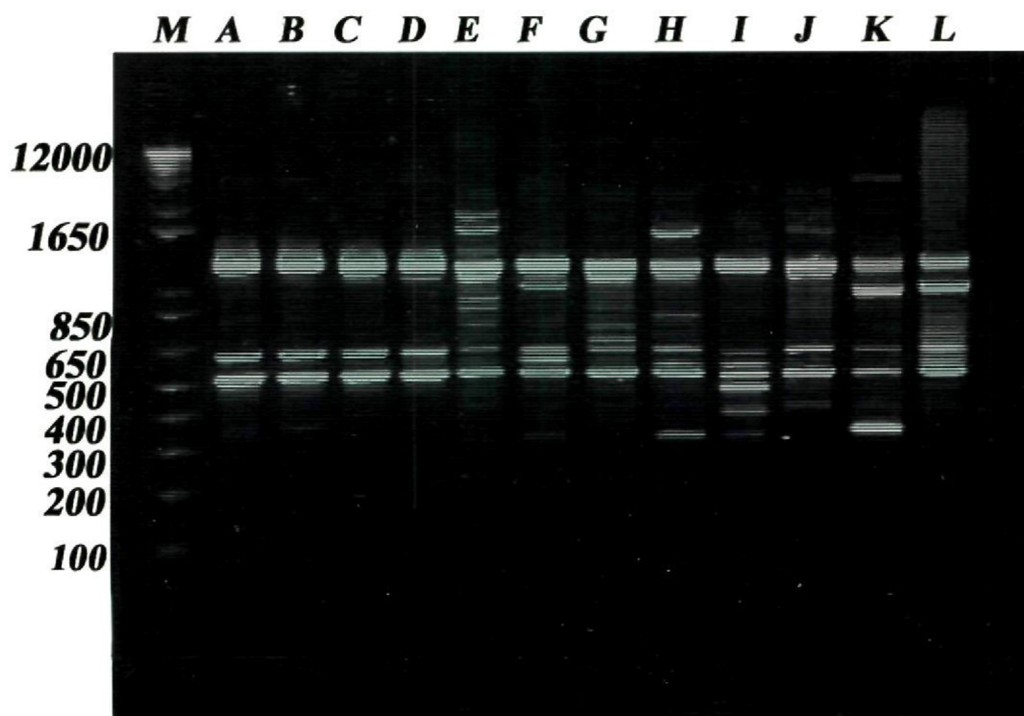


PLATE-22: EcoRI CAPS GENERATED FROM PLANTAGO 03

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

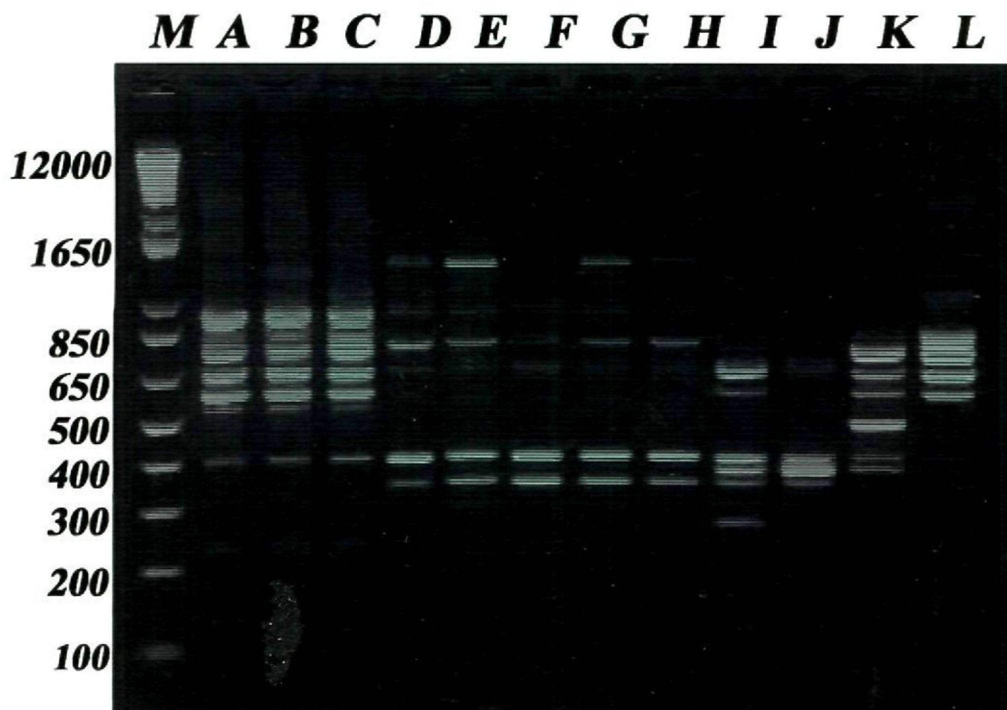


PLATE-23: EcoRI CAPS GENERATED FROM BG50

M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345

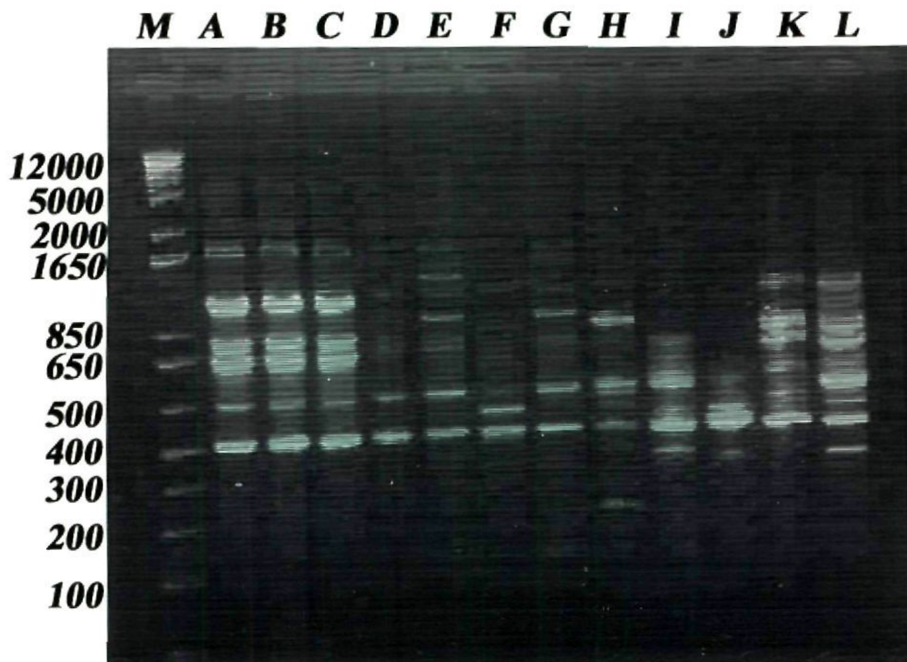


PLATE-24: EcoRI CAPS GENERATED FROM BG51

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

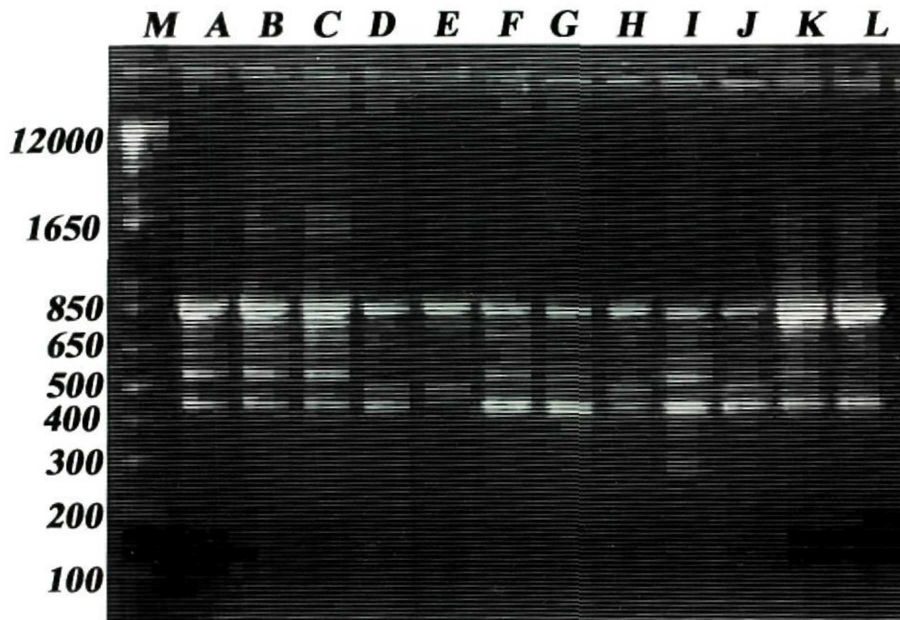


PLATE-25: RsaI CAPS GENERATED FROM PLANTAGO 01

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

PLATE-26: RsaI CAPS GENERATED FROM PLANTAGO 02

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

PLATE-27: RsaI CAPS GENERATED FROM PLANTAGO 03

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42;
G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

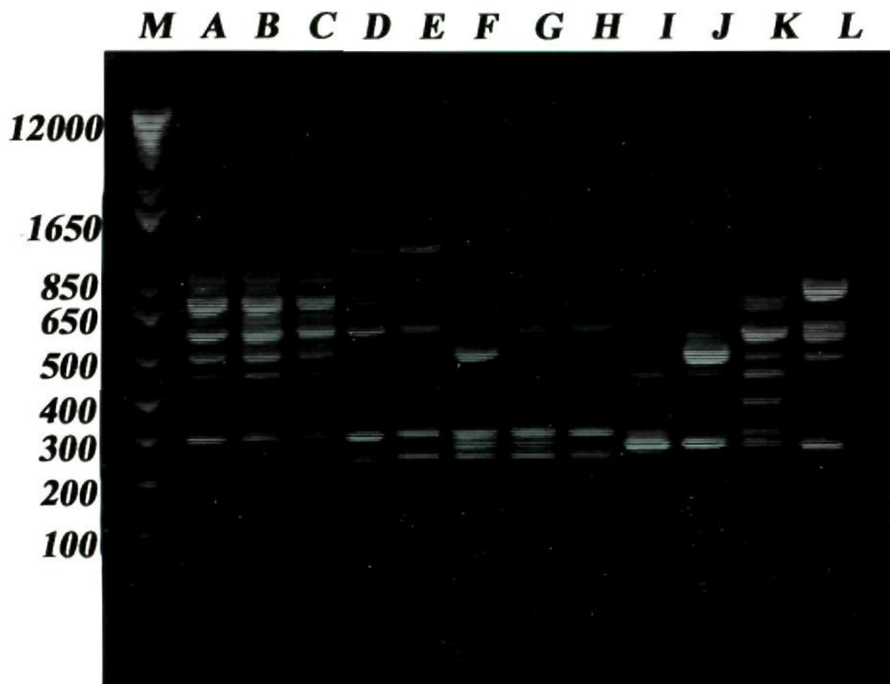


PLATE-28: RsaI CAPS GENERATED FROM BG50

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

PLATE-29: RsaI CAPS GENERATED FROM BG51

(M=1kb plus ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

DM3 was present in RAPD but disappeared during CAPS. This indicates that these fragments harbored the restriction sites for these enzymes.

CAPS profile generated by Plantago 03 showed complete disappearance of 2000 bp and 1650 base pair DNA fragments generated in the RAPD and produced a single band of approximately 1500 base pair. The bands were present in RAPD pattern got disappeared in *EcoR*I and *Rsa*I CAPS (ranged from 450 to 850 base pairs) and two bands were generated in 400-500 base pair range. In DM2 one additional band of 380 base pair was generated. While in *EcoR*I CAPS, one more additional band of 1000 base pair was generated in JI42, DM4 and EC124345.

The band, present in RAPD profile generated with primer BG50, of approximately 1000 base pair disappeared in *EcoR*I and *Rsa*I CAPS and produced four low molecular weight bands ranging from 500 to 600 base pairs. In *Rsa*I CAPS all the four bands were found to be present in GI2, DM1, JI10, DM4 and EC124345 and were absent in rest of the genotypes. Only one band of 550 base pair was present in JI42 and JI65. A 300 base pair band was also generated in all except EC124345, DM2 and DRP46 genotypes. Instead of 300 base pair one band of less than 300 base pair (~280 base pair) was generated in JI42, DM3, DM2, JI65 and EC124345 genotypes.

One band of 280 base pair was found to be present in JI56, JI53, JI42, DM3, DRP46 and DM2 genotypes in the *EcoR*I CAPS profile generated by BG50. A 300 base pair band was also generated in all the genotypes except EC124345, which was absent in RAPD profile.

In RAPD profile of BG51, the bands were ranged from 500 to 1500 base pair but in CAPS they ranged from 300 to 850 base pair. The band of 500 base pair, which

was present in RAPD p-ofile, disappeared in *Rsa*I CAPS and produced a band of 400 base pair in genotypes JI56, JI42, DM3, DRP46 and DM2.

The combined totals of 1440 bands were scored, of which 501 (35%) were polymorphic. The range of marker sizes varied from 250 to 2000 base pairs. The range of scorable bands were from 3-22. The combined matrix of Bs (upper diagonal) and Dxy (lower diagonal) is represented in Table 4.13.1. The primer wise results regarding marker size, scorable bands, total alleles, polymorphic alleles, total bands, total polymorphic bands and Bs, Dxy, PD, APD, PIC, Hav are represented in table 4.5 and 4.6, respectively. The overall average values of PIC and Hav were 0.442 and 0.818, whereas, the MAPD was found to be 33%. The overall lowest genetic distance was found in between genotypes JI10 and DM1 (0.130), while, the highest genetic distance was found between JI10 and JI53 (0.940) genotypes (Table 4.13.1).

Table 4.13.1 : Combined matrix for Bs (Upper Diagonal) and Dxy (Lower Diagonal)

CAPS: (Two Restriction Enzymes and Five RAPD Primers each)

	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	-	0.984	0.970	0.688	0.585	0.637	0.610	0.577	0.629	0.528	0.687	0.737
DM1	0.016	-	0.987	0.708	0.547	0.623	0.608	0.560	0.621	0.530	0.682	0.741
JI10	0.033	0.013	-	0.722	0.559	0.613	0.596	0.548	0.619	0.517	0.670	0.724
JI56	0.408	0.377	0.357	-	0.809	0.669	0.786	0.764	0.559	0.628	0.602	0.655
JI53	0.625	0.600	0.580	0.217	-	0.633	0.753	0.765	0.502	0.607	0.534	0.593
JI42	0.516	0.498	0.516	0.407	0.475	-	0.736	0.731	0.647	0.641	0.637	0.673
DM3	0.562	0.509	0.530	0.278	0.299	0.319	-	0.769	0.62	0.644	0.612	0.701
DRP46	0.623	0.583	0.602	0.276	0.294	0.316	0.282	-	0.582	0.697	0.634	0.662
DM2	0.554	0.501	0.504	0.588	0.702	0.466	0.501	0.570	-	0.753	0.657	0.665
JI65	0.704	0.669	0.695	0.485	0.514	0.450	0.452	0.367	0.292	-	0.687	0.705
DM4	0.414	0.383	0.400	0.585	0.661	0.509	0.579	0.528	0.424	0.377	-	0.814
EC124345	0.327	0.314	0.346	0.443	0.507	0.398	0.364	0.415	0.419	0.398	0.207	-

4.14 ISSR:

The fingerprints generated from three ISSR primers viz. UBC887, UBC889 and UBC891 are shown in Plates 30, 31 and 32, respectively. The combined total bands scored were 372 of which 157 (42%) were polymorphic. All the primers produced scorable bands in the range of 2-12, with in 200-2810 base pair range in different genotypes. The overall matrix for all the three UBC primers for Bs (upper diagonal) and Dxy (lower diagonal) is shown in Table 4.14.1. The information regarding marker size, scorable bands, total alleles, polymorphic alleles, total bands, total polymorphic bands is shown in Table 4.5. Individual primer wise data for Bs, Dxy, PD, APD, PIC and Hav is represented in Table 4.6. The pooled PIC, Hav and MAPD were calculated and these were 0.481, 0.754 and 38%, respectively. The overall lowest genetic distance was between GI2 and JI56 (0.203) and the highest genetic distance was found between DM3 and EC124345 (0.830) (Table 4.14.1).

Table 4.14.1 : Combined matrix for Bs (Upper Diagonal) and Dxy (Lower Diagonal)

ISSR:(3UBC Primers)

	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	-	0.580	0.597	0.810	0.657	0.797	0.653	0.540	0.453	0.497	0.533	0.473
DM1	0.487	-	0.607	0.660	0.573	0.533	0.497	0.487	0.583	0.500	0.547	0.610
JI10	0.587	0.540	-	0.660	0.933	0.730	0.600	0.587	0.557	0.540	0.580	0.573
JI56	0.203	0.390	0.517	-	0.723	0.703	0.657	0.710	0.623	0.643	0.697	0.573
JI53	0.423	0.610	0.067	0.353	-	0.790	0.670	0.657	0.637	0.620	0.660	0.540
JI42	0.223	0.663	0.300	0.357	0.233	-	0.607	0.480	0.500	0.490	0.537	0.463
DM3	0.507	0.760	0.567	0.477	0.403	0.540	-	0.533	0.507	0.590	0.640	0.453
DRP46	0.563	0.727	0.573	0.347	0.410	0.777	0.607	-	0.843	0.803	0.740	0.660
DM2	0.717	0.560	0.597	0.483	0.433	0.660	0.627	0.170	-	0.807	0.800	0.640
JI65	0.663	0.673	0.617	0.463	0.453	0.680	0.510	0.213	0.213	-	0.703	0.663
DM4	0.623	0.620	0.593	0.407	0.430	0.627	0.453	0.303	0.230	0.410	-	0.513
EC124345	0.743	0.487	0.523	0.603	0.593	0.787	0.830	0.487	0.487	0.467	0.683	-

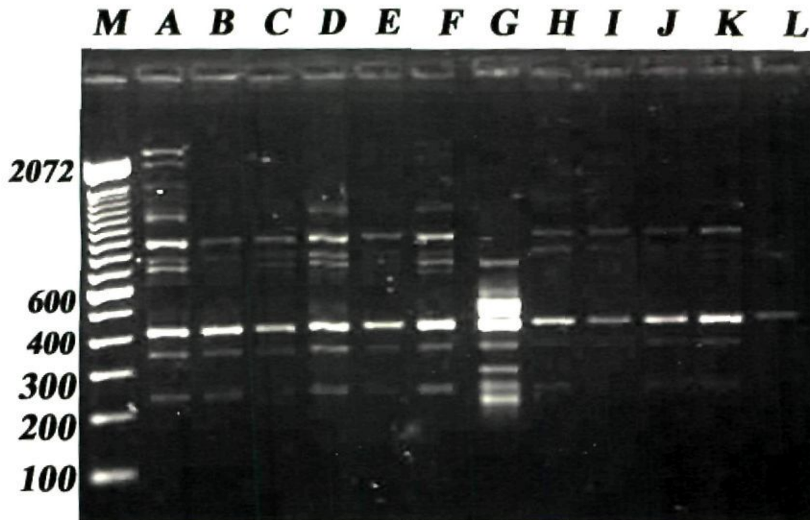


PLATE-30: ISSR PROFILE GENERATED FROM UBC887

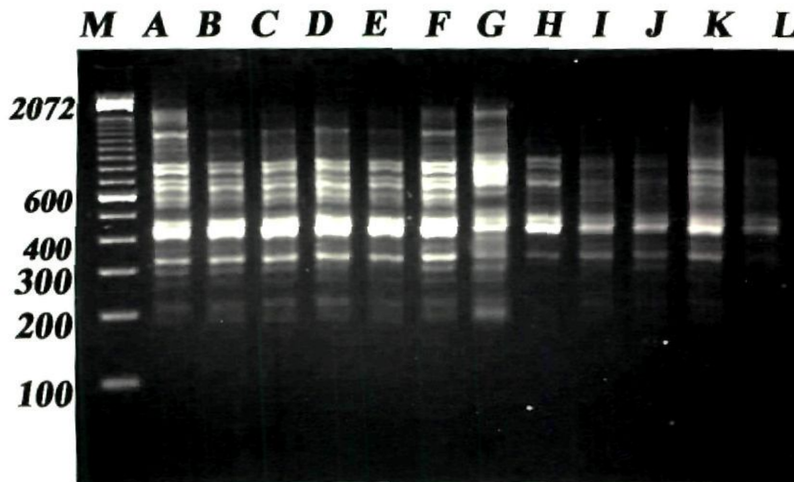


PLATE-31: ISSR PROFILE GENERATED FROM UBC889

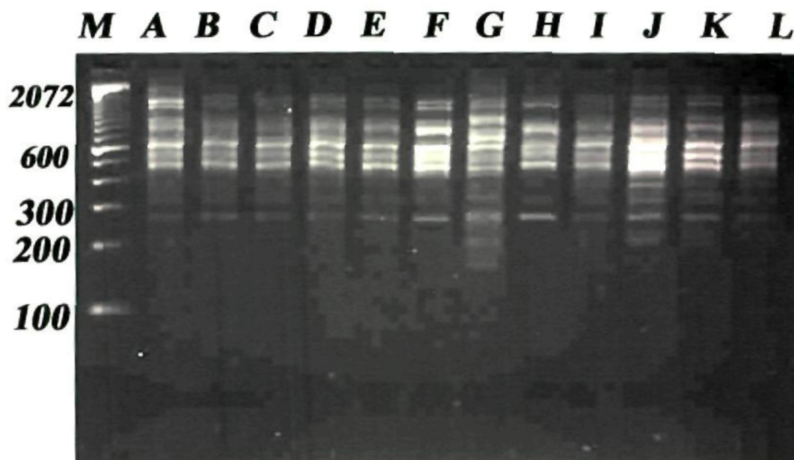


PLATE-32:ISSR PROFILE GENERATED FROM UBC891

(M=100base pair ladder;A=GI2;B=DM1;C=JI10;D=JI56;E=JI53;F=JI42; G=DM3;H=DRP46;I=DM2;J=JI65;K=DM4;L=EC124345)

4.15 AFLP:

The combined total number of bands generated by AFLP (Plate 33), was 1648 of which 700 (42%) were polymorphic. The pooled matrix of three AFLP primer pairs for Bs (upper diagonal) and Dxy (lower diagonal) is shown in Table 4.15.1. The primer wise results showing scorable bands, marker size, total alleles, polymorphic alleles, total bands, polymorphic bands and Bs, Dxy, PD, APD, PIC and Hav are presented in Table 4.5 and 4.6, respectively. The combined PIC, Hav and MAPD were 0.796, 0.752 and 16%. The overall lowest genetic distance was found between GI2 and DM1 (0.230), whereas, the highest genetic distance was found between genotypes JI56 and JI53 (0.349) (Table 4.15.1).

Table 4.15.1 : Combined matrix for Bs (Upper Diagonal) and Dxy (Lower Diagonal)

AFLP: (3 Primer Pairs)

	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	-	0.977	0.913	0.809	0.770	0.866	0.809	0.817	0.766	0.868	0.881	0.869
DM1	0.023	-	0.936	0.829	0.787	0.869	0.819	0.819	0.785	0.881	0.893	0.882
JI10	0.089	0.066	-	0.872	0.769	0.894	0.817	0.816	0.810	0.894	0.912	0.912
JI56	0.210	0.152	0.138	-	0.690	0.804	0.735	0.745	0.760	0.823	0.834	0.844
JI53	0.228	0.288	0.244	0.349	-	0.800	0.905	0.904	0.694	0.793	0.794	0.766
JI42	0.143	0.140	0.114	0.220	0.210	-	0.863	0.863	0.765	0.887	0.904	0.884
DM3	0.206	0.192	0.191	0.295	0.099	0.141	-	0.967	0.739	0.855	0.856	0.829
DRP46	0.197	0.192	0.192	0.282	0.099	0.141	0.033	-	0.751	0.845	0.862	0.835
DM2	0.266	0.242	0.215	0.279	0.347	0.274	0.294	0.278	-	0.790	0.809	0.835
JI65	0.137	0.124	0.111	0.195	0.218	0.124	0.152	0.162	0.239	-	0.955	0.929
DM4	0.124	0.111	0.101	0.181	0.218	0.103	0.149	0.144	0.217	0.047	-	0.956
EC124345	0.137	0.117	0.094	0.172	0.246	0.128	0.175	0.169	0.183	0.074	0.044	-

The technique wise data were pooled on the basis of presence or absence of bands and the combined data were subjected to the phylogenetic as well as network analysis. The data set were bootstrapped to generate 100 multiple data set, which were subjected to parsimony analysis and a consensus tree was constructed (Figures 4.5, 4.6, 4.7, 4.8). The technique wise pooled data in the binary form (0, 1) was subjected

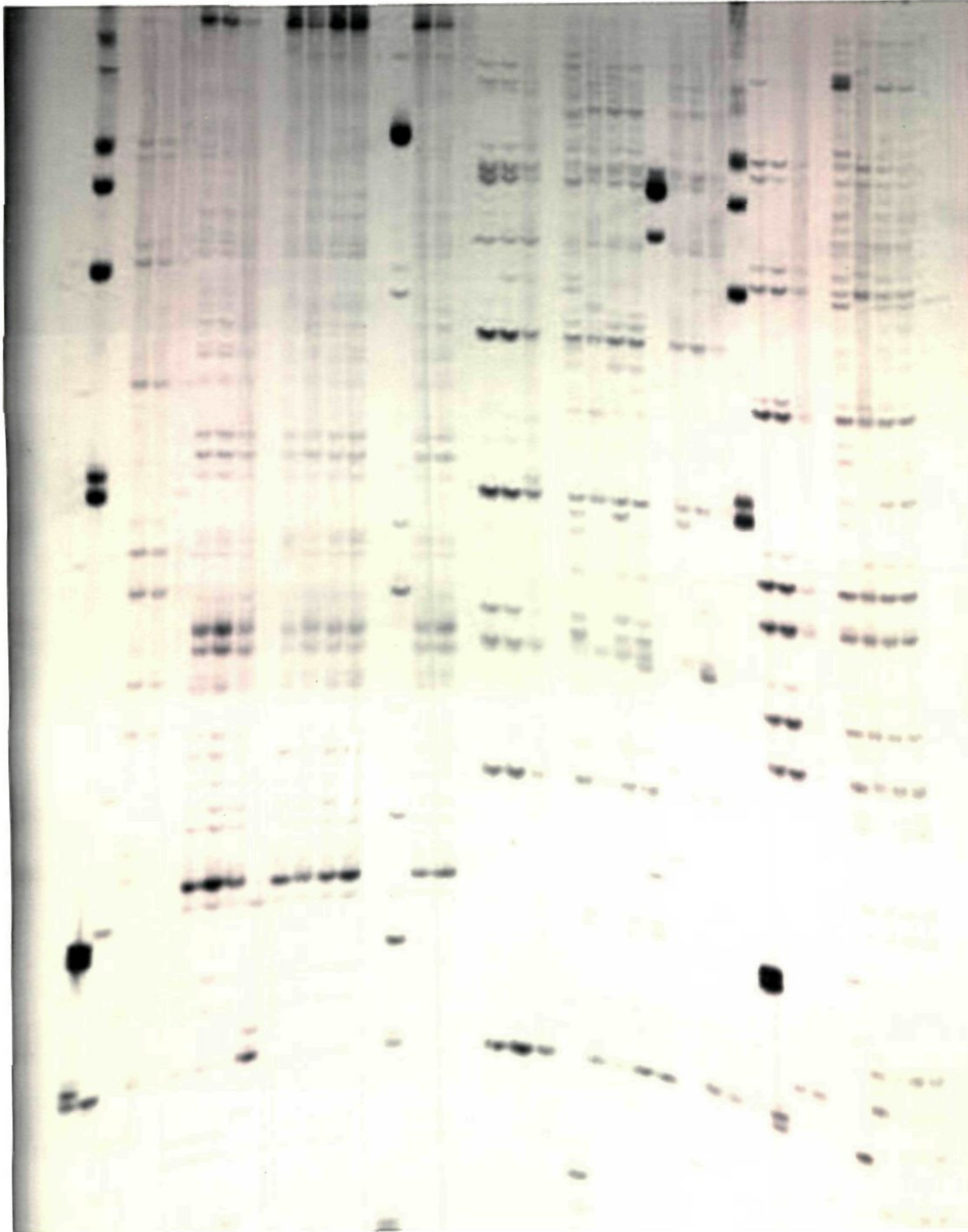


PLATE 33 AFLP PROFILE

01 TO 12: PRIMER SET 1
13 TO 24: PRIMER SET 2
25 TO 36: PRIMER SET 3

01-12: GI2,DM1,JI10,JI56,JI53,JI42,DM3,DRP46,DM2,JI65,EC124345
13-24: GI2,DM1,JI10,JI56,JI53,JI42,DM3,DRP46,DM2,JI65,EC124345
25-36: GI2,DM1,JI10,JI56,JI53,JI42,DM3,DRP46,DM2,JI65,EC124345

to calculate Squared Euclidean Distances (Table 4.12.2, 4.13.2, 4.14.2 and 4.15.2) and the dendrograms were obtained (Figures 4.5, 4.6, 4.7, and 4.8) and compared with Phylogenetic tree and network. (Figures 4.9, 4.10, 4.11 and 4.12).

Table 4.12.2 : Squared Euclidean Distance for RAPD pooled data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.00	8.00	12.00	12.00	21.00	22.00	36.00	35.00	21.00	24.00	14.00	21.00
DM1	8.00	0.00	18.00	18.00	25.00	24.00	40.00	35.00	25.00	24.00	14.00	21.00
JI10	12.00	18.00	0.00	20.00	25.00	26.00	32.00	39.00	27.00	30.00	18.00	27.00
JI56	12.00	18.00	20.00	0.00	17.00	22.00	32.00	33.00	19.00	22.00	16.00	19.00
JI53	21.00	25.00	25.00	17.00	0.00	15.00	31.00	34.00	26.00	29.00	23.00	24.00
JI42	22.00	24.00	26.00	22.00	15.00	0.00	34.00	33.00	29.00	30.00	22.00	21.00
DM3	36.00	40.00	32.00	32.00	31.00	34.00	0.00	45.00	33.00	36.00	32.00	39.00
DRP46	35.00	35.00	39.00	33.00	34.00	33.00	45.00	0.00	30.00	33.00	33.00	32.00
DM2	21.00	25.00	27.00	19.00	26.00	29.00	33.00	30.00	0.00	11.00	19.00	22.00
JI65	24.00	24.00	30.00	22.00	29.00	30.00	36.00	33.00	11.00	0.00	14.00	19.00
DM4	14.00	14.00	18.00	16.00	23.00	22.00	32.00	33.00	19.00	14.00	0.00	13.00
EC124345	21.00	21.00	27.00	19.00	24.00	21.00	39.00	32.00	22.00	19.00	13.00	0.00

This is a dissimilarity matrix

Table 4.13.2 : Squared Euclidean Distance for CAPS pooled data

Proximity matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.00	3.00	5.00	38.00	65.00	56.00	52.00	59.00	51.00	54.00	43.00	35.00
DM1	3.00	0.00	2.00	35.00	64.00	55.00	51.00	56.00	48.00	53.00	40.00	36.00
JI10	5.00	2.00	0.00	33.00	62.00	57.00	53.00	58.00	48.00	55.00	42.00	38.00
JI56	38.00	35.00	33.00	0.00	29.00	46.00	28.00	31.00	51.00	44.00	51.00	45.00
JI53	65.00	64.00	62.00	29.00	0.00	55.00	31.00	40.00	66.00	51.00	66.00	56.00
JI42	56.00	55.00	57.00	46.00	55.00	0.00	32.00	39.00	47.00	44.00	49.00	47.00
DM3	52.00	51.00	53.00	28.00	31.00	32.00	0.00	27.00	45.00	36.00	51.00	37.00
DRP46	59.00	56.00	58.00	31.00	40.00	39.00	27.00	0.00	50.00	35.00	46.00	42.00
DM2	51.00	48.00	48.00	51.00	66.00	47.00	45.00	50.00	0.00	25.00	42.00	40.00
JI65	54.00	53.00	55.00	44.00	51.00	44.00	36.00	35.00	25.00	0.00	35.00	31.00
DM4	43.00	40.00	42.00	51.00	66.00	49.00	51.00	46.00	42.00	35.00	0.00	24.00
EC124345	35.00	36.00	38.00	45.00	56.00	47.00	37.00	42.00	40.00	31.00	24.00	0.00

This is a dissimilarity matrix

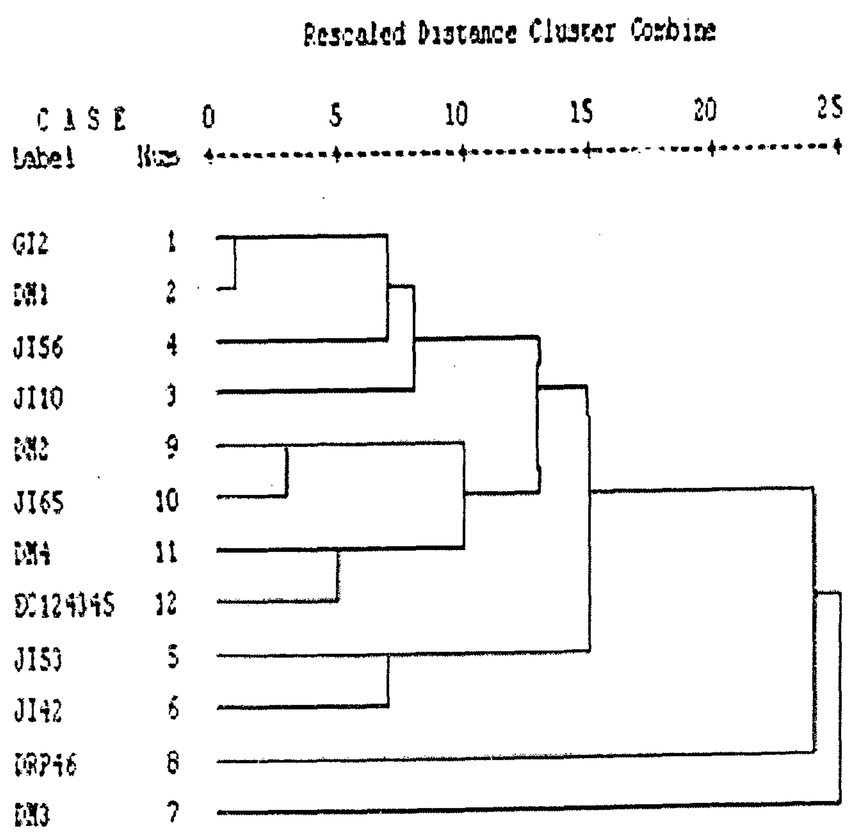
Table 4.14.2 : Squared Euclidean Distance for ISSR pooled data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.00	15.00	14.00	9.00	13.00	9.00	14.00	19.00	21.00	21.00	19.00	19.00
DM1	15.00	0.00	9.00	10.00	10.00	14.00	15.00	14.00	10.00	14.00	12.00	10.00
JI10	14.00	9.00	0.00	9.00	1.00	9.00	12.00	11.00	11.00	13.00	11.00	11.00
JI56	9.00	10.00	9.00	0.00	8.00	12.00	13.00	10.00	12.00	12.00	10.00	12.00
JI53	13.00	10.00	1.00	8.00	0.00	8.00	11.00	10.00	10.00	12.00	10.00	12.00
JI42	9.00	14.00	9.00	12.00	8.00	0.00	15.00	18.00	16.00	18.00	16.00	16.00
DM3	14.00	15.00	12.00	13.00	11.00	15.00	0.00	17.00	17.00	15.00	13.00	15.00
DRP46	19.00	14.00	11.00	10.00	10.00	18.00	17.00	0.00	4.00	6.00	8.00	8.00
DM2	21.00	10.00	11.00	12.00	10.00	16.00	17.00	4.00	0.00	6.00	6.00	8.00
JI65	21.00	14.00	13.00	12.00	12.00	18.00	15.00	6.00	6.00	0.00	10.00	8.00
DM4	19.00	12.00	11.00	10.00	10.00	16.00	13.00	8.00	6.00	10.00	0.00	12.00
EC124345	19.00	10.00	11.00	12.00	12.00	16.00	15.00	8.00	8.00	8.00	12.00	0.00

This is a dissimilarity matrix

DENDROGRAM USING AVERAGE LINKAGE (BETWEEN GROUPS)



PHYLOGENETIC TREE

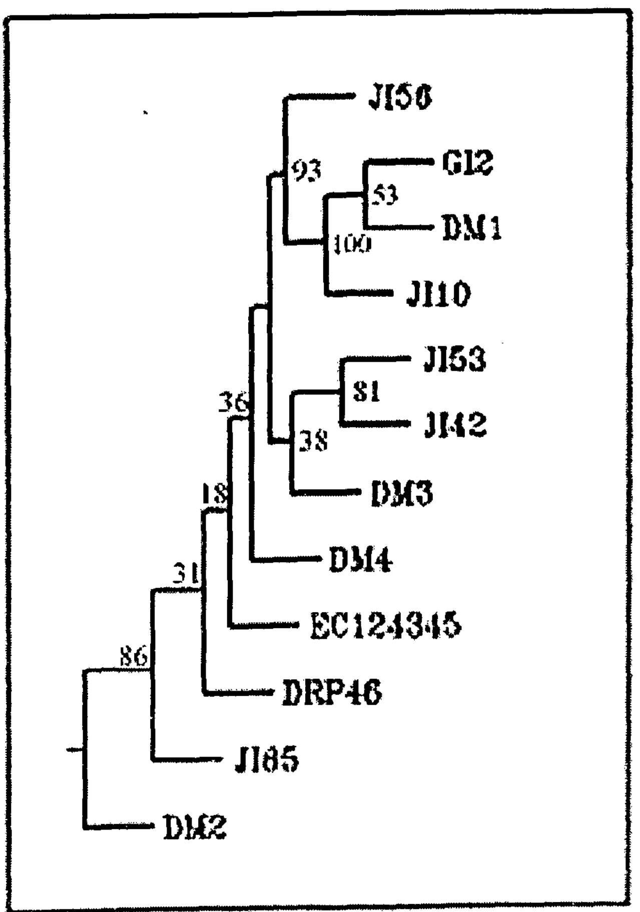
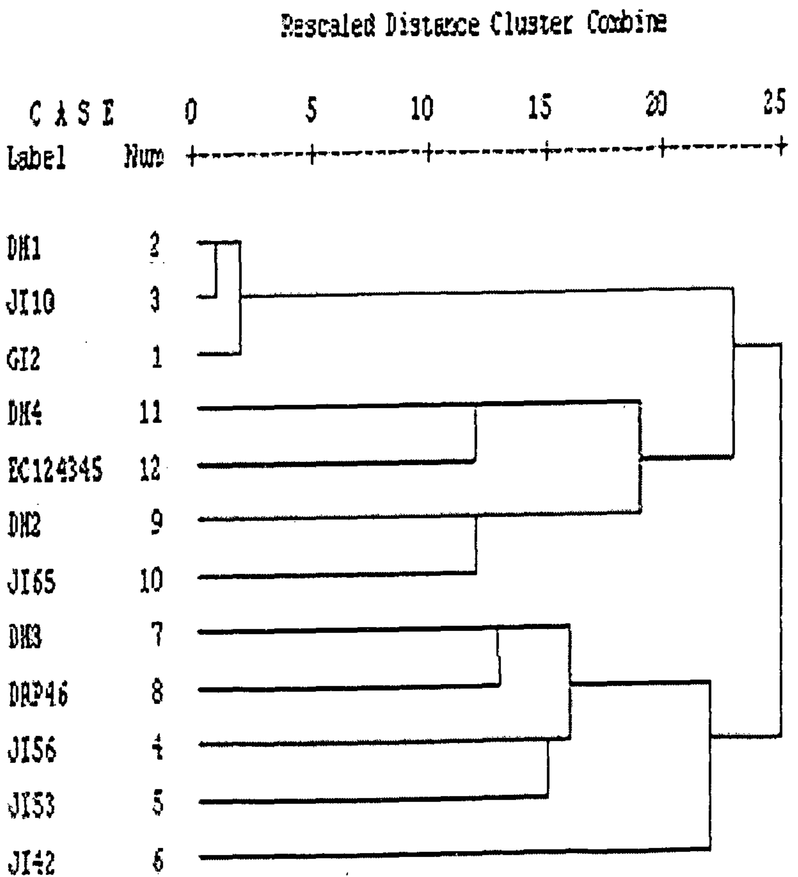


FIGURE 4.5 : DENDROGRAM FROM RAPD POOLED DATA

DENDROGRAM USING AVERAGE LINKAGE
(BETWEEN GROUPS)



PHYLOGENETIC TREE

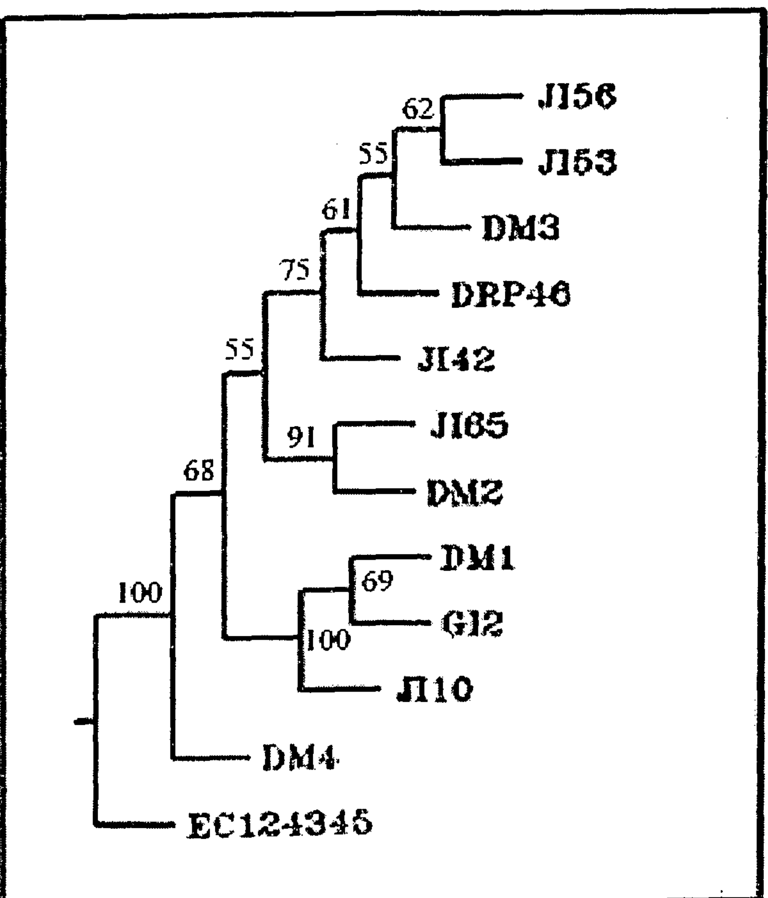
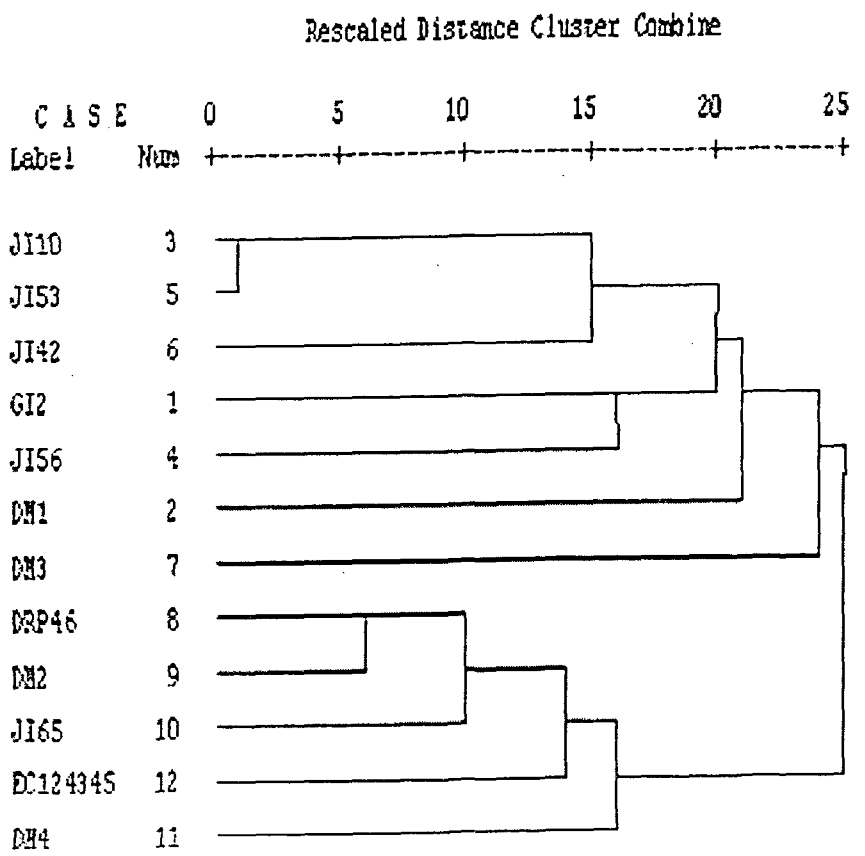


FIGURE 4.6: DENDROM FROM POOLED CAPS DATA

DENDROGRAM USING AVERAGE LINKAGE
(BETWEEN GROUPS)



PHYLOGENETIC TREE

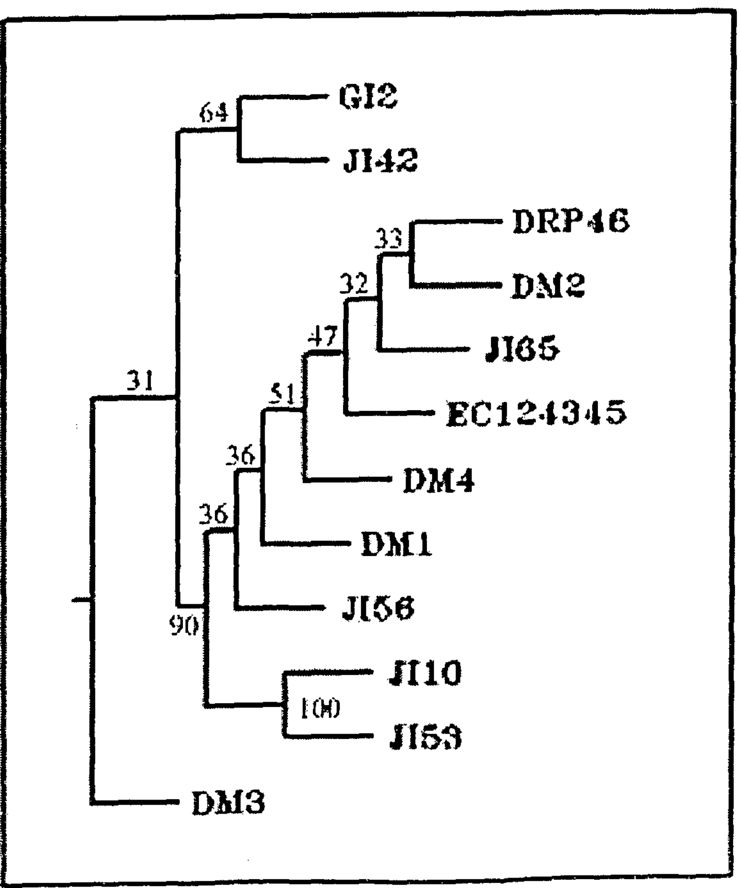
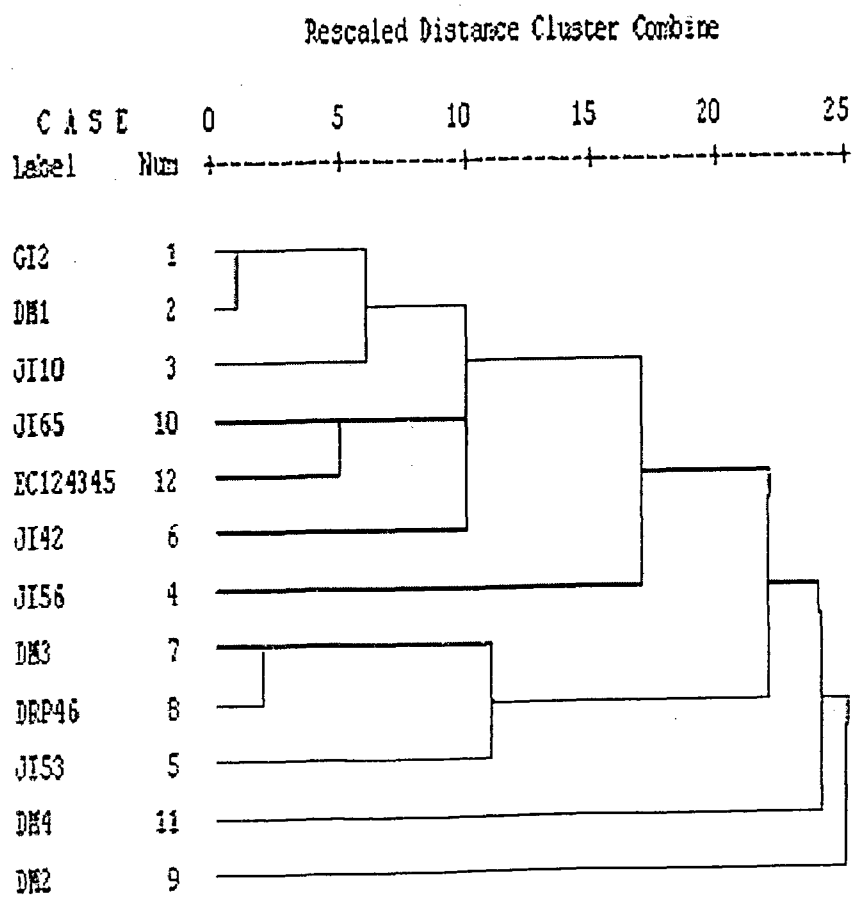


FIGURE 4.7: DENDROGRAM FROM POOLED ISSR DATA

DENDROGRAM USING AVERAGE LINKAGE
(BETWEEN GROUP)



PHYLOGENETIC TREE

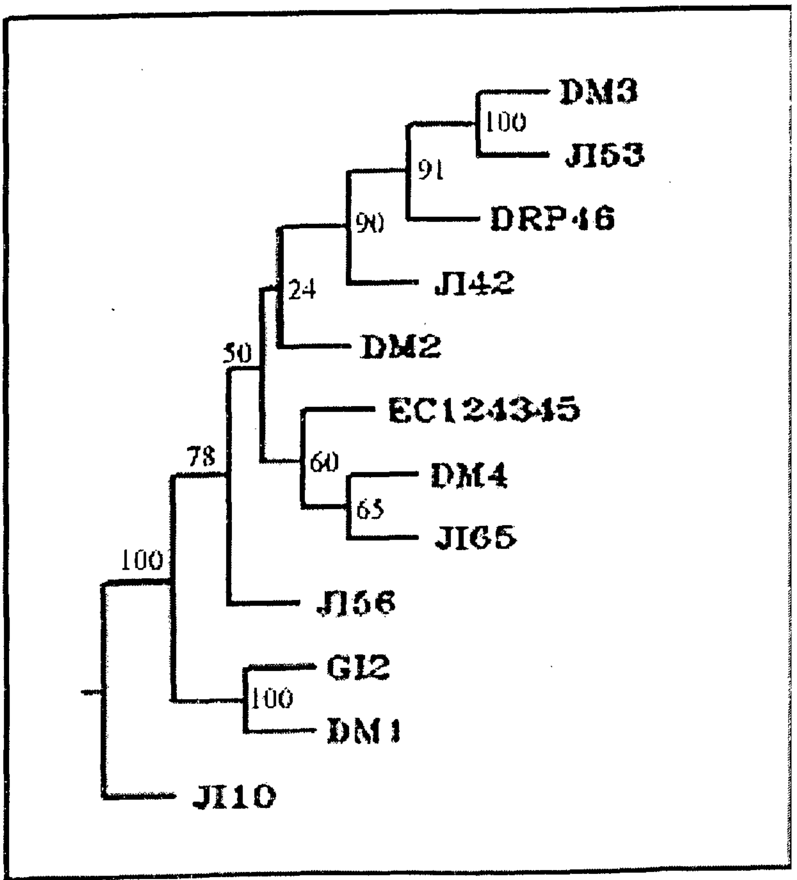


FIGURE 4.8 DENDROGRAM FROM AFLP POOLED DATA

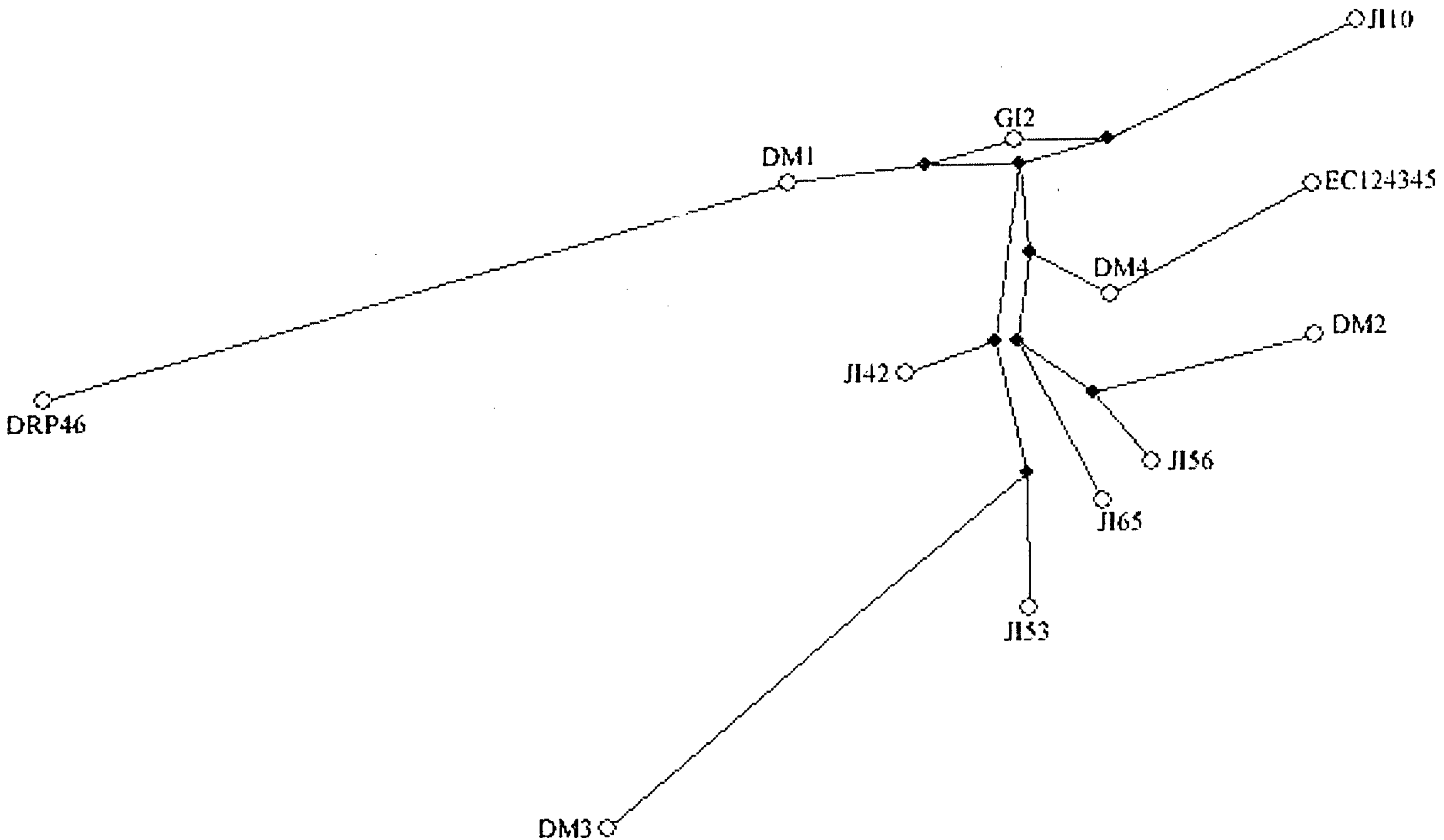


FIGURE 4.9: NETWORK FROM POOLED RAPD DATA

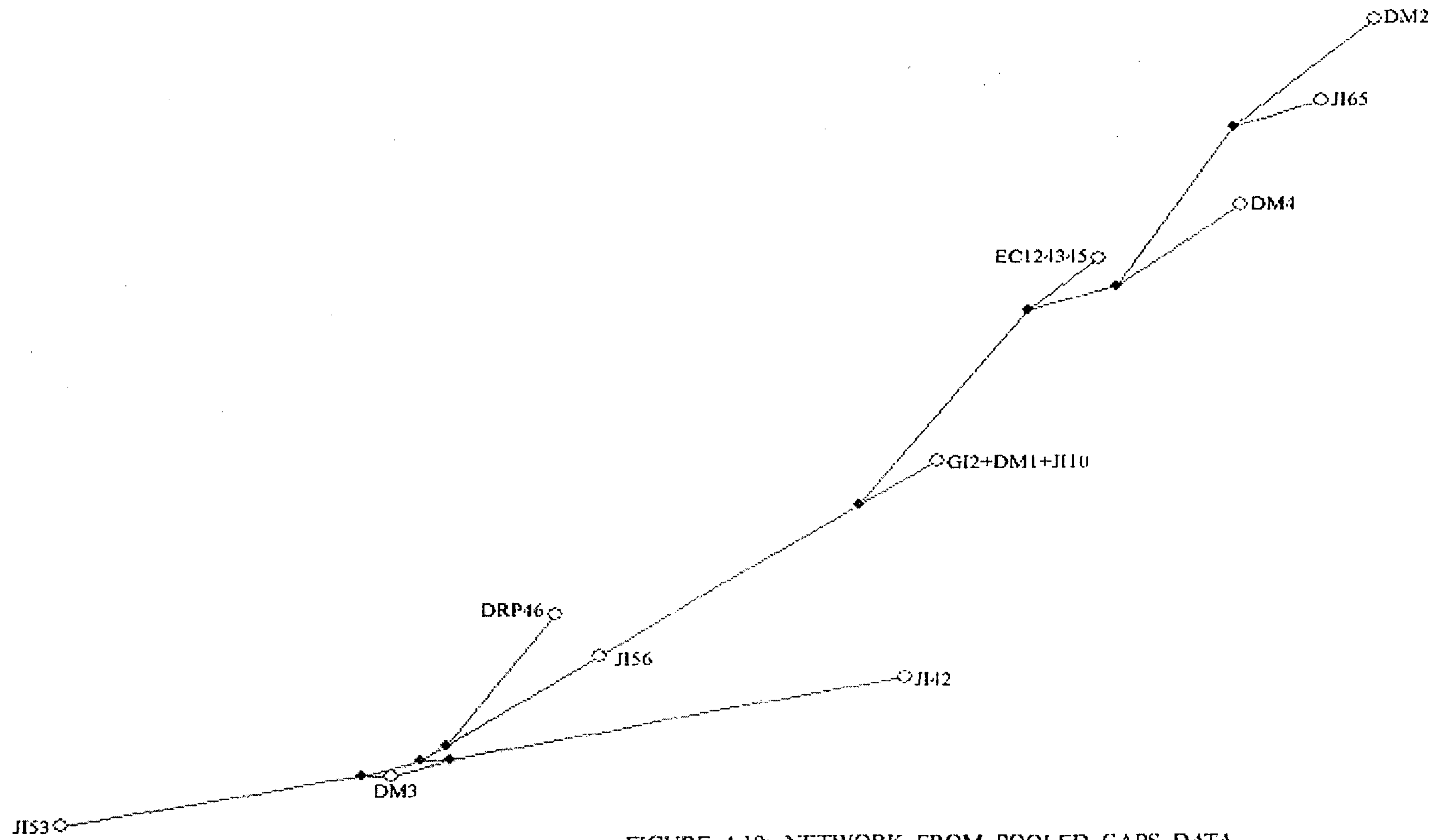


FIGURE 4.10: NETWORK FROM POOLED CAPS DATA

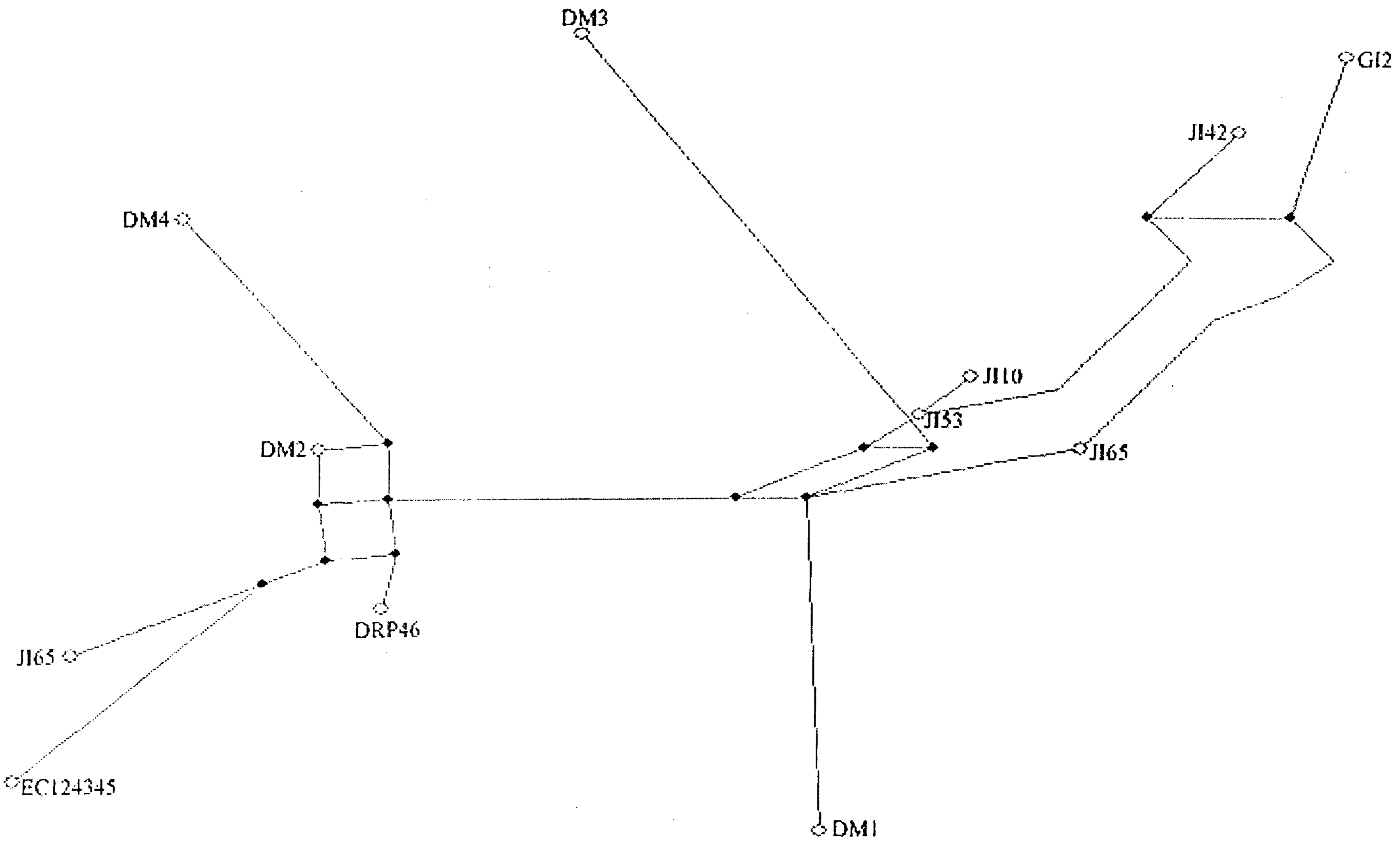


FIGURE 4.11: NETWORK FROM POOLED ISSR DATA

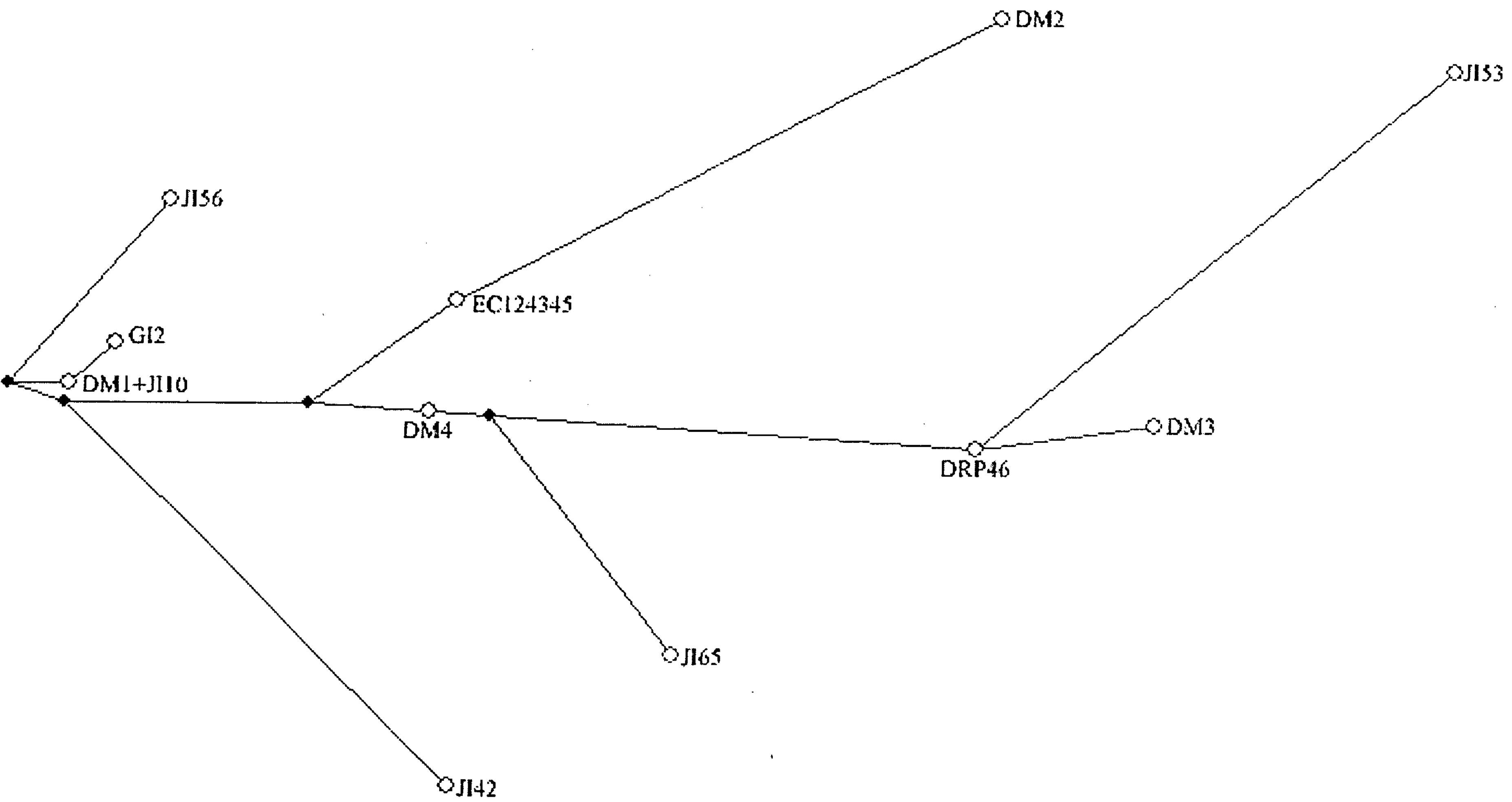


FIGURE 4.12: NETWORK FROM POOLED AFLP DATA

Table 4.15.2 : Squared Euclidean Distance for AFLP pooled data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.00	6.00	22.00	51.00	66.00	33.00	53.00	51.00	62.00	33.00	57.00	32.00
DM1	6.00	0.00	16.00	45.00	62.00	31.00	49.00	49.00	56.00	29.00	53.00	28.00
JI10	22.00	16.00	0.00	33.00	64.00	23.00	47.00	47.00	48.00	25.00	57.00	20.00
JI56	51.00	45.00	33.00	0.00	89.00	48.00	72.00	68.00	61.00	44.00	68.00	37.00
JI53	66.00	62.00	64.00	89.00	0.00	57.00	31.00	31.00	90.00	61.00	75.00	66.00
JI42	33.00	31.00	23.00	48.00	57.00	0.00	36.00	36.00	59.00	28.00	60.00	27.00
DM3	53.00	49.00	47.00	72.00	31.00	36.00	0.00	10.00	73.00	40.00	64.00	45.00
DRP46	51.00	49.00	47.00	68.00	31.00	36.00	10.00	0.00	69.00	42.00	64.00	43.00
DM2	62.00	56.00	48.00	61.00	90.00	59.00	73.00	69.00	0.00	55.00	81.00	42.00
JI65	33.00	29.00	25.00	44.00	61.00	28.00	40.00	42.00	55.00	0.00	44.00	17.00
DM4	57.00	53.00	57.00	68.00	75.00	60.00	64.00	64.00	81.00	44.00	0.00	47.00
EC124345	32.00	28.00	20.00	37.00	66.00	27.00	45.00	43.00	42.00	17.00	47.00	0.00

This is a dissimilarity matrix

4.16 OVERALL POOLED:

The combined total of 4348 bands (RAPD, CAPS, ISSR and AFLP) scored of which 1802 (41%) were polymorphic. A summary of the effectiveness of different markers is given in Table 4.7. The overall lowest genetic distance was found between GI2 and DM1 (0.139) and highest was between DM1 and DM3 (0.478) (Table 4.16.1).

Table 4.16.1 : Overall Pooled (RAPD, CAPS, ISSR and AFLP) matrix for Bs (Upper Diagonal) and Dxy (Lower Diagonal)

	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	-	0.885	0.870	0.827	0.753	0.782	0.701	0.608	0.662	0.673	0.775	0.747
DM1	0.167	-	0.882	0.799	0.727	0.714	0.663	0.591	0.697	0.678	0.781	0.786
JI10	0.223	0.193	-	0.814	0.815	0.767	0.686	0.613	0.696	0.688	0.791	0.780
JI56	0.258	0.259	0.289	-	0.806	0.752	0.719	0.680	0.686	0.724	0.783	0.746
JI53	0.351	0.445	0.297	0.287	-	0.763	0.764	0.706	0.658	0.705	0.747	0.702
JI42	0.261	0.385	0.283	0.277	0.290	-	0.734	0.643	0.628	0.655	0.727	0.688
DM3	0.416	0.490	0.411	0.338	0.293	0.348	-	0.710	0.634	0.690	0.710	0.646
DRP46	0.401	0.451	0.416	0.270	0.293	0.395	0.332	-	0.712	0.754	0.684	0.682
DM2	0.421	0.381	0.361	0.361	0.450	0.385	0.442	0.304	-	0.838	0.766	0.758
JI65	0.433	0.424	0.414	0.326	0.380	0.360	0.372	0.267	0.178	-	0.786	0.797
DM4	0.320	0.329	0.312	0.326	0.425	0.343	0.399	0.339	0.245	0.239	-	0.798
EC124345	0.350	0.290	0.285	0.329	0.411	0.353	0.447	0.335	0.312	0.269	0.268	-

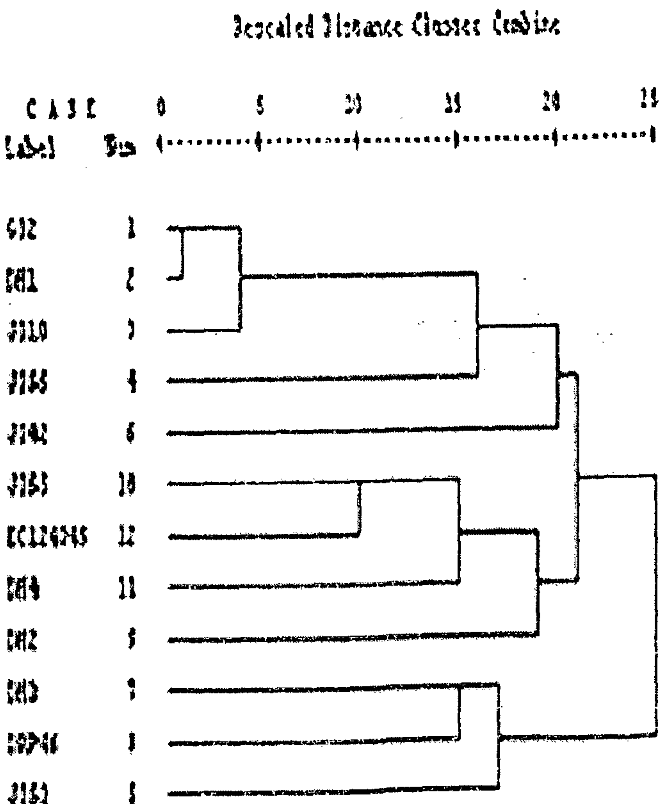
Table 4.7 : Effectiveness of RAPD, CAPS, ISSR and AFLP markers in detecting polymorphism in Isabgol

	RAPD	CAP'S	ISSR	AFLP
TOTAL BANDS DETECTED	891	1440	372	1648
POLMORPHISMS DETECTED	444	501	157	700
% POLMORPHIC BANDS	50%	35%	42%	43%
NO OF PRIMERS USED	12	10	3	3 (PAIRS)
MAXIMUM POLYMORPHISM PER PRIMER/PRIMER PAIR	12	20	11	56
MINIMUM POLYMORPHISM PER PRIMER/PRIMER PAIR	0	4	10	48
AVERAGE POLYMORPHISM PER PRIMER/PRIMER PAIR	6.2	12	10.7	51.3

4.17 PHYLOGENETIC AND NETWORK ANALYSIS:

The data from all the RAPD, CAPS, ISSR and AFLP markers were pooled together for phylogenetic and network analysis. The data were subjected to *bootstrapping* to generate 100 multiple data set. The distances obtained were subjected to neighbor joining analysis and a consensus tree was constructed (Figure 4.17.1). The overall pooled data was also subjected to calculate Euclidean Distances (Table 4.17.1) and a dendrogram was drawn by Average Linkage Method (Figure 4.17.1).

DENDROGRAM USING AVERAGE LINKAGE
(BETWEEN GROUPS)



PHYLOGENETIC TREE

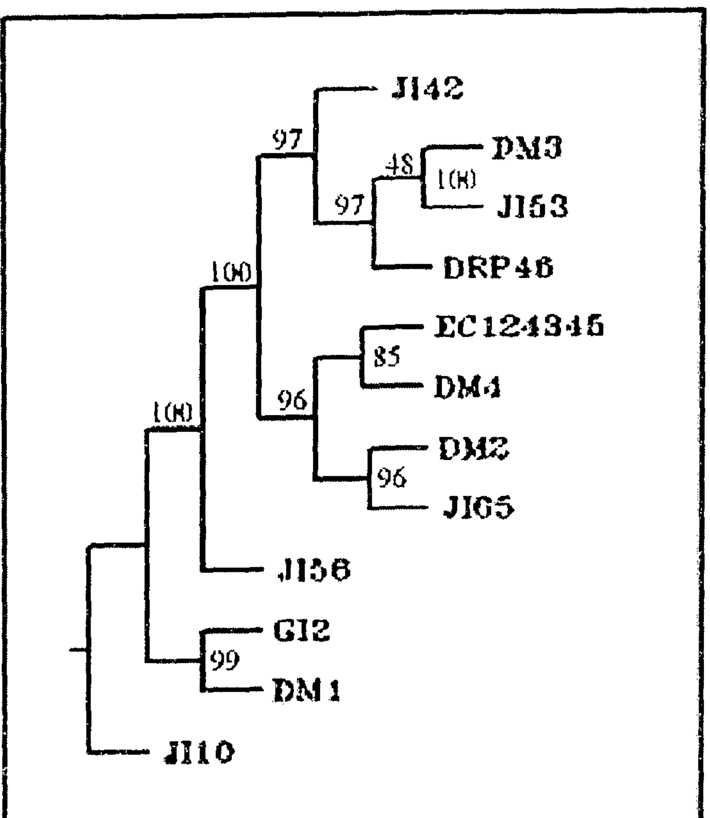


FIGURE 4.17.1: DENDROGRAMS OF MOLECULAR MARKERS OVER OVERALL POOLED DATA
(RAPD, CAPS,ISSR AND AFLP)

Table 4.5 : Data showing marker size, scorable bands, *total alleles*, polymorphic alleles, total bands and total polymorphic bands for individual primer

Technique		Primer	Marker Size (bp) Range	Scorable Bands	Total Alleles	Polymorphic Alleles	Total Bands	Total Polymorphic Bands
RAPD		Plantago 01	300-1000	4-6	07	4	48	28
		Plantago 02	150-850	6-10	10	7	84	47
		Plantago 03	450-2000	6-13	13	11	132	76
		Plantago 04	150-1400	1-8	08	8	96	44
		Plantago 05	600-1500	2-3	03	1	12	6
		Plantago 06	250-700	3	03	-	-	-
		Plantago 07	300-1000	3-8	08	7	84	39
		Plantago 08	500-1200	4-5	05	2	24	12
		Plantago 09	400-1600	7-12	12	8	96	45
		Plantago 10	300-1000	2-12	12	12	144	55
		BG 50	400-1500	4-10	10	9	108	54
		BG 51	500-1500	2-7	07	5	60	38
		Combined	**150-2000**	**2-13**	**98**	**74**	**891**	**444**
CAPS	EcoRI	Plantago 01	300-850	3-13	13	144	144	54
		Plantago 02	300-2000	7-19	19	180	180	61
		Plantago 03	250-1500	3-16	16	180	132	46
		BG 50	250-650	4-12	12	132	144	66
		BG 51	400-850	5-14	14	144	48	49
	RsaI	Plantago 01	400-850	3-6	06	48	240	21
		Plantago 02	300-2000	4-22	22	240	120	95
		Plantago 03	300-1400	4-13	13	120	96	22
		BG 50	350-500	6-11	11	96	156	39
		BG 51	350-850	4-15	15	156	132	48
		Combined	**250-2000**	**3-22**	**141**	**120**	**1440**	**501**
ISSR		UBC 887	350-2810	2-11	11	11	132	49
		UBC 889	215-1447	3-12	12	10	120	61
		UBC 891	487-1868	4-12	12	10	120	47
		Combined	**215-2810**	**2-12**	**35**	**31**	**372**	**157**
AFLP		G1	100-1000	33-78	78	56	672	222
		G2	100-1000	48-85	85	48	576	244
		G3	100-1000	32-69	69	50	600	234
		Combined	**100-1000**	**32-85**	**232**	**154**	**1648**	**700**
OVER ALL POOLED			**150-2000**	**2-85**	**503**	**379**	**4348**	**1802**

Table 4.6 : Data showing Bs, Dxy, PD %, APD %, MAPD, PIC and Hav for individual primer

Technique		Primer	Bs	Dxy	PD %	APD %	PIC	Hav
RAPD		Plantago 01	0.67-1.00	0.00-0.40	0-33	15	0.486	0.604
		Plantago 02	0.77-1.00	0.00-0.69	0-23	12	0.493	0.535
		Plantago 03	0.75-1.00	0.00-0.27	0-25	6	0.489	0.516
		Plantago 04	0.00-1.00	0.11-1.24	0-100	25	0.497	0.729
		Plantago 05	0.80-1.00	0.00-0.20	0-20	10	0.500	0.750
		Plantago 06	1.00	0	0	0	0	0
		Plantago 07	0.29-1.00	0.00-1.24	0-71	14	0.497	0.688
		Plantago 08	0.75-1.00	0.00-0.29	0-25	11	0.500	0.639
		Plantago 09	0.57-1.00	0.00-0.55	0-43	12	0.498	0.701
		Plantago 10	0.00-0.73	0.09-1.35	27-100	18	0.472	0.803
		BG 50	0.22-1.00	0.00-1.61	0-78	16	0.500	0.673
		BG 51	0.50-1.00	0.00-0.69	0-50	13	0.464	0.539
		Combined	**0.64-0.92**	**0.099-0.472**	-	**MAPD=21**	**0.491**	**0.652**
CAPS	EcoRI	Plantago 01	0.17-1.00	0.00-1.79	0-83	48	0.469	0.824
		Plantago 02	0.53-1.00	0.00-0.63	0-47	27	0.448	0.813
		Plantago 03	0.40-1.00	0.00-0.90	0-60	30	0.380	0.864
		BG 50	0.40-1.00	0.00-0.90	0-60	29	0.500	0.653
		BG 51	0.36-1.00	0.00-1.01	0-64	42	0.449	0.849
	RsaI	Plantago 01	0.50-1.00	0.00-0.66	0-50	26	0.492	0.790
		Plantago 02	0.32-0.95	0.05-1.14	5-68	40	0.478	0.768
		Plantago 03	0.50-1.00	0.00-0.69	0-50	30	0.299	0.958
		BG 50	0.46-1.00	0.00-0.77	0-54	30	0.482	0.808
		BG 51	0.33-100	0.00-1.04	0-67	40	0.426	0.859
		Combined	**0.50-0.99**	**0.01-0.70**	-	**MAPD=33**	**0.442**	**0.818**
ISSR		UBC 887	0.22-0.92	0.00-1.24	8-78	53	0.467	0.805
		UBC 889	0.31-1.00	0.00-1.10	0-69	31	0.500	0.685
		UBC 891	0.50-1.00	0.00-0.80	0-50	30	0.477	0.772
		Combined	**0.45-0.93**	**0.21-0.83**		**MAPD=38**	**0.481**	**0.754**
AFLP		G1	0.65-0.97	0.015-0.407	3-35	20	0.442	0.803
		G2	0.68-0.98	0.017-0.386	2-32	13	1.470	0.700
		G3	0.67-0.97	0.030-0.362	3-33	15	0.476	0.753
		Combined	**0.69-0.98**	**0.02-0.29**	-	**MAPD=16**	**0.796**	**0.752**
OVER ALL POOLED			0.61-0.89	0.167-0.490	-	MAPD=27	0.553	0.744

Table 4.17.1 : Squared Euclidean Distance for Overall pooled data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.00	32.00	53.00	110.00	165.00	120.00	155.00	164.00	155.00	132.00	133.00	107.00
DM1	32.00	0.00	45.00	108.00	161.00	124.00	155.00	154.00	139.00	120.00	119.00	95.00
JI10	53.00	45.00	0.00	95.00	152.00	115.00	144.00	155.00	134.00	123.00	128.00	96.00
JI56	110.00	108.00	95.00	0.00	143.00	128.00	145.00	142.00	143.00	122.00	145.00	113.00
JI53	165.00	161.00	152.00	143.00	0.00	135.00	104.00	115.00	192.00	153.00	174.00	158.00
JI42	120.00	124.00	115.00	128.00	135.00	0.00	117.00	126.00	151.00	120.00	147.00	111.00
DM3	155.00	155.00	144.00	145.00	104.00	117.00	0.00	99.00	168.00	127.00	160.00	136.00
DRP46	164.00	154.00	155.00	142.00	115.00	126.00	99.00	0.00	153.00	116.00	151.00	125.00
DM2	155.00	139.00	134.00	143.00	192.00	151.00	168.00	153.00	0.00	97.00	148.00	112.00
JI65	132.00	120.00	123.00	122.00	153.00	120.00	127.00	116.00	97.00	0.00	103.00	75.00
DM4	133.00	119.00	128.00	145.00	174.00	147.00	160.00	151.00	148.00	103.00	0.00	96.00
EC124345	107.00	95.00	96.00	113.00	158.00	111.00	136.00	125.00	112.00	75.00	96.00	0.00

This is a dissimilarity matrix

The clades with at least 75% bootstrap values were considered "highly supported", those with 50-74% were considered "weakly supported" and the clades found in the strict consensus, but with <50% bootstrap values, were considered "not supported". For network analysis data were subjected to Median Joining Analysis and a network was calculated (Figure 4.13).

4.17.1 RAPD:

The pooled data of RAPD was subjected for network analysis to create phylogenetic network (Figure 4.9) between the genotypes which showed that the set of these 12 random oligo primers can differentiate all the 12 different genotypes from each other. The distance between the two genotypes showed that how much they differ or how far they are genetically apart from each other.

DM1 and GI2 had minimum genetic distance because of four mutations but this branching was weakly supported by 53 bootstrap confidence values and hence can be grouped as one. In phylogenetic analysis DM4, EC124345, DRP46, JI65 and DM2 formed one cluster, while DM3, JI42 and JI53 formed another cluster with the internal clades between JI65 and DM2 but their branching was highly supported by 86% of bootstrapped multiple data sets. The second internal clade was formed

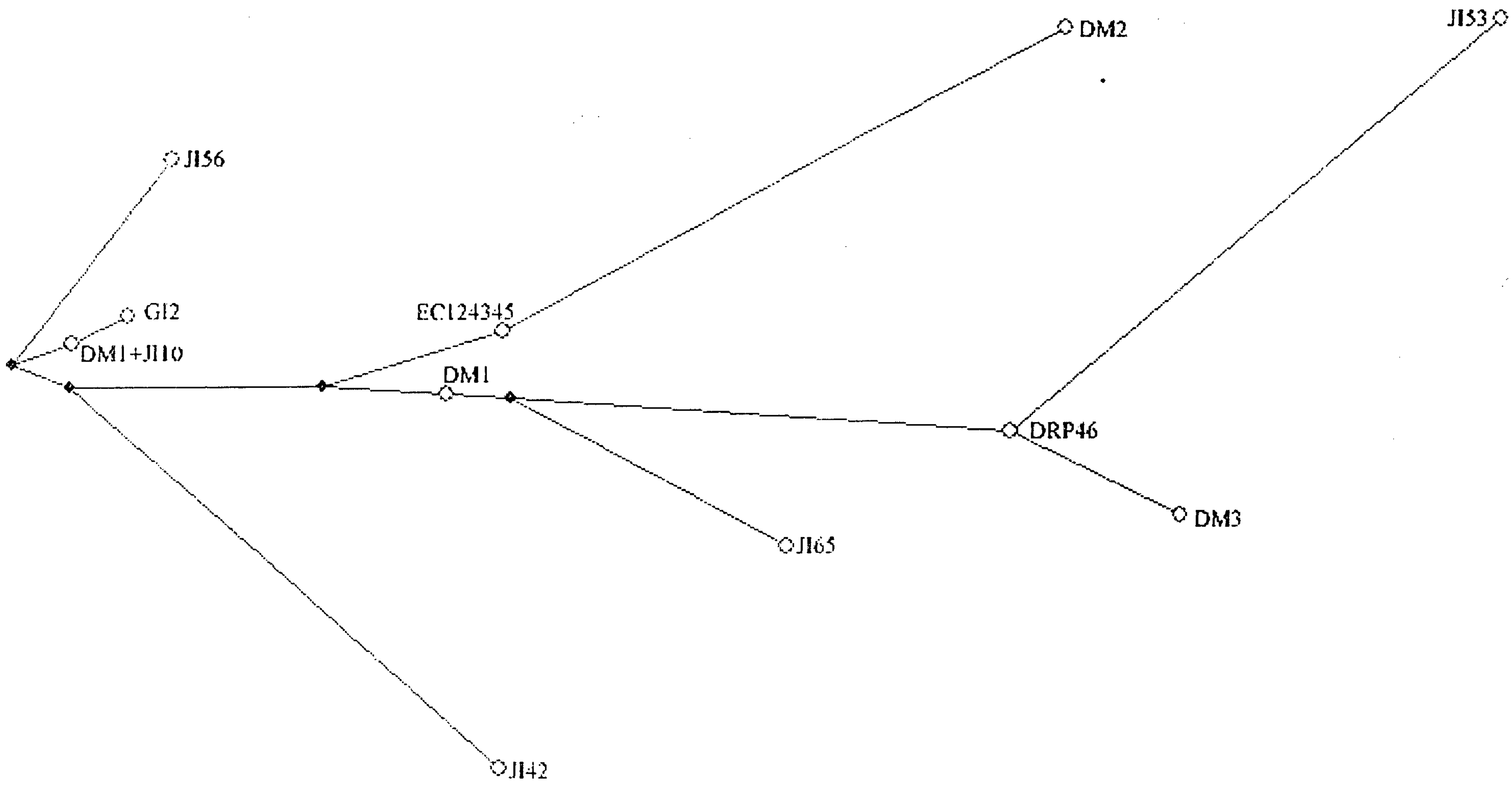


FIGURE 4.13: NETWORK FROM OVERALL POOLED DATA OF MOLECULARS MARKERS

between JI53 and JI42 and they were found to be separated by 7 mutations by network, which was highly supported by 81% bootstrap value.

There was no difference found in the clustering of genotypes as done by PHYLIP, network and Euclidean Distance method except for DM3 and DRP46, which were distinctly separated by Euclidean Distance method.

4.17.2 CAPS:

The technique, CAPS is based on the restriction sites in the DNA sequence. It differentiates the genotypes on the basis of different cutting sites in DNA, which may differ with difference in the nucleotide sequence. In the characterization of *Plantago ovata* CAPS can detect the polymorphism between the genotypes but failed to identify the Genotypes GI2, DM1 and JI10 separately. The probable reason for this may be that they are genetically more similar or their genome is very much similar to the restriction sites for the restriction enzymes used (*EcoR*I and *Rsa*I). In the network drawn, these genotypes formed one big cluster.

In phylogenetic analysis JI10, GI2, and DM1 formed one group and this clustering was also supported by network as they were grouped together. JI42, DRP46, DM3, JI53 and JI56 formed a cluster but the clustering was weakly supported by bootstrap confidence value and they branched out from DM3 by 6 mutations (JI56 and DRP46), 10 mutations (JI53) and 14 mutations (JI42). However, DM2 and JI65 formed one cluster but they are separated by 7 mutations and this branching was highly supported by 91% of multiple data sets. DM4 and EC124345 were genetically apart by 11 mutations.

Practically there was no difference found between the dendrogram from Euclidean Distance method and Phylogenetic tree and network.

4.17.3 ISSR:

The pooled data generated by 3 ISSR primers was subjected to phylogenetic network analysis through median joining analysis and a network was drawn which showed the ISSR had the capability to differentiate all the 12 genotypes of Isabgol under study.

In network JI10 and JI53 had minimum genetic distance because of one mutation. In phylogenetic analysis DM3, GI2, JI42 formed one cluster, whereas, network analysis showed GI2 and JI42 are closer. JI56, DM1, EC124345, JI65, DRP46 and DM2 formed another cluster, however, network plotted JI56 and DM1 relatively away from the other members of the cluster. DM2 and DRP46 were 4 mutations apart.

In the dendrogram obtained through Euclidean Distance method GI2 and JI56 were grouped together whereas, they were branched out in the phylogenetic tree constructed through PHYLIP and network.

4.17.4 AFLP:

The network calculated from pooled data generated from AFLP markers showed that it also failed to differentiate genotype DM1 and JI10.

DM1 and JI10 were grouped together by network and GI2 had minimum genetic distance to this cluster. However, the branching was highly supported by bootstrap value as high as 100. DM3 and DRP46 were separated because of 3 mutations and the branching was highly supported by 91 bootstrap confidence value. In phylogenetic analysis EC124345, DM4 and JI65 formed one clade, but the EC124345 and DM4 were branched out by network with 3 mutations. While, the

DM4 and JI65 are apart by 6 mutations and the branching was weakly supported by bootstrap confidence value.

DM2 genotype was found genetically distinct from rest of the genotypes in the Euclidean Distance method but they formed a cluster in phylogenetic tree constructed through PHYLIP. DM4 also found distinct from rest of the genotypes by Euclidean method.

4.17.5 OVERALL POOLED:

The overall pooled data from RAPD, CAPS, ISSR and AFLP was subjected to median joining analysis through network to create phylogenetic network between the genotypes. The study showed that the DNA markers used could differentiate all the genotypes except DM1 and JI10.

DM1 and JI10 grouped together by network and GI2 had minimum genetic distance because of one mutation. However, the branching was highly supported by bootstrap value as high as 99. With the four mutations JI56 formed another branch and it was also highly supported by bootstrap value 100. EC124345 and DM4 were separated because of 5 mutations and the branching was highly supported in 85% of multiple data sets. Non-significant genetic distance was observed between DRP46 and DM3 because of 3 mutations. However, it was not supported by bootstrap value. Hence, they can be grouped as one. In the phylogenetic analysis, genotypes DM2, JI65, EC124345 and DM4 formed one cluster, whereas, the genotypes JI42, DRP46, DM3 and JI53 formed another cluster which were significantly different from each other.

There was no difference between the clustering as done by PHYLIP and SPSS. All the clusters were found similar except that the JI42 formed cluster with GI2, DM1, JI10 and JI56 instead of DM3, DRP46 and JI53 by SPSS method.

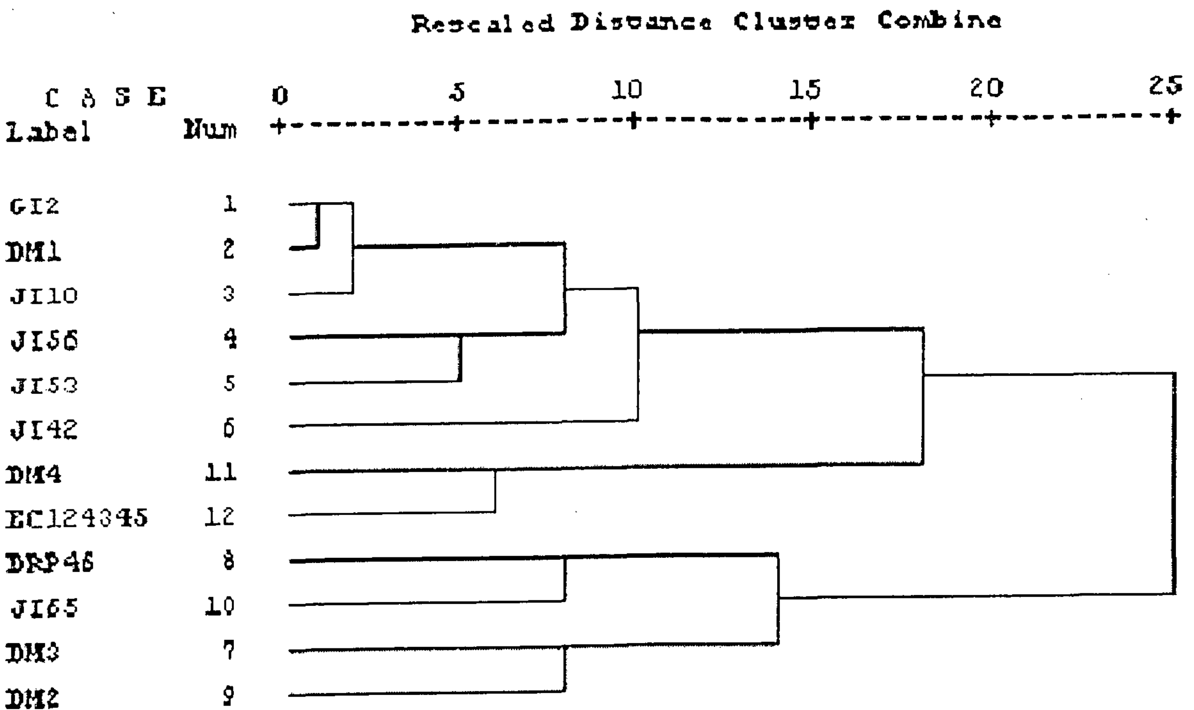


FIGURE 4.18.1 DENDROM CONSTRUCTED FROM BIOCHEMICAL AND POOLED MOLECULAR MARKERS DATA USIG AVERAGE LINKAGE (BETWEEN GROUPS)

4.18 TOTAL OVERALL POOLED EFFECT (Biochemical And Molecular):

The biochemical and molecular data were pooled and subjected to calculate Euclidean Distances (Table 4.18.1) and dendrogram (Figure 4.18.1) was constructed. The overall highest genetic distance was found between the genotypes EC124345 and DM3. While, the lowest genetic distance was found between GI2 and DM1.

Table 4.18.1 : Squared Euclidean Distance for Total Overall pooled data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.000	93.257	103.157	140.552	195.403	197.025	511.443	333.779	472.067	366.960	219.629	254.648
DM1	93.257	0.000	124.216	220.901	244.609	151.781	416.876	413.384	390.638	411.720	358.583	465.348
JI10	103.157	124.216	0.000	129.327	171.401	213.597	319.864	221.903	274.040	225.050	233.946	339.788
JI56	140.552	220.901	129.327	0.000	154.335	278.714	443.172	245.319	406.632	328.391	209.392	260.416
JI53	195.403	244.609	171.401	154.335	0.000	252.036	339.022	198.768	388.138	310.514	242.819	345.680
JI42	197.025	151.781	213.597	278.714	252.036	0.000	470.509	430.691	466.768	388.073	373.984	438.925
DM3	511.443	416.876	319.864	443.172	339.022	470.509	0.000	259.816	185.857	338.440	621.110	935.331
DRP46	333.779	413.384	221.903	245.319	198.768	430.691	259.816	0.000	261.977	182.469	291.410	451.956
DM2	472.067	390.638	274.040	406.632	388.138	466.768	185.857	261.977	0.000	220.509	523.404	805.475
JI65	366.960	411.720	225.050	328.391	310.514	388.073	338.440	182.469	220.509	0.000	288.091	459.871
DM4	219.629	358.583	233.946	209.392	242.819	373.984	621.110	291.410	523.404	288.091	0.000	168.407
EC124345	254.648	465.348	339.788	260.416	345.680	438.925	935.331	451.956	805.475	459.871	168.407	0.000

This is a dissimilarity matrix

4.19 EFFECT OF PLANT DISEASE INDEX OVER TOTAL OVERALL POOLED DATA :

Table 4.8 : Location and PDI of different Isabgol genotypes

Genotype	Location and Nature	PDI I	PDI II
DM1	Susceptible towards Downy mildew (Anand)	48.00	65.33
DM2		34.67	47.33
DM3		50.67	59.33
DM4		44.67	64.00
GI-2	Cultivated variety of Gujarat	24.67	40.00
JI-10	Jagudan	12.67	36.67
JI-42		10.67	38.00
JI-53		13.33	34.67
JI-56		12.67	32.67
JI-65		42.00	53.33
EC-124345	Resistant (Pakistan)	9.33	29.33
DRP-46	Tolerant (Anand)	22.00	37.33
S.Em.		5.81	5.06
C.D. at 5%		21.02	18.33

When Squared Euclidean Distances (Table 4.19.1 and 4.19.2) were calculated and a dendrogram was drawn (Figure 4.19.1) incorporating PDI-1 or PDI-2 (plant disease index) (Table 4.8, Marketa, 2002) in the total overall pooled data, showed the clustering between genotypes of same origin. Genotypes JI10, JI56, JI53 and JI42 formed one cluster and showed very narrow genetic distance. Whereas, GI2, DRP46 and EC124345 formed sub-cluster and were found to be more resistant to downy mildew in the field. DM1, DM2, DM3 and DM4 formed separate cluster and were of same geographical origin and were found to be more susceptible to downy mildew infection.

Table 4.19.1 Squared Euclidean Distance PDI-1 Over Total Overall Pooled Data

Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0000	637.546	247.157	284.552	323.998	393.025	1187.443	340.908	572.067	667.289	619.629	489.964
DM1	637.546	0000	1372.425	1469.110	1446.618	1545.310	424.005	1089.384	568.327	447.720	369.672	1960.717
JI10	247.157	1372.425	0000	129.327	171.837	217.597	1763.864	308.952	758.040	1085.299	1257.946	350.944
JI56	284.552	1469.110	129.327	0000	154.771	282.714	1887.172	332.368	890.632	1188.640	1233.392	271.572
JI53	323.998	1446.618	171.837	154.771	0000	259.111	1733.297	273.937	843.534	1132.483	1225.014	361.680
JI42	393.025	1545.310	217.597	282.714	259.111	0000	2070.509	559.060	1042.768	1369.642	1529.984	440 720
DM3	1187.443	424.005	1763.864	1887.172	1733.297	2070.509	0000	1081.785	441.857	413.609	657.110	2644.326
DRP46	340.908	1089.384	308.952	332.368	273.937	559.060	1081.785	0000	422.506	582.469	805.339	612.485
DM2	572.067	568.327	758.040	890.632	843.534	1042.768	441.857	422.506	0000	274.238	623.404	1447.590
JI65	667.289	447.720	1085.299	1188.640	1132.483	1369.642	413.609	582.469	274.238	0000	295.220	1527.200
DM4	619.629	369.672	1257.946	1233.392	1225.014	1529.984	657.110	805.339	623.404	295.220	0000	1417.323
EC124345	489.964	1960.717	350.944	271.572	361.680	440.720	2644.326	612.485	1447.590	1527.200	1417.323	0000

This is a dissimilarity matrix

Table 4.19.2 Squared Euclidean Distance PDI-II Over Total Overall Pooled Data

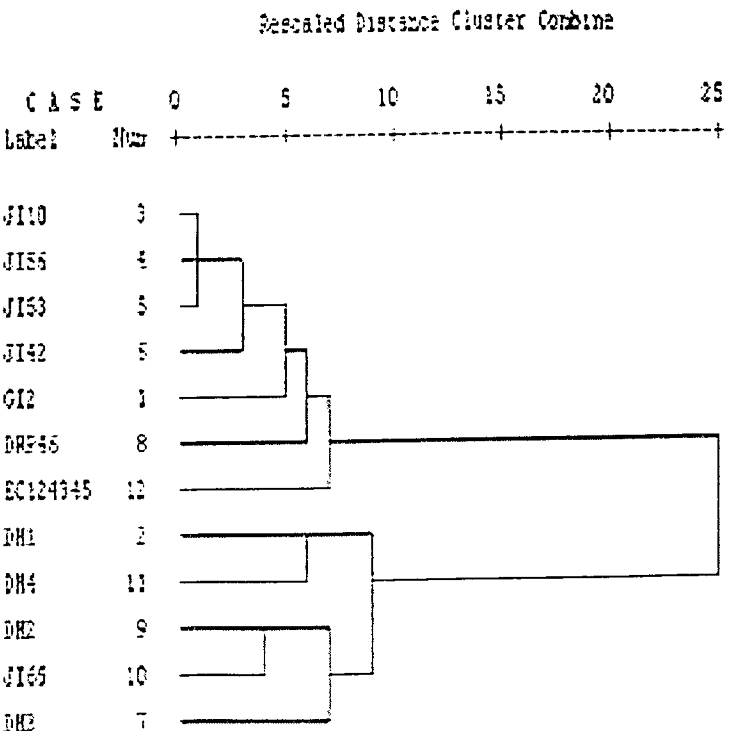
Proximity Matrix

Case	GI2	DM1	JI10	JI56	JI53	JI42	DM3	DRP46	DM2	JI65	DM4	EC124345
GI2	0.000	734.866	114.246	194.281	223.812	201.025	885.091	340.908	525.796	544.649	795.629	261.777
DM1	734.866	0.000	945.612	1287.576	1184.645	898.710	452.876	1197.384	714.638	555.720	360.352	1249.348
JI10	114.246	945.612	0.000	145.327	175.401	215.366	833.340	222.339	387.676	502.606	980.875	340.224
JI56	194.281	1287.576	145.327	0.000	158.335	307.123	1153.927	267.035	621.547	755.227	1190.961	282.132
JI53	223.812	1184.645	175.401	158.335	0.000	263.125	947.137	205.843	548.414	658.710	1103.068	352.755
JI42	201.025	898.710	215.366	307.123	263.125	0.000	925.478	431.140	553.817	623.082	1049.984	439.374
DM3	885.091	452.876	833.340	1153.927	947.137	925.478	0.000	743.816	329.857	374.440	642.919	1419.331
DRP46	340.908	1197.384	222.339	267.035	205.843	431.140	743.816	0.000	361.977	438.469	1002.699	451.956
DM2	525.796	714.638	387.676	621.547	548.414	553.817	329.857	361.977	0.000	256.509	801.293	905.475
JI65	544.649	555.720	502.606	755.227	658.710	623.082	374.440	438.469	256.509	0.000	401.940	715.871
DM4	795.629	360.352	980.875	1190.961	1103.068	1049.984	642.919	1002.699	801.293	401.940	0.000	879.696
EC124345	261.777	1249.348	340.224	282.132	352.755	439.374	1419.331	451.956	905.475	715.871	879.696	0.000

This is a dissimilarity matrix

In another case when disease incidence was maximum the plant disease index was calculated and dendrogram was drawn. Incorporation of these information with the total overall pooled data showed the clustering of JI10, JI56, JI53 and JI42 (same geographical origin) and have very less genetic distance with GI2 (cultivated variety developed at Anand). Between DRP46 and EC124345, less genetic distance was observed, these showed tolerance and resistance to downy mildew in the field, which showed more genetic distance from this group (developed at Anand). Likewise DM2, JI65, DM3, DM1 and DM4 formed one cluster. Deviation in clustering of JI65 (now maintained at Anand) indicated its association with the varieties developed at Anand.

A



B

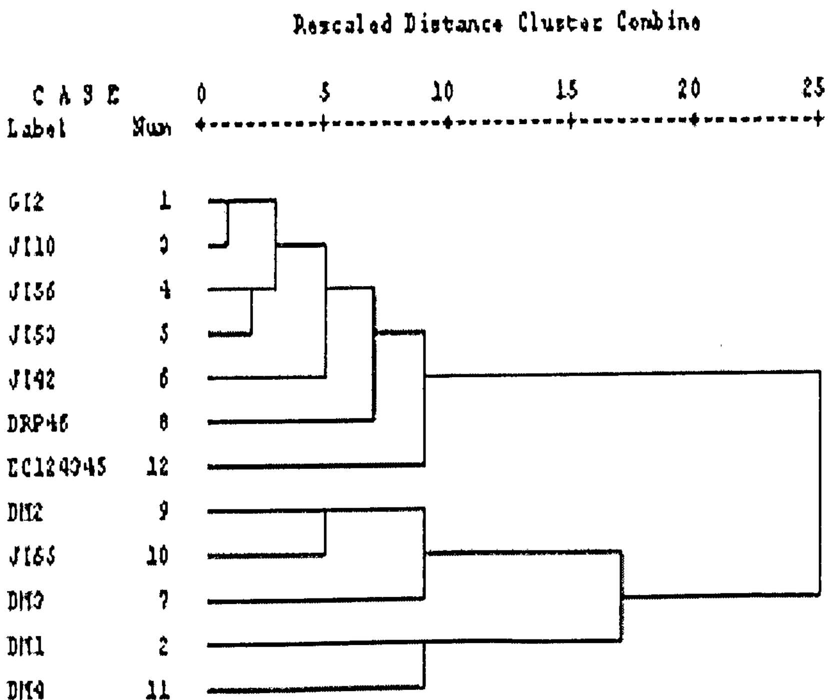


FIGURE 4.19.1 DENDROMS FROM PDI-1 (A) AND PDI-11 (B) OVER COMBINED BIOCHEMICAL AND MOLECULAR DATA

Summary and Conclusion

CHAPTER - V
SUMMARY AND CONCLUSION

Isabgol is an important medicinal crop. In India, it is mainly cultivated in North Gujarat and some parts of Rajasthan and gaining the importance as dollar earner crop. So far it is less exploited crop biochemically as well as at molecular level, so the present investigation was undertaken.

The major constituents of the Isabgol seeds, the oil; husk; total carbohydrate, cellulose and protein were found maximum in the genotypes DM3 (4.06%), EC124345 & JI65 (36%), EC124345 & JI65 (63%), EC124345 (7.7%) and DM3 (18%), respectively. While, the lowest amount of these constituents were found in the genotypes JI10 (3.25%), DM3 (31%), DM3 & DM1 (51%), DM1 (4.2%) and EC124345 & JI65 (15%), respectively.

The downy mildew disease (major disease of this crop) caused by *Peronospora plantaginis* causes severe yield losses. The major constituents/enzymes, which may be, associated with the host-parasite interaction i.e. phenols, peroxidase and PPO were found maximum in the genotypes DM1 & JI42 (0.067%), EC124345 and DM3, respectively. While, DM3 (0.017%), DM3 and EC124345 have the least.

Genotype DM3 had shown the highest oil and protein as compared to the other genotypes. This genotype had shown less husk content and total phenols. This indicated that where oil content was high, the husk content was low, genotype was susceptible to disease (may be to downy mildew) due to low phenol content and increase in total protein.

Genotype EC124345 and JI65 have maximum amount of husk and total carbohydrate and lowest amount of protein. This indicated that the high amount of total carbohydrate and low protein content is desirable for high husk content.

The activity of enzyme peroxidase, which is associated with disease resistance, was shown maximum by EC124345, while the genotype DM3 showed the lowest peroxidase activity. This indicated that the genotype EC124345 is resistant, while the DM3 was found susceptible to downy mildew in the field.

It has been reported that PPO activity has been found to increase following infection by pathogens or mechanical injury (Brueske and Dropkin, 1973). The genotype DM3 showed the highest activity and found to be susceptible to downy mildew in the field. While, the genotype EC124345 showed the least PPO activity and found to have resistance to downy mildew in the field.

No variation was observed in the isoenzyme patterns of Pox and Alkaline phosphatase but the bands were found to be differing in their intensity, which showed different intensity of activity among the genotypes. PPO and esterase were found to be different for their isoenzyme banding pattern, indicating that they can be used as markers to differentiate the genotypes.

The dendrograms constructed from the mean data revealed that the genotypes JI56, JI53 and JI10 were found to be closely related and formed one cluster.

Molecular markers are extremely useful in the characterization of plant germplasm resources. In the characterization of Isabgol, the use of four types of molecular markers (RAPD, CAPS, ISSR and AFLP) were elucidated. The overall maximum polymorphic information was given by AFLP (0.796) followed by RAPD (0.491), ISSR (0.481) and CAPS (0.442). Although, RAPD and ISSR could distinguish all the twelve genotypes. While, AFLP and CAPS could not distinguish between DM1, JI10 and DM1, JI10, GI2, respectively. Each marker type has its strengths and weaknesses. RAPD is technically simple. However, the possibility

exists that same RAPD bands consists of multiple co-migrating products and the scoring of the similar sized bands present in two individuals does not guarantee that the bands have identical sequences. The latter is more of a problem when making inter-specific comparison and should not affect the results presented, as it is intra-specific comparison.

ISSR require small amount of DNA. Technically they are not difficult to develop than RAPD. They are limited by the requirement that SSR sequences must be distributed in the correct orientation and spacing in the genome of the individual being fingerprinted.

In AFLP, a large number of primer combinations can be used and the markers are robust. Technically they are difficult and more expensive to develop than RAPD, CAPS and ISSR.

The basis for detecting each of these markers is different. It would therefore, be expected that the data generated by each would confirm or complement that generated by the other.

When the clustering of the genotypes was done using overall pooled data over the plant or percent disease index. This showed the clustering of genotypes of same geographical origin. The deviation in the clustering of J165 showed close relationship with the genotypes developed at Anand. It may be concluded that the genotypes have narrow genetic distances, which were originated, from the same geographical area than that originated from different geographical locations.

CONCLUSION:

Molecular markers were useful in fingerprinting of Isabgol genotypes. AFLP gave maximum polymorphic information alone but RAPD, CAPS or ISSR required complementation by AFLP data. The broader implications of these results suggest that the success of molecular markers in fingerprinting experiments for some plant species may depend upon which marker type is used and that a combination of marker types may be the best opinion.

The results also suggested that the genotypes JI10, DM1 are not different and they have very close relationship with GI2 and the differences among them were found non-significant. So these can be pooled but confirmation is necessary.

FUTURE RESEARCH NEED:

- The DNA fragments found to be unique can be eluted from the gel, can be sequenced and used as "SCARS" in the identification and differentiation of the genotypes.
- Saturation of genetic map with other molecular markers may give an idea about the linkage of downy mildew resistant gene.
- As very low level of variation is seen and looking into the unavailability of resistant genotypes to downy mildew, *in vitro* methods of inducing variability through somaclonal variation could be a possible method for downy mildew resistance.
- As wild species of Isabgol, such as *P. indica*, *P. lanceolata*, *P. coronopus* and *P. psyllium* have valuable genes for downy mildew resistance and other desirable characters, these could be transferred to *P. ovata*.

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* Original not seen.