BIOCHEMICAL METHODS FOR IDENTIFICATION AND GENETIC PURITY TESTING OF COTTON (*GOSSYPIUM* SPP.) HYBRIDS.

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BIOCHEMICAL METHODS FOR IDENTIFICATION AND GENETIC PURITY TESTING OF COTTON (*GOSSYPIUM* SPP.) HYBRIDS.

By

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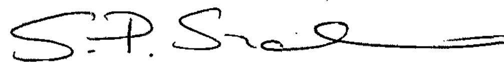
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CERTIFICATE

This is to certify that the thesis entitled "Biochemical methods for identification and genetic purity testing of cotton (*Gossypium* Spp.) hybrids", submitted to Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi in partial fulfillment of DOCTOR OF PHILOSOPHY in Seed Science and Technology embodies the results of bonafide research carried out by Mr. Gowri Sankara Rao Reddy under my guidance and that no part of the thesis has been submitted for any other degree or diploma.

It is further certified that he has duly acknowledged the assistance and help obtained during the course of study.

Place: New Delhi
Date: 8th September

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INTRODUCTION

Chapter I

Introduction

The genus *Gossypium* (cotton) belongs to the natural order Malvales and family Malvaceae. The genus *Gossypium* comprises of 40 species, of which G. *hirsutum*, G. *barbadense*, G. *herbaceum* and G. *arboreum* are under commercial cultivation. India holds the distinction of having developed the first commercial cotton hybrid in the World. The release of intra- hirsutum hybrid cotton 'H-4' in 1970 paved the way to the hybrid cotton era, which is unique in the cotton history. Subsequently, several intra-hirsutum, inter specific (G. *hirsutum* X G. *barbadense*) and desi (G. *herbaceum* X G. *arboreum*) hybrids have been successfully developed for the central and southern zones of India. In India, presently about 9.0 million hectares of land is under cotton cultivation with an average production of 16.8 million bales per annum (Kairon *et al.*, 2000). Of this, hybrid cultivars constitute at least 30 per cent of the total are under cotton cultivation contributing to 48 percent of the total production (Kairon *et al.*, 2000). Thus, there is a huge demand for hybrid cotton seed. This has led to the release of a large number of hybrids both by the private and public sectors. Hence, reliable characterization of cotton hybrids and their parental lines based on physiological or biochemical attributes would be useful in establishing their identity independently or in combination with morphological descriptors.

The benefits of hybrid seeds can be reaped only if their genetic purity is maintained and hybrid seed of high quality are made available to the farmers in time. Identifying the breeding lines and determining the F_1 hybrid purity are important quality requirements. Hybrid seeds of cotton are produced by hand emasculation and pollination technique, which require heavy investment. Cotton hybrids present a unique case, in which commercial seed production is takenup by hand emasculation

and pollination technique. This has not only made the exploitation of hybrid vigour possible in cotton, but also contributed to the generation of considerable employment for the rural people, particularly young women, who perform this task with ease and expertise. However, this also leads to a chance of manual error during the emasculation and pollination, which may lead to selfed female seeds resulting in genetic impurity of the hybrid seed. Considering this, the Indian Minimum Seed certification Standard permits upto 10 percentage of female selfed seeds in cotton hybrid, leaving a minimum purity level of 90 percentage, which is quite achievable. To ensure the genetic purity of hybrid cotton, seed certification agencies follow the field plot technique (grow out test), in which purity of the seed is tested on the basis of morphological descriptors such as the vegetative branching, growth habits, leaf characters, petal colour, petal spot, anther morphology, size and shape of the boll etc., These techniques, though quite reliable, are labour intensive, tedious and time consuming, since the key characters need to be observed until after the flowering (60-80 days) in most cases. Since the time available between seed harvest and sowing is limited (2-3 months), it is difficult for the seed producing companies to ascertain the genetic purity of the seed by conventional methods in such a short period. Rapid and reliable testing techniques are, therefore, necessary for in-house quality control, ascertaining the genetic purity and for decision making by the seed producing agencies

Establishing varietal identity is crucial to the protection of plant varieties. Distinctness (D), uniformity (U) and stability (s) tests have been recommended for more than 160 crops by the UPOV (1991), including cotton, which are based on morphological descriptors. Techniques based on the seed / seedling response to chemicals under controlled conditions, and variation in pigmentation, protein, isoenzyme and DNA polymorphisms have proved useful in characterization of varieties and development of varietal keys in several crop species. These could

possibly serve as supplementary or alternative to morphological markers to establish varietal identity. For the purpose of quick decision making, possible application of these tests and reduction of the sample size to less than 400 seeds need to be worked out.

Hence, the present research project on "Biochemical methods for identification and genetic purity testing of cotton (*Gossypium* spp.) hybrids," was proposed with the following objectives.

1. To standardize a reliable and reproducible laboratory technique for distinguishing parental lines and hybrids of cotton based on the seed and seedling response to chemical application.
2. To distinguish the parental lines and hybrids of cotton using electrophoresis analysis of seed proteins, isoenzymes and RAPD markers.
3. To compare the accuracy of single seed electrophoresis analysis with field grow out test for testing genetic purity of hybrid cotton.
4. To ascertain the appropriateness of reducing the sample size for biochemical analysis by using sequential sampling technique.

REVIEW OF LITERATURE

Chapter-II

Review of literature

The success of agriculture depends largely on the development of genetically improved varieties, which can fulfill the specific requirements with respect to availability of the inputs, climate of the region, marketability of the produce and other related socio-economic parameters. For this reason, crop variety improvement programmes have become highly competitive. To harness the benefits from the commercialization of improved varieties, it is essential for the breeder that there is an appropriate system of plant variety protection. Similarly, highest levels of genetic purity of the seed reaching the farmer also need to be assured for achieving maximum returns by the farmer.

Protection of Plant Varieties and Farmers' Rights Act, 1999 in India (Jain, 1999) has generated a lot of interest both towards the benefits of the breeders and cultivators, while there is some reservation in certain sectors concerning the rise in seed prices as a fall out of the Plant Variety Protection Act. There is a general acceptance of this Act in the public, as it is expected to widen the availability of crop varieties, check unscrupulous businessmen in the seed market and improve the quality of the seed in general. The registration enables a variety to be under the purview of any quality assurance scheme, hence registration of the varieties (including hybrids and parental lines) with appropriate distinguishing characteristics will be useful, both for the seed quality assurance, marketing and also for resolving disputes, if any.

Agriculturally important crops exist as genetically distinct, but related varieties. Cultivar characterization and assessment of genetic purity are crucial for the cultivar development, release, seed production and certification programmes. As per the DUS testing guidelines of UPOV (1991), a new crop variety is to be distinct (D) from other varieties, uniform (U) in its characters and genetically

stable (S). Hence, specific and unambiguous characters are to be precisely described for comparison of new and existing varieties. Relevant literature to cultivar characterization and genetic purity testing are reviewed in this chapter.

Variety identification

Laboratory techniques based on seedling response to chemicals and growth conditions

Characteristic morphological features of seedlings, expressed under defined conditions of growth, are useful in identification of varieties in several species. Chesneaux and Kabilinsky (1982) found the width of the first leaf in fourteen day old seedlings to be a reliable descriptor for characterizing maize cultivars, while Payne *et al.*, (1980) suggested that cultivars of sorghum could be characterized on the basis of anthocyanin pigmentation on seedlings grown in a modified Hoagland nutrient medium. Similarly, Sahoo (1996) and Nagaraja (1998) characterized sunflower and sorghum hybrids and parental lines based on development of anthocyanin pigmentation under controlled conditions. A modified grow-out test on 20 days old seedlings was found as reliable as traditional grow-out test (60days) in pearl millet (Halim and Saxena, 1995) and cotton (Krishna *et al.*, 1996). Genetic variability with respect to sensitivity to high (toxicity) and low (deficiency) doses of micro and macronutrients is inherent to plant species. Though fairly salt-tolerant, cotton varieties at seedling stage are known to exhibit sensitivity to Na^+ (Abul-Naas and Orman, 1974). Varied responses of cotton plant to Mn, Mg and Ca are also reported (Kent and Lauchli, 1985, Foy and Weil, 1995).

Laboratory tests based on colour reactions of seeds

Variability in the colour reaction of the seed subsequent upon application of chemicals provides a simple method to group large number of genotypes into distinct classes or to differentiate between genotypes. The phenol colour reaction depends on the quantity and quality of oxidative enzymes present in the seed coat, which oxidize phenol, producing melanin pigment as the final end product. The colour reaction is fairly consistent with the extent of enzyme activity, conditioned by the variations in the experimental environment. Phenol test had been used to classify wheat cultivars by several workers (Clancy *et al.,* 1982; Singhal and Prakash, 1988). UPOV has included phenol colour reaction as one of the descriptors for DUS testing of wheat varieties. Rao *et al.,* (1989) studied the genetics of phenol colour reaction and concluded it to be controlled by a single gene in pearl millet on the basis of 3:1 segregation of the positive and negative genotypes.

Peroxidase test is one of the standard tests, which is used in the identification of soybean cultivars wherein, cultivars are classified into two groups on the basis of presence and absence of seed coat peroxidase reaction with oxidized guaiacol. Machacek and Gutormson (1990) reported that the efficacy of peroxidase test in soybean could be improved by supplementing with other laboratory test procedures.

AOSA (1989) developed NaOH test to distinguish red from white wheat seeds. Similarly, on the basis of colour reaction between tannic acid in testa with KOH, Payne *et al.,* (1980) classified sorghum cultivars into two colour categories. Fluorescence test could be used in complementation with other laboratory procedures for identifying clover and lucerne cultivars (Stuczynska and Stuczynska, 1994).

Anthocyanin, phenolic compounds and other pigments

A wide array of pigments and colour compounds of plant origin (from different plant parts *viz.*, leaf, petals, berries, seeds etc.,) have been found to be characteristic of different plant species and varieties. Colour intensity and tonality of anthocyanin and phenolic compounds in berries were utilized for varietal characterization of grape wines (Almela *et al.*, 1996). The use of tocopherol and fatty acid composition was suggested as biochemical markers for genetic purity testing of hybrid seeds in sunflower (Loskutov *et al.,* 1994). In gerbera, cultivars were identified on the basis of phenolic compounds in the ray florets (Booy, 1995).

Biochemical markers

Development of cultivar-specific genetic markers is desirable for variety identification and protection as well as for seed purity determination. Proteins (including isoenzymes) being the products of gene translation can be regarded as markers for the respective structural genes. A comparison of the polymorphism, which occurs due to variation in protein composition among individuals could be reflected to the gene expression.

Electrophoresis is a technique in which electric current is applied across a medium (polyacrylamide or agarose gel) for the separation of the charged molecules. The current accelerates the mobility and consequent separation of compounds, which appear as distinct bands on staining. The separation is due to differences in the size and charge of proteins involved (Davis, 1964). The relevance of electrophoresis of the seed and seedling proteins or isoenzymes for cultivar identification and genetic purity testing has been well established and

thoroughly reviewed by Cooke (1988,1995), Smith and Smith (1992) and several others.

Seed proteins for cultivar identification

Electrophoresis of denatured seed proteins is one of the simplest and routine laboratory techniques used for cultivar identification. Considering its applicability and relevance for cultivar identification, UPOV included the electrophoresis of glutenein as a supplementary test for DUS testing of wheat and barley. ISTA (1992) recommended PAGE technique for cultivar characterization of wheat, barley, pisum and lolium. SDS-PAGE of the tris soluble proteins and salt soluble globulins have been extensively used for cultivar identification in crops like rice (Bhowmik *et al.,* 1990; Abdel *et al.,* 1993d), sunflower (Varier *et al.,* 1992; Sahoo et al., 2000), wheat (Abdel *et al.,* 1993b), sorghum (Abdel *et al.,* 1993a), maize (Wang *et al.,* 1994a) castor (Varier *et al.,* 1999), chinese cabbage (Zheng *et al.*, 1997), pea (Mishra *et al.*, 1996), Soybean (Goyal and Sharma, 1999), Pepper (Odeigah *et al.*, 1999) etc.

Protein markers for cultivar identification of cotton

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)of tris soluble proteins and salt soluble globulins has been used by a number of workers for identification of cotton cultivars. Kapse and Nerkar (1985) studied electrophoretic profiles of four intra-hirsutum hybrids, two G. *hirsutum* X G. *barbadense* hybrids, their parental lines and two varieties each of G. *hirsutum* and G. *barbadense* species of cotton. Agrawal *et al.* (1988) reported the use of SDS-PAGE of soluble seed proteins as well as isoenzymes in identification of two cotton hybrids and their parental lines.

SDS-PAGE of salt soluble globulins was recommended as more useful than tris-soluble proteins in distinguishing cotton cultivars by Rao *et al.* (1990) and

Nerkar and Rao (1993). They reported that the G. *arboreum* and G. *hirsutum* varieties, hybrids like H-4, Godavari and Varalakshmi were easily identified on the basis of globulin profiles, whereas hybrids NHH-44, AHH-478 and DCH-32 were identified better on the basis of tris soluble protein profiles. Shen *et al.* (1994) analyzed alcohol soluble seed proteins of 30 varieties of 6 geographic races of G. *arboreum* by SDS-PAGE. They reported 9 bands of different patterns distributed in common in each electrophoregrams revealing the existence of both differences and affinity among the varieties and races of G. *arboreum*. Zang *et al.* (1998) have reported AUG-PAGE (acetic acid, urea, glycine-polyacrylamide gel electrophoresis) to be a simpler, better and rapid method. They reiterated that the water-soluble proteins were plentiful, clear and polymorphic, hence could be used for cultivar identification. Similarly, Abdel *et al.*, (1993c) identified eleven cotton cultivars on the basis of electrophoretic profiles of seed proteins and some isoenzymes.

Wang *et al.* (1990) evaluated 73 accessions of G. *herbaceum* by electrophoretic analysis of water-soluble seed proteins. They classified the accessions into 18 groups according to the electrophoretic banding pattern. They also suggested that SDS-PAGE of soluble proteins could be used in identifying the variations and their inheritance among the plant species for their classification. Hence, SDS-PAGE of seed proteins demonstrate good potential to be used as a marker for identification of cotton genotypes.

Isoenzymes for cultivar identification

An isoenzyme is one of the multiple forms of an enzyme having the similar / identical catalytic activities. Electrophoretic analysis of isoenzymes has been performed in a large number of species and in several cases, an isoenzyme banding pattern was found to be controlled by a single gene (Schwartz and Endo, 1968). Consequently, isoenzymes have been more widely used for identification

of crop plants, as they are under relatively simple and well understood genetic control (Goodman and Stuber, 1983). They do not suffer the limitations, as morphological traits, such as variations due to environmental fluctuations, need to grow plants till maturity, limited range of expression etc. (Ainsworth and Sharp, 1989). The use of isoenzyme markers for estimating genetic variability, identification of cultivars and confirming hybridity was adequately reviewed by Cooke (1988, 1995) and Smith and Smith (1992). Cultivar identification and genetic purity testing on the basis of polymorphic isoenzyme banding patterns have been found useful in crops like sorghum (Schertz *et al.,* 1990; Nagaraja, 1998), sunflower (Quillet *et al.,* 1992, Sahoo *et al.*, 2000), pearl millet (Varier *et al.,* 1993), soybean (Pinto *et al.,* 1995), tomato (Vodonicharova *et al.,* 1996), maize (Yu *et al.,* 1998) and rice (Santhy *et al.,* 1998).

Isoenzymes for identification of cotton cultivars

Cherry *et al.,* (1972) observed esterase, leucine amino peptidase and catalase isoenzyme variation between the species of the different cotton genome groups. They suggested that the isoenzyme polymorphism could evaluate intra-specific as well as inter-specific variations. Hybrids H-4, H-6 and their parental lines were characterized on the basis of esterase isoenzyme profile (Agrawal *et al.,* 1988). They found that the presence of female selfed seed within the seed lot could possibly be detected and hence such markers can be employed in genetic purity testing. Abdel *et al.,* (1993c) identified eleven Egyptian cotton cultivars on the basis of esterase, catalase, glutamate oxalo acetate transaminase, peroxidase, leucine amino peptidase and indophenol oxidase isoenzymes.

Genetic diversity studies between the accessions of various *Gossypium* spp. on the basis of isoenzyme polymorphism were attempted by several workers (Wendel *et al.,* 1992; De Joode and Wendel 1992; Saha *et al.,* 1998) with varying degrees of success.

Molecular markers

Varietal identification based on specific genetic markers could provide a suitable system for the purpose of identification and protection of plant varieties and control of seed purity. Conventionally, morphological characters, and in some cases protein and isoenzyme markers are used in most cultivated species (Tanksley and Orton, 1983). However, the ability of isoenzymes to discriminate between genotypes is generally limited due to a small number of loci and insufficient polymorphism among closely related genotypes. Further, morphological markers and isoenzymes have the disadvantages of expression at specific developmental stages. The recently developed DNA technologies, which can assay a greater proportion of the plant genome, provide a plentiful array of additional genomic markers. (Heun *et al,* 1994).

Molecular markers offer a number of advantages over isoenzyme markers in demonstrating the distinctness. These are consistent in every living tissue of the plant, at any stage of growth (Morell *et al.,* 1995) and being unlimited in number, a thorough sampling of genome is possible (Helentjaris *et al.,* 1985). The advent of several methods for DNA analysis has widened the possibilities of applying such technologies for the purpose of variety identification and protection (Smith, 1995). The two commonly adopted approaches in the use of molecular markers are essentially either probe-based restriction fragment length polymorphism (RFLP) (Botstein *et al.,* 1980) or detection of amplification based polymorphic DNAs (Williams *et al.,* 1990).

The potential value of RFLPs in crop improvement, varietal characterization, protection and its ability to generate highly specific fingerprints have been pointed out by a number of workers (Helentjaris *et al.,* 1985; Jeffrys *et al.,* 1985). Despite their usefulness, the application of RFLP markers for routine

variety identification purpose is limited being time consuming, expensive, the need for short lived radio isotopes for detection and requirement of a large amount of DNA (Winter and Kahl, 1995).

Williams *et al.* (1990) proposed the use of random amplified polymorphic DNAs (RAPDs) as an additional form of molecular markers. The advantages of this technique over RFLPs include faster data production, a protocol that requires less DNA, and no radioactivity. Problems concerning reproducibility of results can be eliminated by optimizing the experimental protocol (Williams *et al.,* 1990; Heun and Helentjaris, 1993). RAPD analysis involves polymerase chain reaction (PCR) technique which works on the principle of amplification of random DNA sequences, in which short DNA primers of known sequences, chosen on arbitrary basis are used to amplify those regions of genome where the primer binds sufficiently close on opposite strands to allow amplification of the intervening DNA (Williams *et al.,* 1990). No prior knowledge of the DNA sequence is necessary. Morell *et al.* (1995) observed a consistent relationship between the variability observed through RAPD analysis and isoenzyme and / or protein markers. RAPD technique has been exploited for the purpose of variety identification in many crop species by several workers *viz.*, in canola (Marshall *et al.*, 1994), red gram (Ratnaparkhe *et al.*, 1995), tomato (Arens *et al.,* 1995), wheat (Farooq *et al.,* 1994), pepper (Prince et *al.,* 1995), cichorium (Bellamy *et al.*, 1996), maize (Yu *et al.,* 1998), soybean and maize (Zhang *et al.*, 1996), carrot (Grzebelus *et al.*, 1997), peanut (Huang and Tsai, 1997), pearl millet (Prasada Rao, 1999), rice (Santhy, 1999) etc.

Molecular markers for the cultivar identification in cotton

Geng *et al.* (1995) applied RAPD protocol for characterizing cotton (*Gossypium hirsutum*) cultivars *viz.*, Chuan kang-77 and Chuanmian-109 (which are resistant to aphids), cv. Chuan 98 (resistant to mites) and Jiang Sumian. Out of the 14

primers tried, one primer produced a polymorphic fragment of about 350 bp, which varied among the cultivars. Thus, RAPD markers could not only characterize the cultivars but also could tag the gene of interest. Similarly, Multani and Lyon (1995) produced genetic fingerprints of twelve Australian cotton cultivars using 30 random decamer primers in RAPD analysis. They characterized ten of the G. *hirsutum* cultivars individually on the basis of cultivar specific RAPD markers, thus indicating that there exists good possibility of differentiating closely related cultivars by molecular markers. Subsequently, Wajahtullah *et al.*, (1997) analyzed the genomic relatedness among the Australian cotton cultivars using RAPD technique. Convincingly, the classification of varieties based on the morphological and RAPD markers produced similar results in 16 elite US cotton cultivars (Tatineni *et al.*, 1996).

Evaluation of genetic diversity of some elite cotton varieties of Pakistan was studied by Iqbal *et al.*, (1997). They evaluated 22 varieties belonging to *Gossypium hirsutum* L. and one to *Gossypium arboreum* L. using 50 random decamer primers following the polymerase chain reaction. Forty-nine primers detected polymorphism in all the 23 cotton varieties. They concluded that the inter-varietal genetic relationships of several varieties are related to their centre of origin and most of the varieties have a common genetic base. Similarly, Wang *et al.*, (1997) used the RAPDs for detecting the genetic diversity of 25 short seasoned cotton (G. *hirsutum*) cultivars. They reported that 25 cultivars could be classified into 4 sub groups on the basis of cluster and similarity analysis of RAPD fingerprinting of each cultivar amplified with 18 random primers.
Krishna and Jawali (1997) studied the applicability of RAPD technique for distinguishing the hybrid NHH-44 from its parental lines.

Genetic purity testing

Better agronomic performance of a hybrid results from the heterotic effects obtained by crossing inbred lines, which differ genetically, but possess good combining ability (Sprague, 1977). Superior hybrids are the products of superior inbreds selected by the plant breeders for desired agronomic traits. Maintenance of the genetic make up of the inbreds used in hybrid seed production is essential, if performance gains achieved by plant breeders are to be passed on to growers (Lambert and Sprague, 1984). The objective of genetic purity testing is to determine the extent to which the submitted seed sample confirms to the kind specified.

Proper field isolation and purity of the initial seed stock are critical requirements for successful multiplication of the parental lines and production of high quality hybrid seed. Contamination by foreign pollen either from neighboring fields or off type plants within the inbred plot itself can result in a segregating seed lot, unfit for use in hybrid seed production. Hence, observing the uniformity of morphological traits in control plot tests assesses the genetic purity of inbred seed lots. To determine the genetic purity of hybrid seed, it is put through stringent quality control tests to verify that

i. the designated cross occurred,
ii. the number of self or sib pollinations between plants of the female parents meets the necessary purity standards required by law and,
iii. the product has an adequate quality level of germination.

In a field grow out test, a representative sample of the F_1 seed is examined to differentiate the true hybrid seed plants from the off types on the basis of morphological descriptors.

Biochemical markers for genetic purity testing

Good and unambiguous morphological markers are generally limited in number, and their expression depends on the developmental and growth stages in the plant. In hybrid seed production, the time between the seed harvest and subsequent sowing is normally short, and so it is difficult for seed producers to test the genetic purity of the seed by conventional methods in such a short period. The characteristics of open pollination also make control of seed purity difficult thus seed contamination can cause heavy losses for farmers. Fast, simple and reliable methods are, therefore desired for assessing the genetic purity of hybrid seed samples. For this purpose, polymorphic markers based on electrophoretic patterns of proteins and isoenzymes have been used for genetic purity testing of various crop species.

Wang *et al.* (1994bb) developed a high resolution PAGE system (discontinuous PAGE under acidic conditions) for maize cultivar identification and genetic purity assessment. They separated maize salt soluble proteins into numerous components showing high heterogeneity. Among 23 tested maize cultivars, which included 10 hybrid combinations, each cultivar had a characteristic PAGE pattern, which could be useful in assessing the genetic purity of hybrid cultivars and parental lines. Tanyolac *et al.* (1996) studied the PAGE banding pattern of gliadin sub units of two wheat crosses for assessing the genetic purity, in which one off type cross seed in both the hybrid samples could be detected.

Dadlani *et al.* (1994) compared field grow out test with electrophoresis of seed proteins for testing the genetic purity of cotton hybrid NHH-44. Out of 400 single seeds tested using SDS-PAGE, 45 exhibited banding pattern different from standard NHH-44 sample. Similarly, in the field grow out test, 48 plants out of 424 were detected as off-types. Thus, in both the cases, the percentage of

off-types were almost equal, showing the reliability of the electrophoresis technique vis-à-vis the field grow out test.

Electrophoresis of isoenzyme analysis has been demonstrated to be useful for the genotypic characterization (Smith, 1988) and superior to field grow out tests for detecting the female selfs in hybrid seed lots (Smith and Wych, 1986). Isoenzyme analysis by electrophoresis for detection of various types of genetic contamination has been attempted by various workers in most field crops *viz.*, maize (Orman *et al.*, 1991; Bilgen *et al.*, 1995), tomato (Zlokolika *et al.*, 1997), pearl millet (Varier *et al.*, 1993), wheat (Tanyolac et *al.*, 1996), pepper (Zheng *et al.*, 1997). In cotton, Agrawal *et al.* (1988) suggested the possible use of esterase isoenzyme profile in genetic purity testing of hybrids H-4 and H-6.

Orman *et al*., (1991) determined the accuracy of laboratory and field results by comparing the square root of sum of squared differences. On average, laboratory isoenzyme purity observations were 1.6 times more accurate than morphological field observations for outcross contamination measurement. The accuracy of outcross detection did vary depending upon the inbred. Whereas, for off-type detection, laboratory results were on average 4 times more accurate than field observations. They concluded that there were no statistically significant differences in accuracy between the field and laboratory test results.

Molecular markers for genetic purity testing

DNA markers are independent of environmental conditions and are present in all plant cells, in contrast to markers based on morphological and biochemical characteristics. RAPD markers have been successfully used for genetic finger printing. Because of the Mendelian segregation (dominant markers) of the molecular markers, RAPD polymorphic profile could be applied to evaluate the genetic purity of F_1 hybrid seed samples. Hence, it has been used for testing the

genetic purity of F_1 hybrid seed samples in most of the crop species *viz.*, pepper (Choe *et al.,* 1998, Ballestar *et al.,* 1998); watermelon (Hashimuze *et al.,* 1993); tomato (Hashimuze *et al.,* 1993, Rom *et al.,* 1995, Paran *et al.,* 1995); chicorium (Bellamy *et al.,* 1996); rice (Xie *et al.,* 1997, Arti *et al.,* 1998); carrot (Grzebelus *et al.,* 1997).

Bellamy *et al.,* (1996) characterized hybrids and parental lines of chicorium using 100 decamer primers. They observed that genetic purity testing on the basis of RAPD analysis was easier and less time consuming. Meng *et al.* (1998) determined the genetic purity of two F_1 hybrids and their parental lines in Chinese cabbage using RAPD markers. For obtaining the standard DNA samples of F_1 hybrids and their parents, pooled samples from 30 individual seeds were used. One person could test more than 100 single seeds per day using this method. The whole procedure of DNA extraction and PCR amplification for the genetic purity testing of F_1 hybrid could be completed in about four days. They suggested that this approach greatly improves the level of seed quality assurance for Chinese cabbage hybrid seed production.

Sequential sampling

Agrawal (1985) reported that for one per cent off-type plants, which is the maximum permissible limit, at least 400 plants need to be tested during the field plot test. An equal number of seeds (i.e., 400) therefore, need to be tested individually in electrophoresis analysis. However, for the total replacement or supplementing the field grow-out test by electrophoretic analysis, Agrawal *et al.* (1988) found the sample size to be the major constraint. They suggested that sequential analysis technique needs to be followed for the purpose of reducing the sample size to a batch of 20 or more, which would mean upto 95 percent saving in workload. Singh and Agrawal (1995) devised a statistical model of sequential sampling procedure for testing genetic purity, if specifications are predetermined. They illustrated the possible application of sequential

sampling procedure by limiting the sampling size to 109 individual seeds in case of hybrid cotton purity analysis, wherein the specification of 1:100 off type seeds are maximum permissible. They suggested that the minimum number of seeds required is 109 (1.09N) for acceptance, but can be rejected at any stage due to higher number of contaminants. The identification of individual seeds is analyzed by an appropriate laboratory technique and the number of selfed seeds and male / off types are counted separately.

Singh and Singhal (1999) studied the comparative efficiency of sample sizes using statistical tools for obtaining significant results in field grow out test. They observed that a sample size of 300 seeds gave significantly similar results as that based on 400 seeds. Hence, a sample size of 300 seeds was assumed to be sufficient for conducting field grow out test in hybrid cotton.

MATERIALS AND METHODS

Chapter III
MATERIALS AND METHODS

The experiments were conducted with an objective to determine the usefulness of chemical tests and biochemical and molecular markers for identification and genetic purity testing of cotton (*Gossypium hirsutum.*) hybrids. For the purpose of identification, genotypes were characterized on the basis of seedling response to modified nutrient medium under controlled conditions, colour of the seed extract in organic solvents and biochemical (protein and isoenzymes) and molecular (RAPD) markers.

The genetic purity of the commercial seed samples of cotton hybrids was evaluated by field grow-out trials and laboratory evaluation using standardized electrophoresis technique. The results obtained in the laboratory tests were compared with those of the field grow-out trials for testing the reliability of the techniques.

To reduce the sample size for genetic purity analysis, sequential sampling procedure was tested.

Seed material

The seed material for the present study consisted of five inter-hirsutum cotton (*Gossypium hirsutum*) hybrids and their parental lines. Seeds were collected through the Project Coordinator (cotton), CICR from the following sources. Parentage of the hybrids used for characterization is given below:

Hybrid	Female parent	Male parent	Source
Savitha	T-7	M-12	CICR, Regional Station, Coimbatore
PKHy-2	AK-32	DHY-286-2	P.D.K.V., Akola
NHH-44	BN-1	ACC-738	Cotton Research Centre, Nanded.
H-10	BC-68/2	LRA-5166	CICR, Regional Station, Coimbatore
PHH-316	PH-93	PKV-081	P.D.K.V., Akola

Of these, hybrid PHH-316 is based on CMS system of the female parent PH-093.

3.1 Identification of cotton hybrids and their parental lines

3.1.1 Chemical tests

3.1.1.1 Seedling response to modified nutrient medium and chemical applications under controlled conditions:

Experiments were conducted during 1997-98 in controlled growth cabinet and the selected tests were performed in 1998-99 in the glass house, Division of Seed Science and Technology, IARI, New Delhi. Seeds of five hybrids and their female parents were sown in sand medium in three replications of 15 seeds per pot. Sterilized sand was filled $3/4^{th}$ in plastic pots of 4.0" diameter and 4.0" height. Growth conditions were maintained at 25°C and continuous light. The inert sand was 70% saturated with normal or modified Hoagland solutions (Appendix -1) *viz.*, to create

I. deficiency and toxicity of sodium,

II. deficiency and toxicity of calcium, and

III. toxicity to 2,4-D. (5ppm and 10ppm) .

Table 1: Morphological descriptors of cotton hybrids and their female parental lines

Characters	Savitha (H)	T-7 (F)	PKHy-2 (H)	AK-32 (F)	NHH-44 (H)	BN-1 (F)	H-10 (H)	BC-68/2 (F)
Plant habit	Open, short internodes	Pyramid	Bushy	Semi erect	Bushy, open	Open	Erect	Open
No. of monopodia	0-3	1-3	3-4	2-4	2-3	1-2	3-4	2-3
Leaf colour	Light green	Dark green	Light green	Dark green	Light green	Light green	Light green	Light green
Leaf hairiness	Medium	Moderate	Dense	Short dense	Medium hairy	Medium hairy	Medium hairy	Hairy
Leaf nectaries			Present	Present	Present	Present		
Leaf lobes	3-5	3-5	3-5 (Broad)	3-4 (Medium)	3-5	3-5	3-5	3
Petal colour	Cream	Cream	Sulphur yellow	Yellow	Light yellow	Cream	Creamy white	Basal colour
Anther colour	Yellow	Yellow	Pale yellow	Pale yellow	Yellow	Yellow	Yellow	Yellow
Petal spot	Absent	Absent	Purple	Purple	Absent	Absent	Absent	Absent
Bracts			Less serrated	Medium serrated				
Boll shape & size	Medium ovate, beaked tip	Medium ovoid	Medium ovate	Medium	Medium round	Slightly elongated	Oval, pointed tip	Big, Oval

Source: Seed Certification Diary, 1999, A.P. Seed State Certification Agency, Hyderabad.

The sand was moistened with respective solutions on every fourth day. Ten seedlings were maintained per pot and data were recorded on 20th day on shoot length, proportion of shoot-root growth, leaf area, leaf shape, leaf colour, trichome density.

3.1. .2 Colour intensity of the seed extract in organic solvents

3.1. .2.1 Anthocyanin intensity

Estimation of Anthocyanin content (Oleze-Karow and Mohr, 1978)

200 mg of the seed material, hydrated for 72 hours and decoated, was crushed in a pestle and mortar and transferred to 10mL test tubes. To this 5mL of acidified methanol (Conc. HCl was added to make up 1% acidified methanol reagent) was added. Test tubes were wrapped in black paper and maintained at 4°C overnight. The supernatant was decanted and sediments, if any, were separated by centrifugation.

The colour variation of the methanolic extract (ranging from yellow to wood) was recorded by visual comparison (Ridgway, 1912) and documented photographically for quick comparison of cultivars. The intensity of the anthocyanin colouration in the methanolic extract was determined by measuring the OD of the decanted extract at 653 nm in a spectrophotometer.

3.1. .2.2 Gossypol intensity

Estimation of Gossypol content (Sadasivam and Manickam, 1997)

Reagents

Phloroglucinol reagent: 5g Phloroglucinol was dissolved in 100mL of 80% ethanol.

The gossypol content was estimated following the method of (Sadasivam and Manickam, 1997). 5gm seed imbibed in water for 48hr was decoated and homogenized in 95% ethanol in a pestle and mortar. Homogenized material was transferred into clean test tubes and heated in boiling water bath for 5 minutes. Extract was collected by filtering through Whatman no.1. The same was repeated until the extraction from the residues was complete. The extracts were pooled and, the pH was adjusted to 3.0 using 1N HCl and it was diluted with 40% ethanol to a volume of 10mL. 1.5 volumes of diethyl ether was added to the ethanolic extract and incubated at 10°C for an hour. Ether phase was separated following several washes with distilled water. Ether extract was evaporated to dryness and redissolved in 95% ethanol to a volume of 5 mL.

The colour variation (from pale buff to purple) was recorded by visual comparison (Ridgway, 1912) and documented photographically for quick comparison of cultivars. 1mL of the extract was pipetted out in test tubes and added with 0.5 ml of Phloroglucinol reagent, followed by 1mL of conc. HCl to each tube. Samples were incubated for 30 min. at room temperature with occasional stirring. Volume was made up to 10mL with 80% ethanol. The relative amount of gossypol was estimated by measuring the OD of the samples at 550 nm against a ethanol blank.

3.1.3 Biochemical markers

Electrophoresis of soluble seed proteins and isoenzymes were performed following standard techniques.

3.1.3.1 Total soluble seed proteins

Total tris soluble proteins were electrophoresed by the modified procedure of Lammaeli (1971) described by Dadlani and Varier (1993).

Reagents for extraction and electrophoresis are given in Appendix 2.

Ten seeds from each genotype were decoated, powdered and defatted using 20 ml of defatting solvent mixture (A1) for 48 hrs with atleast 5 solvent changes. Fifty milligrams of ground material, air dried at room temperature, was taken in a clean 1.5 ml Eppendorf tubes, to which 0.5 ml of working protein extraction solution (A-3) was mixed well and kept overnight at room temperature. The samples were heated in a boiling water bath for 10 minutes, cooled and centrifuged at 15,000 rpm for 10 min. The supernatant was collected for electrophoresis.

Electrophoresis

Electrophoresis was conducted in a Bio-Rad Protean II vertical Electrophoresis Unit. 20 μl of protein extract was carefully loaded on a 15 % denaturing polyacrylamide gel using a micro syringe. A tracking dye (Bromophenol blue) was added to the upper tank buffer. The gel was run at maximum volt and constant power of 30amp per plate till the tracking dye reached the bottom of the gel.

Fixing and staining

The gel was fixed overnight in 15% Trichloroacetic acid (TCA). After fixing, the gel was rinsed with distilled water and immersed in a mixture of 15 ml of 2 per cent coomassie blue (in rectified spirit) and 100 ml of 15

per cent TCA. Staining was done till the bands developed. Destaining was done in distilled water till the background was clear. Later the gels were photographed.

Evaluation and documentation

The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

Relative mobility (Rm) of each band was calculated as

$$\text{Rm.} = \frac{\text{Distance traveled by the band}}{\text{Distance traveled by the tracking dye}}$$

Bands were numbered on the basis of increasing Rm values.

3.1.3.2 Salt soluble Globulins

Globulins were analysed by the procedure described by Anisimova et al. (1991).

Reagents for extraction and gel electrophoresis are given in Appendix 3

50 mg of defatted seed material was taken in a clean Eppendorf tube. To that 0.5 ml of extraction buffer (1.1) was added and mixed thoroughly. The sample was incubated for 5-6 hours in room temperature. The contents were then centrifuged at 10,000 rpm for 20 minutes. The supernatant was transferred to a centrifuge tube, to which a 10-fold volume of chilled distilled water was added and kept at +4°C overnight for cryoprecipitation. The following day the contents were centrifuged for

30 minutes at 10,000 rpm. The supernatant was discarded and the residue was dried at room temperature. Dried pellet (globulin) was transferred to clean Eppendorf tubes. The globulin was then dissolved with a minimal volume of tris glycine buffer (1.2). 40-50 μL of dissolved globulin was taken in another tube and mixed with equal volume of working sample buffer (1.3.2) to be used for electrophoresis.

Electrophoresis

Electrophoresis was conducted using Bio-Rad Protean II vertical electrophoresis unit.
10-15 μL of the sample (globulin extract) was carefully loaded on a denaturing polyacrylamide gel using a micro syringe. The gel was run at maximum voltage and constant power of 30amp per plate till the tracking dye reached the bottom of the gel.

Fixing, staining and evaluation was done as described in 3.1.2.1

3.1.3.3 Isoenzyme markers

For the purpose of the identification of hybrids and parental lines, polymorphism among genotypes with respect to the following isoenzymes was analyzed.

i) Acid phosphatase
ii) Estérase
iii) Superoxide dismutase
iv) Alcohol dehydrogenase
v) Malate dehydrogenase
vi) Peroxidase
vii) Catalase

Reagents for extraction and gel electrophoresis of isoenzymes are given in the appendix 3

Preparation of sample

For extraction of ADH isoenzyme, five seeds of each genotype were soaked in distilled water in airtight tubes for 48 hours. Outer seed coat was removed and seeds were ground using chilled mortar and pestle over ice with 0.30 ml extraction buffer. After fine grinding, the sample was taken in a clean 1.5 ml Eppendorf tubes and centrifuged at 12,000 rpm for 30 minutes at 4°C. The supernatant was transferred to another 1.5 ml Eppendorf tube and centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was used for electrophoresis.

For the rest of the isoenzymes, seedlings were raised at 25°C, and 5-7 days old coleoptiles were used for extraction of enzymes. 5 coleoptiles were crushed with 0.25-0.3 ml extraction buffer at 4°C in a chilled pestle and mortar. The extracts were centrifuged at 10,000 rpm for 20 min at 4°C. The clear supernatant was collected and used for loading.

Electrophoresis

Electrophoresis was conducted using a Bio-Rad Protean II electrophoresis unit in an alkaline native polyacrylamide gel system (Davis, 1964). 70 μl of sample was loaded in each well. Bromophenol blue was added to the upper tank buffer as tracking dye. The electrophoresis unit was connected to the powerpack and electrophoresis was conducted at 4°C. Voltage was set to maximum and constant current of 30 amps per gel was given till the tracking dye reached the bottom of the gel. The gels were stained in appropriate solutions.

Reagents for staining of all isoenzymes are given in the appendix-5

Evaluation of the gels

The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

Relative mobility (Rm) of each band was calculated as

$$\text{Rm.} = \frac{\text{Distance traveled by the band}}{\text{Distance traveled by the tracking dye}}$$

Bands were numbered on the basis of increasing Rm values.

3.1.4 Molecular markers

3.1.4.1 DNA isolation

DNA was isolated from the seeds following the method of Krishna and Javali (1997) with some modifications. This method is particularly suitable for single seed analysis in such crops where the quality of DNA gets adversely affected during the process of isolation due to presence of polyphenols, polysaccharides etc. due to their ability to bind with nucleic acids. The present method gave good quality DNA following a simple protocol.

Reagents

Reagents for DNA extraction, purification and electrophoresis are given in Appendix 5.

Protocol

1. Seeds were soaked overnight at room temperature.
2. Seed coat was removed and 3-4 grams of cotyledon tissue were crushed in a prechilled mortar and pestle.
3. 15 ml of DNA extraction buffer (solution A) and 1.5 ml of 20% SDS (solution B) was added to the sample which was transferred to a centrifuge tube and pulverized.
4. The sample was incubated at 65°C for 10 min with occasional gentle swirling and then cooled on ice for 10 min.
5. 5.0 ml of potassium acetate (solution C) was added and mixed thoroughly.
6. The mixture was centrifuged at 13,000 g for 20 min at 4°C.
7. 700 μl of clear supernatant was collected in 1.5 ml Eppendorf tube.
8. Same quantity of isopropanol and ammonium acetate (3:1) mixture (solution D) was added to precipitate nucleic acids.
9. Contents were mixed thoroughly and centrifuged at 10,000 rpm for 20 min to pellet the DNA.
10. Supernatant was discarded and DNA pellets were washed twice with 70% alcohol to remove the salts.
11. Pellet was air dried at room temperature in aseptic conditions and dissolved in 500 μl of TE buffer (solution F).

3.1.4.1.1 Purification of DNA

Inclusion of SDS at the time of DNA extraction helps in the precipitation of proteins. Potassium acetate facilitates the SDS-protein precipitation. RNA was removed by treating the sample with RNAase.

Treatment of RNAase

1. RNAase was added to DNA sample @ 50 µg/500 µl and incubated at 37°C for one hour.
2. An equal volume of phenol-chloroform (1:1) was mixed and the tubes were centrifuged at 10,000 rpm for 5 min at room temperature.
3. The aqueous phase was transferred into a fresh microcentrifuge tube.
4. Extraction with chloroform : isoamyl alcohol (24:1) was done twice and aqueous phase separated out.
5. 0.5 volume of 3M sodium acetate buffer (pH 4.8) was added and mixed properly.
6. 2.5 times chilled absolute alcohol was added and mixed to precipitate the DNA.
7. DNA was pelleted by centrifugation at 10,000 rpm for 5 min.
8. The supernatant was decanted carefully and the pellet was washed with 70% ethanol, dried aseptically and dissolved in 50-100 µl buffer.

3.1.4.1.2 Checking the quality and quantity of DNA

This was done by agarose gel electrophoresis.

0.8 per cent gel was prepared by mixing 1.2 g of agarose in 150 ml of distilled water. It was heated in a microwave oven till agarose was dissolved. After cooling to room temperature, ethidium bromide was added @ 50 µg/ml. This was poured into the gel casting tray in which combs were set. After one hour, the gel was solidified and the combs were removed to form the wells.

To 2 μl of DNA sample, 2.5 μl of dye and 10.5 μl TE buffer was mixed and loaded into the wells. Gene ruler DNA ladder plus was loaded as control in the corner well (3 μl of marker DNA ladder plus and 2 μl of dye). Gel was run at 50V for 1 h. The quality of the DNA was judged by the nature of the band at the corresponding position of the control. Presence of a single compact band indicated that isolated DNA was of high molecular weight and good quality. The approximate quantity of the DNA was estimated in the sample by comparison with control.

After quantifying the DNA of each genotype, the samples were diluted with TE buffer so that final concentration of DNA was 12.5 ng/ml. Depending on the quantity of DNA in the samples, different volumes of TE buffer was added to get the above mentioned concentration of DNA.

3.1.4.2 RAPD analysis

RAPD analysis was carried out using standard Polymerase Chain Reaction (PCR), which is a technique, used to selectively amplify *in vitro* a specific segment of the total genomic DNA a billionfold (Mullis *et al.*, 1986). The random oligonucleotides obtained from Operon Tech. USA were used as primers. Sixty random primers from Operon kits *viz.*, OPN 1-20, OPO 1-20 and OPR 1-20 were used to produce amplification products in the DNA from five hybrids and parental lines.

List and details of primers are given in appendix-6.

Setting the PCR Reaction

1. In a sterile 0.2 ml thin walled PCR tube, the following components were mixed in the order as given below:

Sterile double distilled water	18 μl
10 x buffer	2.5 μl
dNTP mix (Bangalore Genei, India) (containing 200 μM each)	0.5 μl
Primer (Operon Tech, USA)	1 μl
Taq DNA polymerase enzyme (Bangalore Genei, India)	0.5 units
Template DNA	2 μl

2. The reaction was carried out in a thermocycler (Perkin-Elmer, Model 9600, USA) with the following specifications:

Cycle 1	Denaturation (92°C)	4 min
	Primer annealing (37°C)	1 min
	Primer extension (72°C)	2 min
Cycle 2-44	Denaturation (92°C)	1 min
	Primer annealing (37°C)	1 min
	Primer extension (72°C)	2 min
Cycle 45	Denaturation (92°C)	1 min
	Primer annealing (37°C)	1 min
	Primer extension (72°C)	7 min

3. At the end of the run, the tubes were taken out and 2.5 μl of loading dye was added.
4. The amplification products were stored at 4°C till their electrophoretic separation.

Agarose gel electrophoresis

The amplification products were separated by electrophoresis in 1.5 per cent agarose gel. Gel and TAE buffer were prepared as mentioned earlier. Gel was run at 50V for 3 h till the amplified products are separated. The sizes of the amplified fragments were determined by using size standard molecular weight marker (Gene Ruler 100 bp DNA Ladder plus, MBI). DNA fragments were visualized under UV light and photographed.

Scoring and evaluation RAPD

The amplification products resolved on agarose gels were scored separately for each primer. The bands observed on the gels were compared across the lanes for products with similar molecular weight. The bands were scored for the presence or absence by assigning a value of '1' for presence and '0' for absence in a lane.

3.2 Genetic purity analysis

3.2.1 Testing homogeneity of the parental lines

For the purpose of testing the homogeneity of the parental lines, 30 individual seeds from parental lines of two hybrids Savitha and H-10 were analyzed using electrophoretic band profiles of globulins and EST and ADH isoenzymes.

Reagents and protocol were same as for cultivar identification.

3.2.2 Genetic purity testing of commercial hybrid samples

3.2.2.1 Field Grow-Out Test

Samples from four commercial seed lots each of three hybrids *viz.*, Savitha, H-10 and NHH-44 were obtained from Andhra Pradesh State Seed Certification Agency (APSSCA), Hyderabad. The samples were drawn from the lots which were subjected to field grow out trials at GOT farm, Amaravati in 1998 by the APPSCA for testing the genetic purity. A second field grow out trial was conducted during *kharif* 98 in Genetics "H" block, IARI farm in two replications. Plants were raised under standard field conditions, maintaining row-to-row distance of 60cm and plant-to-plant distance of 40cm and observations were taken in 300-400 plants (depending on the survival of plants from 600 seeds sown per sample) as per the descriptors given in table 1. Presence of selfed female and off type plants was evaluated separately in every row of 25 plants.

3.2.2.2 Biochemical markers

200 individual seeds from each of the four commercial seed samples of three hybrids *viz.*, Savitha, H-10 and NHH-44 were tested for genetic purity using the standardized electrophoresis technique for salt soluble globulin markers.

Reagents and protocol are same as described for cultivar identification.

3.2.2.3 Molecular markers

30 individual seeds of one commercial seed sample of H-10 were tested for the genetic purity analysis using RAPD profile with primer OPN-2 following the standard protocols.

Reagents and protocol were same as described for cultivar identification.

3.3 Sequential sampling

Results of field and laboratory genetic purity analyses of the four commercial seed samples of the three hybrids were analyzed using the sequential sampling procedure (Singh and Agrawal, 1995). For the purpose, a number of 20 individual seeds / plants were examined for the selfed females and offtypes / male types separately. Data were compared with the working tolerance table till a decision is arrived at whether to continue or terminate the testing procedure.

The comparative efficiency of sample sizes (50, 100, 150 & 200 seeds) was tested in the statistical environment on the field grow out tests besides the laboratory test (Singh and Singhal, 1999). Correlation studies between the threshold levels of contaminants observed in different cumulative sub-sample sizes as well between the field grow-out tests and laboratory test.

All data were statistically analyzed using SAS package following the procedures of Panse and Sukhatme (1961).

RESULTS

Chapter-IV
Results

Characterization of fifteen cotton genotypes, comprising of five hybrids and their parental lines, was done on the basis of rapid chemical tests and protein, isoenzyme and molecular markers. Genetic purity testing of commercial seed lots of three cotton hybrids was tested on the basis of protein markers and the results were compared with those of field grow out test. The results of these studies are detailed below.

4.1. Identification of cotton hybrids and their parental lines.

4.1.1 Characterization based on seedling response to nutrient medium and chemical applications

Pot culture experiments were conducted under controlled growth conditions in the glass house. Seedlings were raised in nutrient media, which were deficient or toxic for certain elements or containing a low dose of herbicide 2,4-D.

Seedling characters of the hybrids and their female parents grown under controlled conditions were recorded. The observations on seedling characters *viz.*, foliage colour, leaf area, shoot length and stem diameter for different hybrids and their female parents are presented in table 2 to table 6.

4.1.1.1 **Savitha and T-7**

Seedling response to high concentration and deficiency of sodium

Under high sodium concentrations in the growth medium, hybrid Savitha showed normal seedling growth with slight yellowing of leaves. Seedlings were marginally smaller than control with 3.82 cm of shoot length, 1.26 cm of stem diameter and 20.95 cm^2 of leaf area as compared to 4.18 cm, 1.27 cm and 21.35 cm^2, respectively in the control treatment (Table2). The female parent T-7, on the other hand, exhibited pale yellow seedlings with 2.14 cm of stem diameter, 2.49 cm of shoot length and 13.34 cm^2 of leaf area as compared to 2.25cm, 2.62 cm and 18.27 cm^2, respectively in the control.

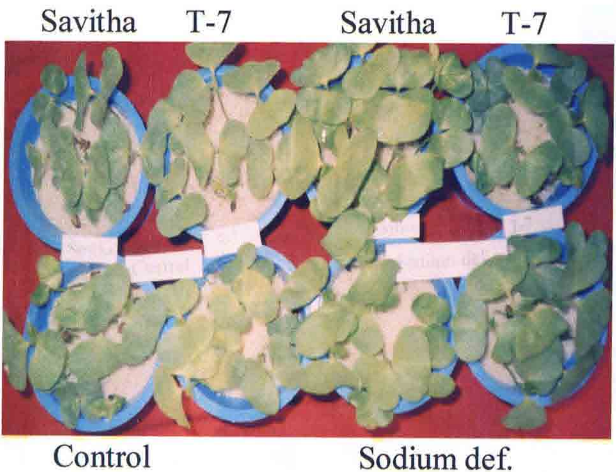
Conversely, both Savitha and T-7 showed normal and vigorous growth under sodium deficiency condition. There was no significant variation in case of stem diameter, shoot length and leaf area. But, the leaves of T-7 were dark green in colour (Fig. 1a &1b).

Seedling response to high concentration and deficiency of calcium

Savitha exhibited normal seedling characters under high calcium conditions, whereas under the calcium deficiency, the seedlings were pale yellow. Seedlings with reduced growth and dark green leaves were observed in T-7 under high calcium conditions with stem diameter of 1.9 cm, leaf area of 14.35 cm^2as compared to 2.25 cm and 18.27 cm^2 in control treatment. Under calcium deficiency treatment, the growth of T-7 seedlings was highly reduced with dark green and wrinkled leaves. The leaf area, stem diameter and shoot length were 10.7 cm^2, 1.38 and 1.98 cm as compared to 18.27 cm^2, 2.62 cm and 2.25 cm, respectively in

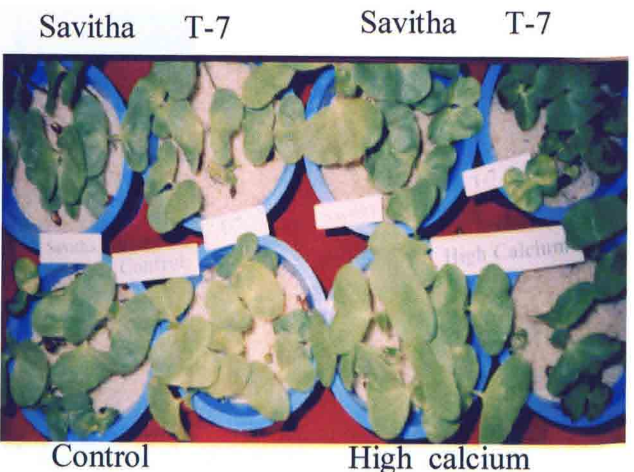
Fig 1a : Seedling growth of cotton hybrid Savitha and its female parent T-7 in different nutrient media

Savitha T-7 Savitha T-7



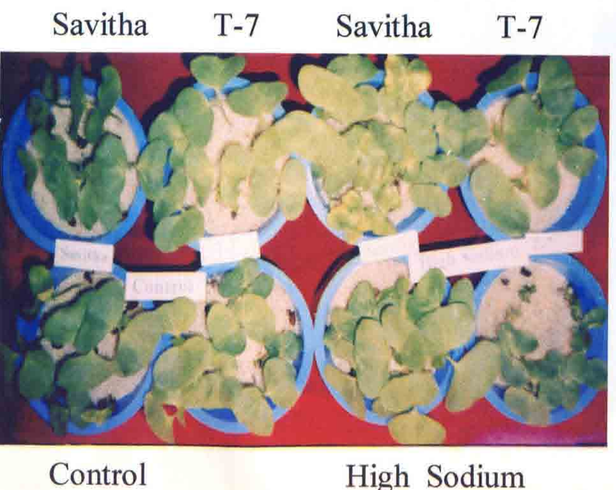
Control Sodium def.

Savitha T-7 Savitha T-7



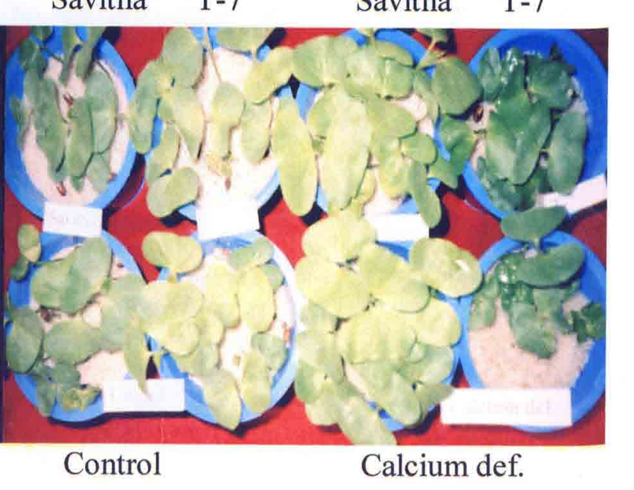
Control High calcium

Savitha T-7 Savitha T-7



Control High Sodium

Savitha T-7 Savitha T-7



Control Calcium def.

Fig 1b : Seedling growth of cotton hybrid Savitha and its female parent T-7 in different nutrient media

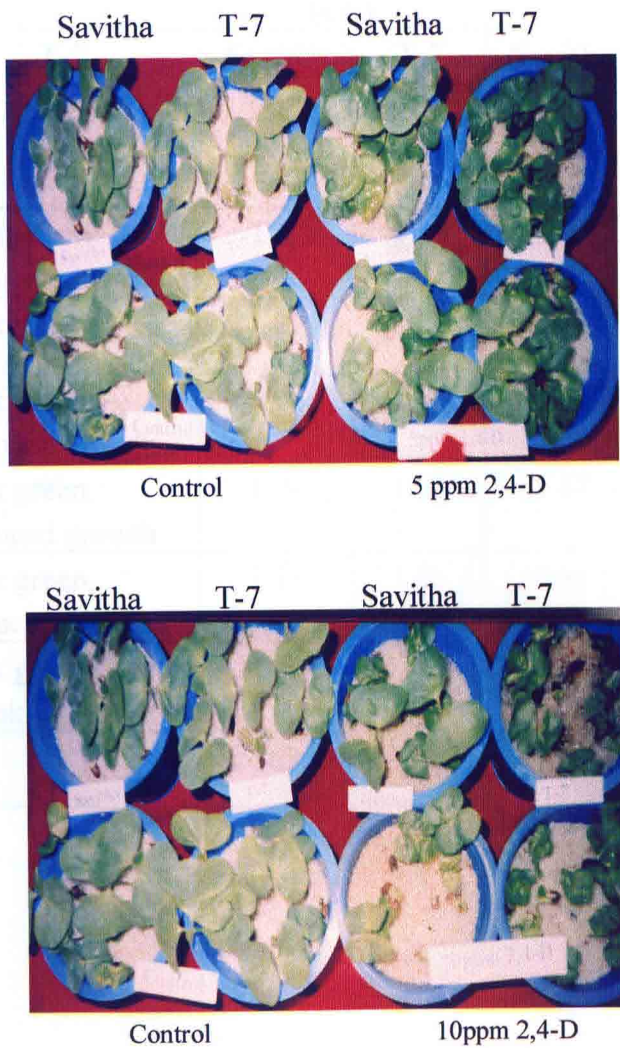


***Table 2*: Seedling response of Savitha and T-7 to modified nutrient media**

Treatment	Colour and growth of seedling		Stem diameter (cm)		Shoot length (cm)		Leaf area (cm^2)	
	Savitha	T-7	Savitha	T-7	Savitha	T-7	Savitha	T-7
Control	Green	Pale yellow	1.27	2.25	4.18	2.62	21.35	18.27
High sodium	Pale yellow	Pale yellow Reduced growth	1.26	2.14	3.82	2.49	20.95	13.34
Sodium def.	Green	Dark green	1.27	2.13	3.76	2.32	24.8	16.7
High calcium	Green	Dark green, Reduced growth	1.32	1.93	3.98	2.52	22.22	14.35
Calcium def.	Pale yellow	Dark green, Reduced growth	1.24	1.38	3.67	1.98	24.62	10.7
5 ppm 2,4-D	Green	Dark green, Reduced growth	1.19	1.21	3.68	1.19	21.35	9.5
10 ppm 2,4-D	Green	Dark green, Wrinkled leaves	1.03	0.98	3.13	1.02	18.31	6.69
C.D.(0.05)			0.0243	0.0239	0.1192	0.0273	0.0380	0.1794

High sodium : 440 ppm
High calcium : 320 ppm
Sodium and calcium deficiency : 0 ppm

control (Table 2) hence, seedling response to calcium deficiency clearly distinguished T-7 from Savitha.

Response to 2,4-D application

With 5-ppm 2,4-D application, seedling growth in Savitha was normal except reduction in shoot length (3.68 cm, as compared to 4.18 cm in control). In case of 10 ppm 2,4-D treatment Savitha exhibited reduced seedling growth with stem diameter of 1.03 cm, shoot length of 3.13 cm and leaf area of 18.31 cm^2as compared to 1.27 cm, 4.18 cm and 21.35 cm^2, respectively in control. Conversely, In T-7, the female parent of Savitha, seedlings in 5 ppm 2,4-D exhibited reduced growth and dark green leaves with 1.21 cm of stem diameter, 1.19 cm of shoot length and 9.5 cm^2 of leaf area as compared to 2.25 cm, 2.62 cm and 18.27 cm^2 in control. At 10 ppm 2,4-D application, T-7 seedlings showed dark green and highly wrinkled leaves with 0.98 cm stem diameter, 1.02 cm shoot length and 6.69 cm^2 leaf area as compared to 2.25 cm, 2.62 cm and 18.27 cm^2 in control (Table 2), which clearly distinguished it from the hybrid Savitha.

Hence, hybrid Savitha and its female parent T-7 could be distinguished on the basis of their response to calcium deficiency and 10 ppm 2,4-D application.

4.1.1.2 PKHY-2 and AK-32

Seedling response to high concentration and deficiency of sodium

The response of the hybrid PKHy-2 and AK-32 to high concentration of sodium was marginal with no significant variation in terms of seedling appearance though the colour of PKHy-2 seedlings were green and that of AK-32 were pale yellow

Table 3: Seedling response of PKHy-2 and AK-32 to modified nutrient media

Treatment	Colour and growth of seedling		Stem diameter (cm)		Shoot length (cm)		Leaf area (cm^2)	
	PKHy-2	AK-32	PKHy-2	AK-32	PKHy-2	AK-32	PKHy-2	AK-32
Control	Green	Pale yellow	1.27	2.25	3.18	3.28	24.8	20.9
High sodium	Green	Pale yellow	1.19	2.10	3.01	3.0	21.6	19.8
Sodium def.	Green	Reduced growth	1.20	1.19	3.20	3.10	20.8	16.5
High calcium	Reduced growth	Pale yellow	1.11	2.10	2.7	3.13	19.2	19.8
Calcium def.	Green	Reduced growth	1.26	2.20	3.10	2.97	25.4	19.2
5 ppm 2,4-D	Green	Reduced growth, Dark green	1.10	1.9	2.95	2.65	22.5	16.8
10 ppm 2,4- D	Reduced growth	Dark green, Wrinkled leaves	1.03	1.10	2.72	1.95	20.3	11.3
C.D.(0.05)			0.1192	0.273	0.0133	0.3273	0.1987	0.0394

High sodium : 440 ppm
High calcium : 320 ppm
Sodium and calcium deficiency : 0 ppm

(Table 3). In PKHy-2, stem diameter, shoot length and leaf area were 1.19 cm, 3.01 cm and 21.6 sq cm as compared to 1.27 cm, 3.18 cm and 24.8 cm^2 in control, whereas in T-7, stem diameter, shoot length and leaf area were 2.10 cm, 3.0 cm and 19.8 sq cm as compared to 2.25 cm, 3.28 cm and 20.9 cm^2in control.

Under the conditions of sodium deficiency, a significant reduction in leaf area and stem diameter of T-7 seedlings was observed *i.e.*, 16.5 $cm^2$and 1.19 cm as compared to 20.9 cm^2 and 2.25 cm in control. In case of PKHy-2, no such variation was observed.

Hence, significant reduction in seedlings growth of AK-32 under sodium deficiency condition could be used for distinguishing AK-32 from PKHy-2.

Seedling response to high concentration and deficiency of calcium

A significant reduction in the seedling growth of PKHy-2 was seen at high concentrations of calcium as its leaf area (19.2 cm^2) and shoot length (2.7 cm) were significantly lower as compared to control i.e., 24.08 cm^2 and 3.18 cm, respectively. In case of AK-32, seedlings were normal, pale yellow with no significant reduction in seedling growth.

Under the conditions of calcium deficiency seedling growth in PKHy-2 was as vigorous as in control with 1.26 cm stem diameter, 3.10 cm shoot length and 25.4 cm^2leaf area as compared to 1.27 cm, 3.18 cm and 24.8 cm^2, respectively in control. There was no significant variation in the performance of AK-32 seedlings under calcium deficiency. Hence, the variation observed in PKHy-2 in response to high concentration of calcium treatments could be used for distinguishing the PKHy-2 and AK-32.

Response to 2,4-D application

On treatment with 5 ppm 2,4-D, PKHy-2 seedlings exhibited almost normal growth with stem diameter, shoot length and leaf area of 1.10 cm, 2.95 cm and 22.5 cm^2 as compared to 1.27 cm, 3.18 cm and 24.8 cm^2, respectively in control. But, AK-32 showed marked reduction in seedling growth, with dark green leaves and stem diameter of 1.9 cm, shoot length of 2.65 cm and leaf area of 16.8 cm^2as compared to 2.75 cm, 3.28 cm and 20.9 cm^2, respectively in control treatment.

Under the conditions of 10 ppm 2,4-D treatment, the seedling growth of both PKHy-2 and AK-32 were reduced, wherein the response of AK-32 was quite significant in terms of highly wrinkled dark green leaves with leaf area of 11.3 cm^2 as compared to 20.9 cm^2 in control. Similarly, the shoot length was also reduced to 1.95 cm in opposition to 3.28 cm in control. Thus, response to 2,4-D application, both at 5 and 10 ppm distinguished AK-32 from PKHy-2.

Hence, hybrid PKHy-2 and its female parent AK-32 could be distinguished on the basis of their response to sodium deficiency, high concentration of calcium, 5 ppm and 10 ppm 2,4-D application.

4.1.1.3 NHH-44 and BN-1

Seedling response to high concentration of sodium and deficiency

Hybrid NHH-44 showed a marked reduction in seedling growth at high concentration of sodium with stem diameter, shoot length and leaf area of 1.06 cm, 3.01 cm and 24.5 cm^2 as compared to 1.22cm, 3.74 cm and 28.38 cm^2, respectively in control (Table 4). Similar reduction was also seen in BN-1 with reduced leaf area

Table 4: Seedling response of NHH-44 and BN-1 to modified nutrient media

Treatment	Colour and growth of seedling		Stem diameter (cm)		Shoot length (cm)		Leaf area (cm^2)	
	NHH-44	BN-1	NHH-44	BN-1	NHH-44	BN-1	NHH-44	BN-1
Control	Green	Pale yellow	1.22	1.25	3.74	3.38	28.38	24.3
High sodium	Pale yellow, Reduced growth	Reduced growth	1.06	1.14	3.01	3.02	24.3	16.7
Sodium def.	Green	Green	1.27	1.21	3.72	3.0	28.45	22.8
High calcium	Green	Reduced growth	1.19	1.13	3.62	2.91	27.5	19.8
Calcium def.	Pale yellow	Green	1.20	1.21	3.46	3.12	27.8	22.9
5 ppm 2,4-D	Green	Reduced growth, Reduced growth	1.16	1.00	3.52	2.89	27.4	19.4
10 ppm 2,4-D	Reduced growth, Dark green	Dark green, Wrinkled leaves	0.95	0.88	2.76	2.39	24.6	12.6
C.D.(0.05)			0.0532	0.0380	0.0423	0.0339	0.0572	0.1496

High sodium : 440 ppm
High calcium : 320 ppm
Sodium and calcium deficiency : 0 ppm

(16.7 cm^2), shoot length (3.02 cm) and stem diameter (1.14 cm) as compared to 24.3 cm^2, 3.38 cm and 1.25 cm, respectively in control.

Under the conditions of sodium deficiency, NHH-44 showed vigorous seedling growth with 28.43 cm^2 leaf area, 3.72 cm shoot length and 1.27 cm stem diameter, which were at par with control. In case of BN-1, seedlings exhibited slight reduction in the leaf area (22.8 cm^2), shoot length (3.0 cm) and stem diameter 1.21 cm) as compared to 24.3 cm^2, 3.38 cm and 1.25 cm, respectively in control.

Seedling response to high concentration and deficiency of calcium

Under conditions of high calcium treatment, seedlings of NHH-44 were normal with leaf area, shoot length and stem diameter comparable to that in control. Whereas, seedlings of BN-1 showed highly reduced growth with 19.8 cm^2 of leaf area, 2.91 cm of shoot length and 1.13 cm of stem diameter as compared to 24.3 cm^2, 3.38 cm and 1.25 cm, respectively in control. Conversely, under the conditions of calcium deficiency, both NHH-44 and BN-1 performed normal except yellowing of leaves in NHH-44. The observations on leaf area, shoot length and stem diameter were 27.8 cm^2, 3.46 cm and 1.20 cm in NHH-44 and 22.9 sq cm, 3.12 cm and 1.21 cm in BN-1, respectively, which were at par with those in control.

Response to 2,4-D application

On treatment with 5 ppm 2,4-D, the appearance of NHH-44 seedlings were normal with 27.4 cm^2 leaf area, 3.52 cm of shoot length and 1.16 cm of stem diameter as compared to 28.38 cm^2, 3.74 cm and 1.72 cm, respectively in the control treatment. But, BN-1 seedlings were dark green in colour with reduced growth *i.e.*, 19.4 cm^2

leaf area, 2.89 cm shoot length and 1.00 cm stem diameter as compared to 24.3 cm^2, 3.38 cm and 1.25 cm, respectively in control.

Seedling response to 10 ppm 2,4-D treatment was marked both in NHH-44 and BN-1. The performance of BN-1 was distinct from that of NHH-44 as the seedlings turned dark green and highly wrinkled with significantly reduced leaf area (12.6 cm^2), shoot length (2.89 cm) and stem diameter (0.88 cm) as compared to control. Seedlings of NHH-44 were dark green with reduced growth *i.e.*, 24.64 cm^2 leaf area, 2.76 cm shoot length and 0.95 cm stem diameter as compared to 28.38 cm^2, 3.74 cm and 1.22cm, respectively in control but the leaves were not wrinkled.

Hence, hybrid NHH-44 and its female parent BN-1 could be distinguished on the basis of their response to high calcium, 5 ppm and 10 ppm 2,4-D application.

4.1.1.4 H-10 and BC-68/2

Seedling response to high concentration of sodium and deficiency:

The seedling growth of the hybrid H-10 and BC-68/2 under high concentration of sodium was normal with slight yellowing and reduction in stem diameter, shoot length and leaf area. Under the conditions of sodium deficiency also, no significant variation in seedling growth was seen in both H-10 and BC-68/2. However, the seedlings exhibited dark green leaf colouration (Table 5).

Table 5: Seedling response of H-10 and BC-68/2 to modified nutrient media

Treatment	Colour and growth of seedling		Stem diameter (cm)		Shoot length (cm)		Leaf area (cm^2)	
	H-10	BC-68/2	H-10	BC-68/2	H-10	BC-68/2	H-10	BC-68/2
Control	Green	Pale yellow	1.31	1.16	4.24	3.98	18.5	18.2
High sodium	Pale yellow	Pale yellow	1.27	1.03	4.22	3.83	18.2	17.3
Sodium def.	Green	Dark green	1.29	1.18	4.18	3.78	17.9	17.9
High calcium	Green	Pale yellow	1.19	1.20	4.13	3.7	18.1	16.6
Calcium def.	Pale yellow, Reduced growth	Green, Reduced growth	1.18	1.11	4.02	3.40	16.2	14.1
5 ppm 2,4-D	Reduced growth	Dark green, Reduced growth	1.10	1.12	3.99	3.30	16.1	12.0
10 ppm 2,4-D	Reduced growth	Dark green, Wrinkled leaves	1.00	0.98	3.89	2.99	15.2	9.21
C.D.(0.05)			0.023	0.0285	N.S.	N.S.	N.S.	N.S.

High sodium : 440 ppm
High calcium : 320 ppm
Sodium and calcium deficiency : 0 ppm

Seedling response to high concentration and deficiency of calcium

H-10 showed no reduction in seedling growth under high concentration of calcium as its leaf area, stem diameter and shoot length were at par with control. In case of BC-68/2, seedlings were pale yellow in colour with reduced leaf area compared to control. Under the conditions of calcium deficiency significant reduction in seedling growth was observed in H-10 and BC-68/2 in terms of stem diameter, shoot length and leaf area as compared to control. Hybrid H-10 also exhibited some degree of yellowing of the seedlings.

Response to 2,4-D application:

On treatment with 5 ppm 2,4-D, both H-10 and BC-68/2 exhibited reduction in seedling growth, which was more pronounced in BC-68/2. Seedlings also showed dark greening of leaves with stem diameter of 1.9 cm, shoot length of 2.65 cm and leaf area of 16.8 cm^2 as compared to 2.75 cm, 3.28 cm and 20.9 cm^2, respectively in control treatment. With 10-ppm 2,4-D treatment, the seedling growth was drastically reduced in both H-10 and BC-68/2, wherein the response of BC-68/2 was quite significant in terms of highly wrinkled dark green leaves with leaf area of 11.3 cm^2 as compared to 20.9 cm^2 in control.

Hence, hybrid H-10 and its female parent BC-68/2 could be distinguished on the basis of their response to calcium deficiency and 10 ppm 2,4-D application.

4.1.1.5 PHH-316 and PH-093

Seedling response to high concentration and deficiency of sodium

Under the conditions of high sodium treatment, the seedling growth of hybrid PHH-316 and its female parent PH-093 were normal and at par with respective controls. Similarly, under the conditions of sodium deficiency, both PHH-316 and PH-093 produced normal seedlings with no variation in seedling appearance (Table 6).

Seedling response to high concentration and deficiency of calcium

Under the conditions of high calcium treatment, seedling growth of PHH-316 were normal with only slight reduction in leaf area (19.8 cm^2), shoot length (4.13 cm) and stem diameter (1.18 cm) as compared to control. The seedlings of PH-093 also showed slightly reduced growth with 15.0 $cm^2$of leaf area, 4.0 cm of shoot length and 1.13 cm of stem diameter as compared to 16.3 sq cm, 4.1 cm and 1.35 cm, respectively in control but the leaves were darker green in colour. No significant variation was observed in both PH-316 and PH-093 with calcium deficiency treatment.

Response to 2,4-D application

On treatment with 5 ppm 2,4-D, both PHH-316 and PH-093 seedling exhibited reduction in growth and dark green leaves. But, the growth of PH-093 seedlings was more pronounced with 14.0 cm^2 leaf area, 3.68 cm shoot length and 1.01 cm stem diameter as compared to 16.3 sq cm, 4.1 cm and 1.35 cm, respectively in control.

Table 6: Seedling response of PHH-316 and PH-083 to modified nutrient media

Treatment	Colour and growth of seedling		Stem diameter (cm)		Shoot length (cm)		Leaf area (cm^2)	
	PHH-316	PH- 093	PHH-316	PH-093	PHH-316	PH 093	PHH-316	PH 093
Control	Green	Green	1.27	1.35	4.44	4.1	22.8	16.3
High sodium	Green	Green	1.24	1.11	4.31	3.9	21.66	15.9
Sodium def.	Green	Green	1.19	1.21	4.20	4.10	20.6	15.1
High calcium	Green, Reduced growth	Dark green	1.18	1.13	4.13	4.0	19.8	15.0
Calcium def.	Normal	Dark green	1.00	1.10	4.21	3.99	21.3	14.8
5 ppm 2,4-D	Normal,	Reduced growth	1.00	1.01	4.11	3.68	20.5	14.0
10 ppm 2,4-D	Dark green, Reduced growth	Dark green, Wrinkled leaves	0.98	0.93	4.02	2.41	19.8	10.4
C.D.(0.05)			NS	NS	0.0021	0.0416	0.0349	3.086

High sodium : 440 ppm
High calcium : 320 ppm
Sodium and calcium deficiency : 0 ppm

Table 7: Seedling response of cotton hybrids and their female parents with respect to leaf area in modified nutrient media

	Control	High sodium	Sodium def.	High calcium	Calcium def.	5ppm 2,4-D	10ppm 2,4-D	C.D.(0.05)
Savitha	21.35	20.95	24.8	22.22	24.62	21.35	18.31	0.1869
T-7	18.25	13.34	16.70	14.35	10.7	9.5	6.69	0.1794
PKHy-2	24.8	21.6	20.6	19.2	25.4	22.5	20.3	0.1987
AK-32	20.9	19.8	16.5	19.8	19.2	16.8	11.3	0.0394
NHH-44	28.38	24.3	28.45	27.5	27.8	27.4	24.6	0.0572
BN-1	24.3	16.7	22.8	19.8	22.9	19.4	12.6	0.1496
H-10	18.5	18.2	17.9	18.1	16.2	16.1	15.2	0.1541
BC-68/2	18.2	17.3	17.9	16.6	14.1	12.0	9.21	0.1563
PHH-316	22.8	21.66	20.6	19.8	21.3	20.5	19.8	0.0349
PH-093	16.3	15.9	15.1	15.0	14.8	14.0	10.4	3.0867
C.D.(0.05)	0.1569	0.2583	5.6896	0.1013	0.1322	0.06350	0.1288	

C.D. (Genotypes) (0.05) : 1.4703
C.D. (Treatments) (0.05) : 0.2302
C.D. (Genotypes X Treatments) :1.93

Under conditions of 10 ppm 2,4-D treatment, the performance of PH-093 was distinct from that of PHH-316 as the seedlings were dark green and highly wrinkled with significantly reduced leaf area, shoot length and stem diameter as compared to control. Seedlings of PHH-316 were also dark green with reduced growth but without leaf wrinkling.

Hence, hybrid PHH-316 and its female parent PH-093 could be distinguished on the basis of their response to 10 ppm 2,4-D application.

The differential response of the growing seedlings to the nutrient medium, particularly to high concentration of sodium, calcium and application of 2,4-D, could clearly distinguish all five hybrids from their female parents, and was also useful in characterization of cotton genotypes. Effect was most pronounced on leaf area of the seedlings grown in different media (Table7).

4.1.2. Characterization of genotypes based on anthocyanin and gossypol contents.

4.1.2.1. Anthocyanin content

The colour of seed extracts due to anthocyanin content varied from reed yellow to olive yellow in different genotypes (fig.2). The optical density of extracts (*i.e.*, the colour intensity) ranged from 0.034 in H-10 to 0.116 in PHH-316 (Table 8). This test was proved very useful as all five hybrids were distinct from their respective female parents, though the difference in colour (O.D. value) was most prominent between NHH-44 (0.100) and its female parent BN-1 (0.035), followed by PHH-316 (0.116) and PH-093 (0.073). The visual classification was more clear-cut than the O.D. determination. Hence, it could be useful in characterization of cotton

Fig 2: Colour of seed extracts for anthocyanin contents in cotton hybrids and parental lines

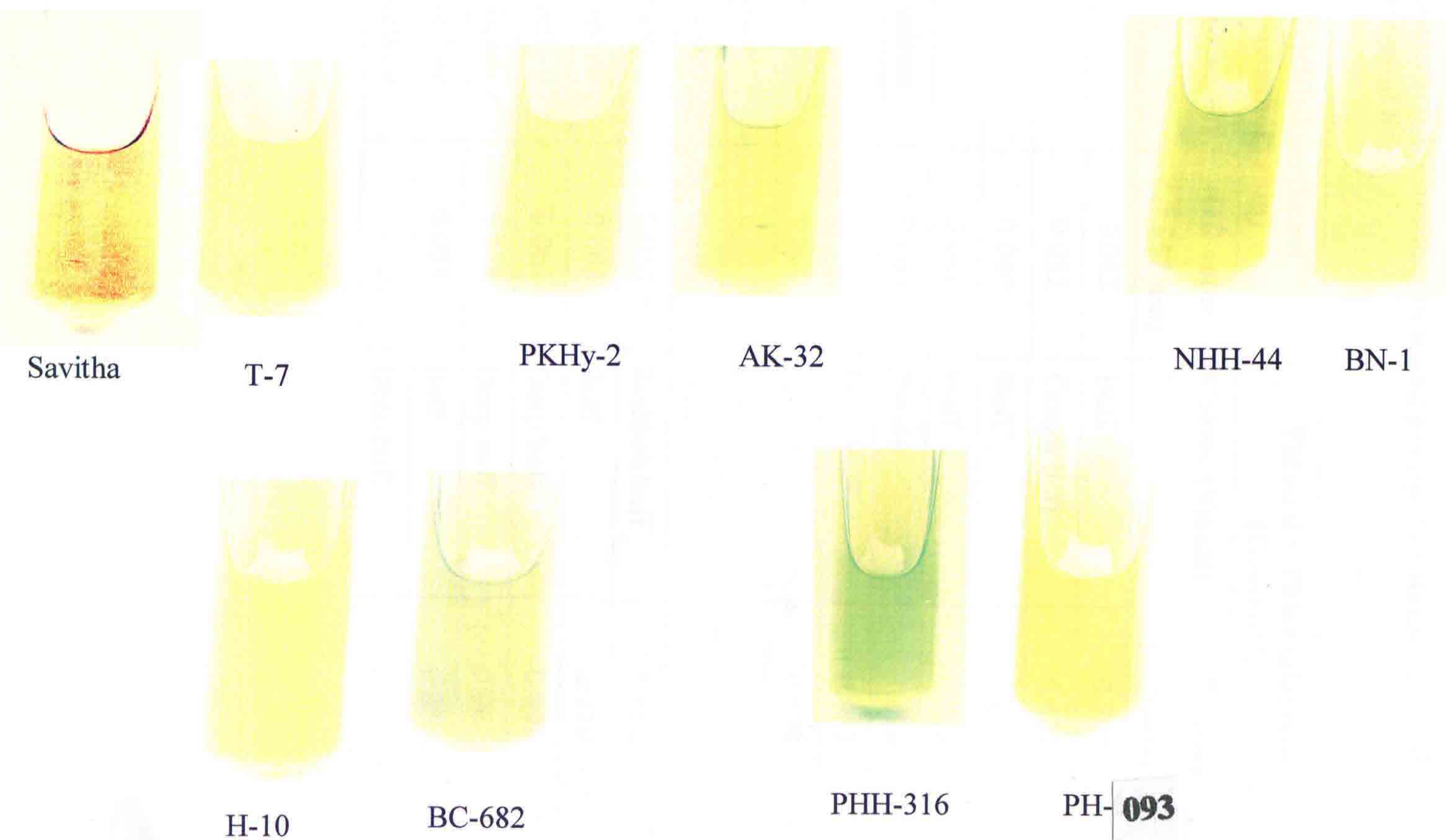


Table 8: Colour of seed extracts for anthocyanin and gossypol contents in cotton hybrids and parental lines

Genotype	Acidified methanol (Anthocyanin)		Ethanol + Phloroglucinol (Gossypol)	
	Colour (Visual)	OD value (at 657 nm)	Colour (Visual)	OD value (at 550nm)
Savitha	Wood	0.082	Buff	0.704
T-7	Yellow	0.052	Cream buff	0.421
M-12	Yellow	0.040	Buff	0.698
PKHy-2	Yellow	0.041	Buff	0.758
AK-32	Lemon yellow	0.060	Purple	1.098
DHY-286	Yellow	0.047	Deep buff	0.967
NHH-44	Dark yellow	0.100	Deep Buff	0.868
BN-1	Reed yellow	0.035	Buff	0.771
ACC-738	Dark yellow	0.108	Buff	0.793
H-10	Reed yellow	0.034	Reddish buff	1.105
BC-286	Lemon yellow	0.065	Buff	0.779
LRA-5166	Lemon yellow	0.061	Deep buff	0.893
PHH-316	Olive yellow	0.116	Deep buff	0.986
PH 093	Lemon yellow	0.073	Buff	0.856
PKV-081	Lemon yellow	0.070	Deep buff	0.971

genotypes, for differentiating and distinguishing hybrids from their respective female parents.

4.1.2.2Gossypol content

The colour variations of the seed extracts due to gossypol content and its intensity, which was taken as O.D. values at 550 nm among the genotypes are presented in table 8 and fig 3. The colour of the gossypol extract varied from cream buff to reddish buff in different genotypes. Seven of the genotypes, 2 hybrids *viz.*, Savitha and PKHy-2 were classified as buff, whereas six genotypes including three hybrids *viz.*, NHH-44, H-10 and PHH-316 were classified as reddish buff and deep buff. Only one genotype *viz.*, T-7 showed cream buff colour and one genotype AK-32 showed purple colour. Three of the hybrids *i.e.*, Savitha, PKHy-2 and H-10 were distinct from their female parents, whereas the distinction between PHH-316 and PH-081 was less prominent and that between NHH-44 and BN-1 was negligible. The difference in optical densities, which ranged from 0.042 to 1.105, was more marked among the genotypes than the visual classification.

Thus, the colour difference of the seed extract due to anthocyanin and gossypol content was a useful parameter for characterization of cotton genotypes, particularly in distinguishing the hybrids from their female parents.

Possible application of gossypol content test for testing the genetic purity of hybrids was assessed by individual seed analysis of hybrid PKHy-2 and its female parent AK-32 (Table 9). The O.D. of single seed extracts ranged from 0.310 to 0.448 in PKHy-2 and from 0.509 to 0.661 in AK-32 (values were lower than the corresponding pooled seed samples due to a dilution factor in case of single seed

Fig 3: Colour of seed extracts for gossypol contents in cotton hybrids and parental lines

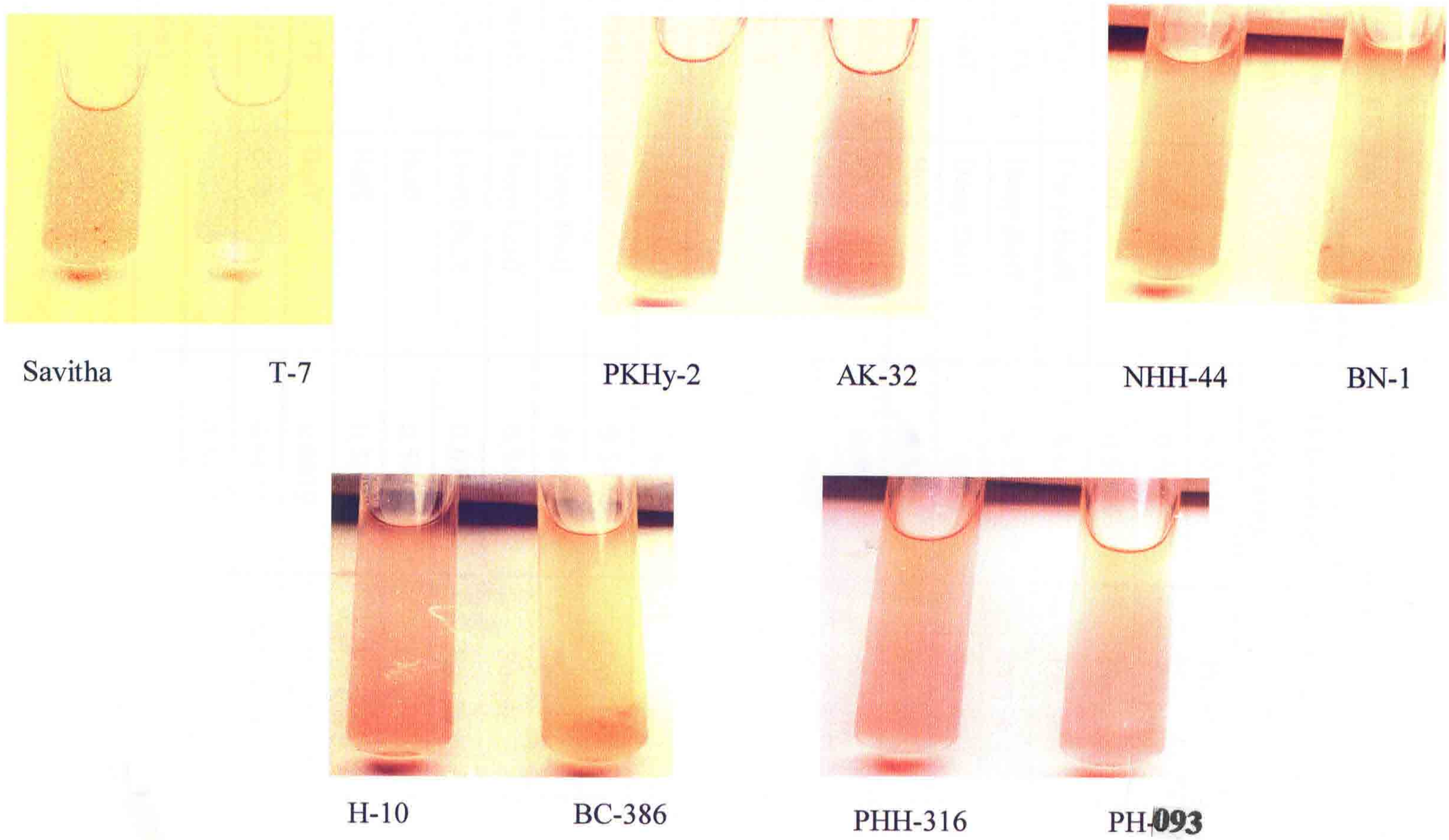


Table 9: Gossypol pigment variation in 20 single seeds of PKHy-2 and its female parent

Genotype	PKHy-2		AK-32	
Observations	**O.D.value (550 nm)**	**Colour of the extract**	**O.D.value (550 nm)**	**Colour of the extract**
1	0.3369	Buff	0.5958	Purple
2	0.3108	Light Buff	0.5479	Purple
3	0.3629	Buff	0.5236	Purple
4	0.4098	Deep Buff	0.6089	Purple
5	0.4073	Deep Buff	0.5363	Purple
6	0.4487	Deep Buff	0.6174	Purple
7	0.3884	Buff	0.5306	Purple
8	0.3636	Buff	0.5672	Purple
9	0.3836	Buff	0.5092	Purple
10	0.3397	Buff	0.6158	Purple
11	0.3487	Buff	0.5672	Purple
12	0.3198	Light Buff	0.6079	Purple
13	0.3641	Buff	0.5476	Purple
14	0.3981	Deep Buff	0.6072	Purple
15	0.4016	Deep Buff	0.5407	Purple
16	0.3928	Deep Buff	0.6116	Purple
17	0.3687	Buff	0.5946	Purple
18	0.3848	Buff	0.5937	Purple
19	0.3673	Buff	0.6019	Purple
20	0.3863	Buff	0.6613	Deep Purple
Mean	0.3741		0.5793	
c.v.	0.0883		0.00688	

extract). Thus, though the visual differentiation was possible, for precise quantification, there is a need to refine the technique further.

.13.Characterization based on electrophoresis profiles

4.1.3.1Tris soluble proteins

The electrophoresis profile of tris soluble proteins from seeds revealed a good degree of polymorphism among the genotypes. The results of the SDS-PAGE analysis of seed proteins are represented in fig 4. The scoring was done for the presence and absence of bands, which are identified by their relative mobility values and numbered in sequence from the cathodal origin (Table 10). A total of 20 bands were observed with number ranging from 13 (PKHy-2) to 20 (PHH-316) in different genotypes. Of these, 11 bands were common to all the fifteen genotypes. Hybrid Savitha exhibited a pattern nearly similar to that of its male parent M-12, except for the absence of three bands (Rm value: 0.23, 0.46 and 0.58), whereas the female parent T-7 was distinct from the hybrid by the presence of a band having Rm value of 0.50. Hybrid PKHy-2 was also found to be distinct from its female parent AK-32 by the absence of four bands (Rm values: 0.23, 0.46, 0.50 and 0.58) and the presence of a band having Rm value of 0.53, whereas its male parent DHY-286 was very similar to the hybrid differing only by the absence of one light band (Rm value: 0.52). Hybrid NHH-44 was distinct from its female parent BN-1 by the absence of a band having the Rm value of 0.56, which was intense in BN-1. The banding patterns of NHH-44 and its male parent were very similar except for one band having Rm value of 0.56, which was present in ACC-738, while bands with Rm of 0.52 and 0.83 were absent in it. The banding pattern of the hybrid H-10 exhibited complimentary bands by the female (Rm value 0.23 and 0.54) and male (Rm value 0.46) parents. The banding profiles of hybrid PHH-316 and its parents

Fig 4: Total tris soluble seed protein profile in five hybrids and parental lines of cotton

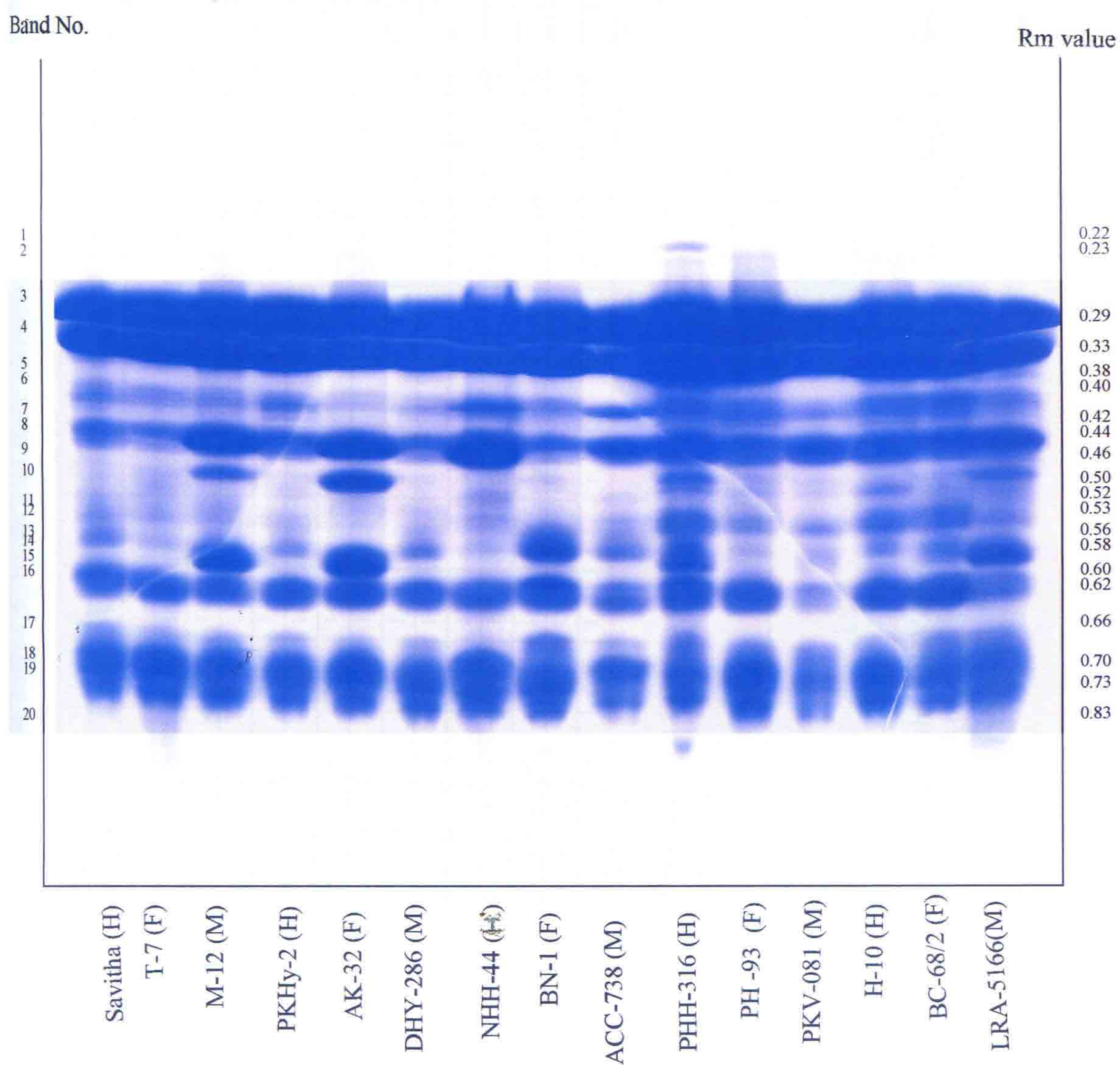


Table 10: Tris soluble protein profiles of five cotton hybrids and their parents

l No:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total	Band absent
Value	0.22	0.23	0.29	0.33	0.38	0.40	0.42	0.44	0.46	0.50	0.52	0.53	0.56	0.58	0.60	0.62	0.66	0.70	0.75	0.83		
ha (H)	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	0	**14**	1,2,9,10,14,20
F)	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	**16**	1,2,9,14,
2 (M)	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	**17**	1,10,20
ly-2(H)	0	0	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	1	0	**13**	1,2,9,10,11,14,20
32 (F)	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	**16**	1,11,12,20
Y-286 (M)	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	0	**14**	1,2,9,10,14,20
H-44 (H)	0	0	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1	0	**14**	1,2,9,13,14,20
1 (F)	0	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	**15**	1,2,10,11,14
C-738 (M)	0	0	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	0	**14**	1,2,9,11,14,20
0 (H)	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	**16**	1.10.14.20
68/2 (F)	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	**17**	1.9.10.20
A 5166 (M)	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	**17**	1.2.10.11
H-316 (H)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**20**	-----
-093 (F)	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	**17**	1.12.20
V-081 (M)	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	**16**	1.2.11.20

"1" represents presence "0" and represents absence of a band

were very similar except for the high intensity of band numbers 1, 9, 10 and 14 having Rm values of 0.22, 0.46, 0.50 and 0.58 in the hybrid.

Thus, on the basis of tris soluble protein profiles, 13 genotypes could be distinguished individually, all hybrids were distinguishable from their female parents and hybridity was exhibited in H-10.

4.1.3.2 Salt soluble globulins

The SDS-PAGE profile of salt soluble globulins is presented in fig 5. A total of 15 bands were observed amongst the genotypes ranging from 10 (BN-1) to 15 (H-10). Of these 7 bands having Rm of 0.22, 0.27, 0.59, 0.63, 0.74, 0.88, and 0.91 were common to all genotypes.

The banding profile of each genotype is tabulated for comparison (Table11). The banding profile of hybrid Savitha was complimented by both of its parents. The female parent T-7 was characterized by the presence of an intense band having Rm value of 0.56, which is absent in the male parent M-12, which was distinguished by a band having Rm value of 0.97. Similarly, hybrid PKHy-2 was complimented by two bands contributed by each of its parents AK-32 (Rm value: 0.44 and 0.56) and DHY-286 (Rm value: 0.95 and 0.97). In case of hybrid NHH-44, its female parent BN-1 contributed one prominent band having Rm values of 0.97, whereas the male parent ACC-738 contributed only four bands with Rm of 0.56, 0.71, 0.93 and 0.95. The complimentarity of the bands contributed by the two parents was characteristic of the profile of the hybrid H-10 too. The male parent LRA-5166 contributed three bands having Rm values of 0.38, 0.57 and 0.97 and two bands having Rm value 0.56 and 0.71 was contributed by the female parent BC-68/2. Hybrid PHH-316 showed a pattern similar to its male parent PKV-081.

Fig. 5: Salt soluble globulin profile in 5 hybrids and parental lines of cotton

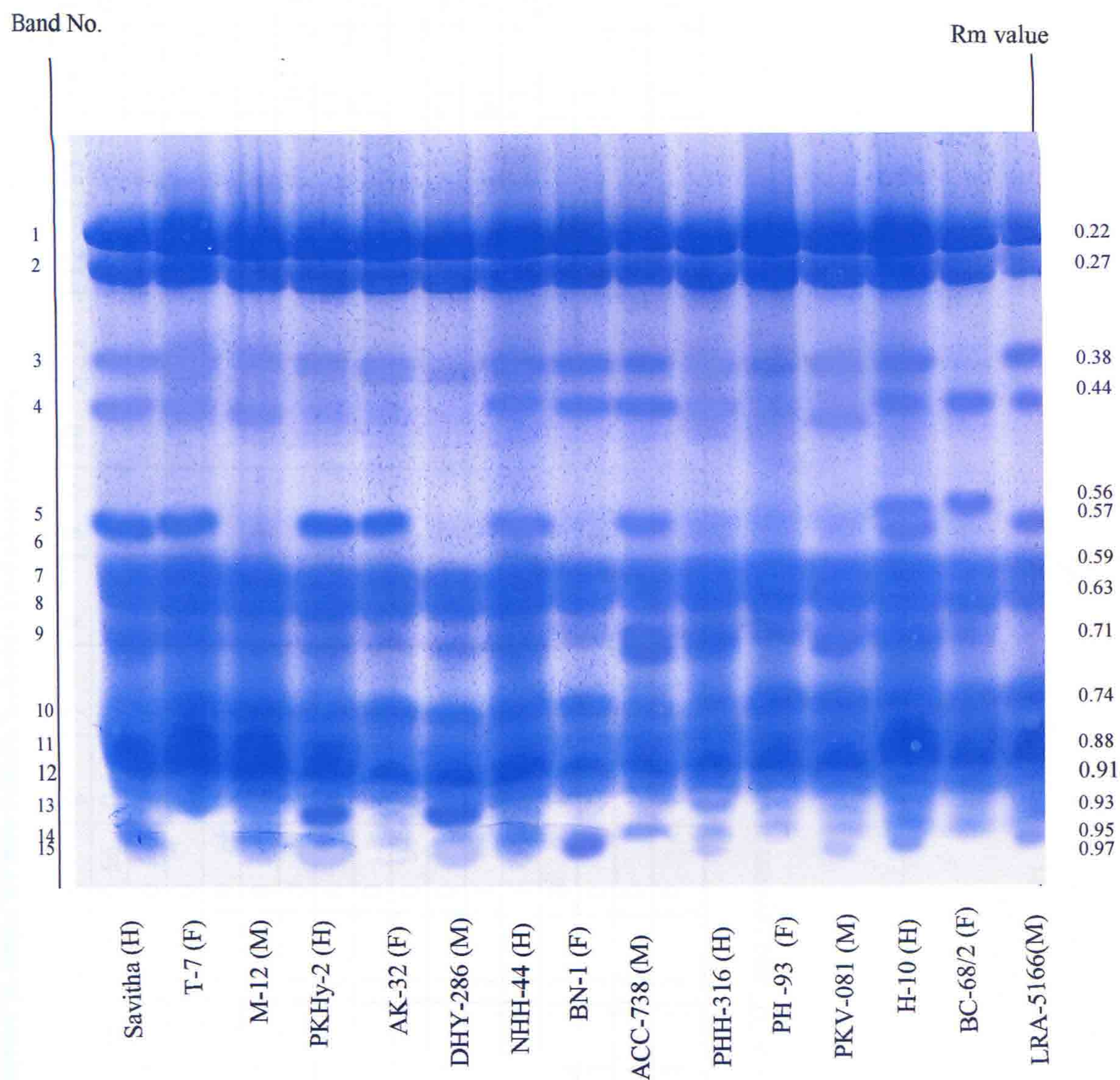


Table11: Salt soluble globulins profiles of five cotton hybrids and their parents

and no.:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total	Band absent
n Value	0.22	0.27	0.38	0.44	0.56	0.57	0.59	0.63	0.71	0.74	0.88	0.91	0.93	0.95	0.97		
vitha (H)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	**14**	5
7 (F)	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	**12**	5,13,14
-12 (M)	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	**13**	5,6
Hy-2 (H)	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	**13**	5,12
-32 (F)	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	**11**	5,12,13,14
HY-286 (M)	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	**11**	4,5,6,12
HH-44 (H)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	**14**	5
N-1 (F)	1	1	1	1	0	0	1	1	0	1	1	1	0	0	1	**10**	5,6,9,12,13
CC-738 (M)	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	**12**	5,6,14
-10 (H)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	**15**	------
C-68/2 (F)	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	**12**	3,6,14
RA 5166 (M)	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	**13**	5,9
HH-316 (H)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	**14**	5
H-093 (F)	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	**12**	4,5,14
KV-081 (M)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	**14**	5

"1" represents presence "0" and represents absence of a band

Distinction of genotypes

Savitha, NHH-44,
PHH-316, PKV-081
(Band no. 8 absent)

Rest of the genotypes
are distinct

Thus, SDS-PAGE profile of the salt soluble globulins provided an efficient marker system, which could distinguish all hybrids from their parental lines. This not only established the hybridity (except PHH-316), but also was efficient in distinguishing the hybrids from their respective female parents. However, three of the hybrids i.e., Savitha, NHH-44 and PHH-316 and male parent PKV-081 exhibited similar patterns.

4.1.3.3 Acid phosphatase (ACP)

The acid phosphatase isoenzymes exhibited a good degree of polymorphism among the genotypes studied. A total of 9 bands were observed with 4 (BC-68/2) to 9 (Savitha, PKHy-2, NHH-44, ACC-738) bands present in individual genotypes (fig.6). Except PHH-316 all the hybrids were distinguishable from their respective female parents. This could be of particular use in detecting the selfed females in hybrid seed lots. Hybridity could be established in case of Savitha and PKHy-2 by the presence of marker band(s) contributed by each of the parental lines (Table12). Hybrid Savitha was distinguishable by the presence of an intense band having Rm value of 0.30 and a medium intensity band having Rm value of 0.60, which were contributed by its female parent T-7 and two medium intensity bands having Rm values of 0.74 and 0.81, contributed by the male parent M-12. Similarly, a characteristic ACP profile of the hybrid PKHy-2 was resultant of a medium intensity band having the Rm values of 0.60, which was contributed by its female parent AK-32 and two bands having Rm value of 0.74 and 0.81 by the male parent DHY-286.

Hybrid NHH-44 showed an ACP profile similar to its male parent ACC-738 and hence, was clearly distinguishable from its female parent BN-1 by the presence of two bands having Rm values of 0.30 and 0.81, which were absent in the female

Fig.6: Acid phosphatase isozyme profile in five hybrids and parental lines of cotton

Band No.

Rm value

Band No.	Rm value
1	0.30
2	0.36
3	0.41
4	0.51
	0.60
5	0.67
6	0.74
7	0.81
8	

Savitha (H) T-7 (F) M-12 (M) PKHy-2 (H) AK-32 (F) DHY-286 (M) NHH-44 (H) BN-1 (F) ACC-738 (M) PHH-316 (H) PH -93 (F) PKV-081 (M) H-10 (H) BC-68/2 (F) LRA-5166(M)

Table 12: Acid phosphatase isoenzyme profiles of five cotton hybrids and their parents

Band no:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Total**	**Band absent**
Rm Value	0.30	0.36	0.41	0.44	0.51	0.60	0.67	0.74	0.81		
Savitha (H)	1	1	1	1	1	1	1	1	1	**9**	----
T-7 (F)	1	1	1	1	1	1	1	0	0	7	8,9
M-12 (M)	0	1	1	1	1	0	1	1	1	7	1,6
PKHy-2 (H)	1	1	1	1	1	1	1	1	1	**9**	----
AK-32 (F)	1	1	1	1	1	1	1	0	0	7	8,9
DHY-286 (M)	1	1	1	1	1	0	1	1	1	**8**	6
NHH-44 (H)	1	1	1	1	1	1	1	1	1	**9**	----
BN-1 (F)	0	1	1	1	1	1	1	1	0	7	1,9
ACC-738 (M)	1	1	1	1	1	1	1	1	1	**9**	-----
H-10 (H)	1	1	1	1	1	1	1	1	0	**8**	9
BC-68/2 (F)	1	1	1	1	0	0	0	0	0	**4**	5,6,7,8,9
LRA 5166 (M)	1	1	1	1	1	1	1	1	0	**8**	9
PHH-316 (H)	1	1	1	1	1	1	1	1	0	**8**	9
PH-093 (F)	1	1	1	1	1	1	1	1	0	**8**	9
PKV-081 (M)	1	1	1	1	1	1	1	0	0	7	8,9

"1" represents presence "0" and represents absence of a band

Distinction of genotypes

Savitha, PKHy-2,
NHH-44, ACC-738
(All bands present)

Rest of the genotypes are distinct

parent BN-1. Similarly, the presence of four bands having Rm of 0.51, 0.60, 0.67 and 0.74 in hybrid H-10, which were characteristic of its male parent LRA-5166 and absent in the female parent BC-68/2, distinguished it clearly from its female parent.

Thus, ACP isoenzyme pattern was useful in characterization, establishing the hybridity in Savitha and PKHy-2 and distinguishing female parents from hybrids Savitha, PKHy-2, NHH-44 and H-10.

4.1.3.4 Esterase (EST)

Esterase isoenzymes exhibited a moderate degree of polymorphism with a total of 12 bands among all the genotypes (Fig 7, Table13). All hybrids were characterized by a very prominent, darkly stained thick band (no.7) having a Rm value of 0.56, which was also prominent in three of the female parents *i.e.*, BN-1, PH-093 and BC-68/2. Similarly, an active zone comprising three bands having Rm of 0.08, 0.27 and 0.35, was also seen in all the hybrids and these three female parents. A light band (no. 9) having Rm value of 0.66 was contributed by the female parent of the hybrid Savitha. Hybrids Savitha and H-10 were distinguishable from their female parents by the presence of a combination of band nos. 7, 10 and 11 and 2 and 9 respectively.

Thus, esterase isoenzyme could only provide useful information for characterization of genotypes, distinguishing three of the hybrids from their female parent and establishing the hybridity by complimentary banding profiles.

Fig. 7: Esterase isozyme profile in five hybrids and parental lines of cotton

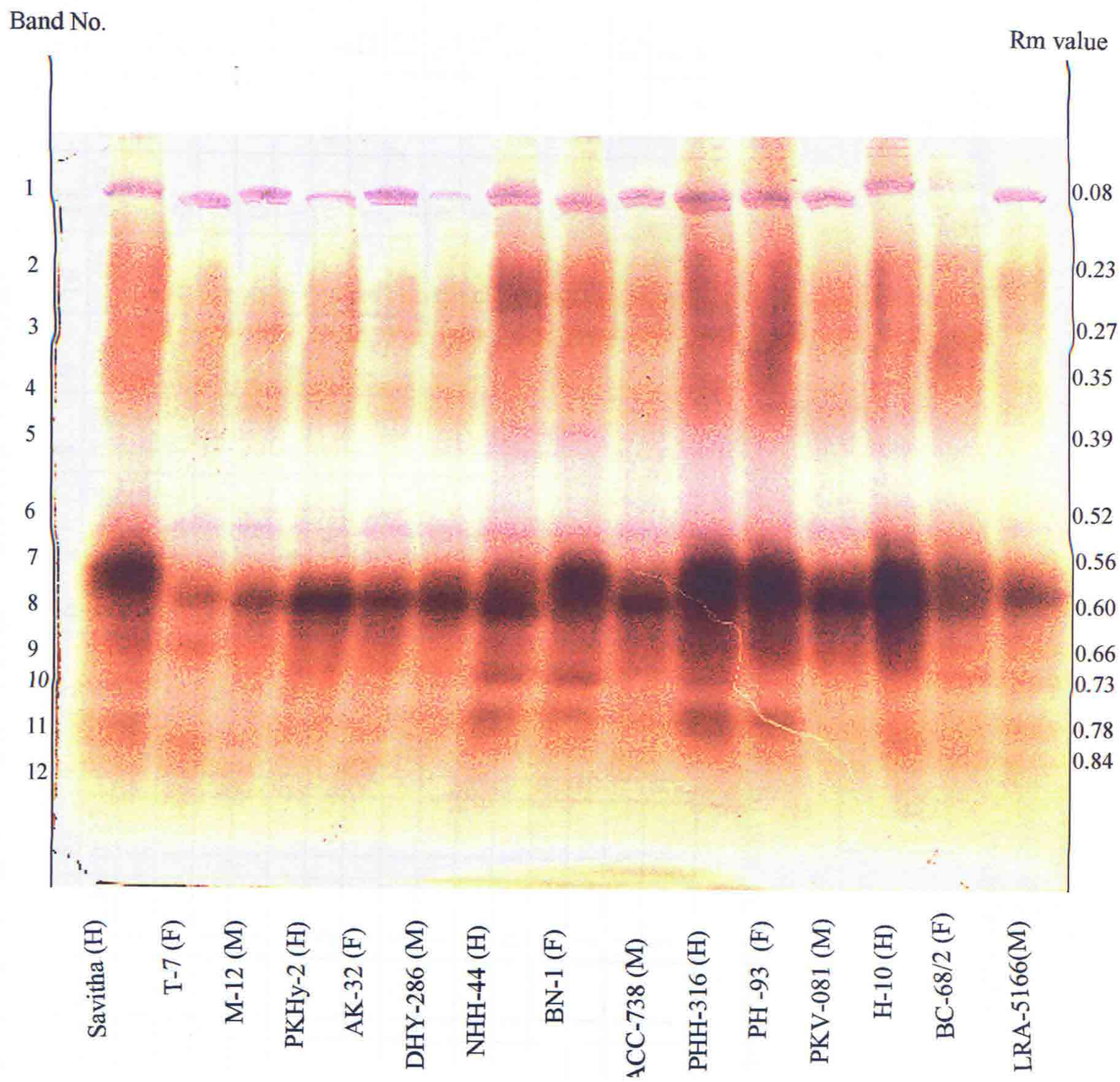


Table 13: Esterase isoenzyme profiles of five cotton hybrids and their parents

Band no.:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Total**	**Band absent**
Rm Value	0.08	0.23	0.27	0.35	0.39	0.52	0.56	0.60	0.66	0.73	0.78	0.84		
Savitha (H)	1	1	1	1	0	1	1	1	1	1	1	1	11	5
T-7 (F)	1	1	1	1	0	1	0	1	1	0	0	1	8	5,7, 10,11
M-12 (M)	1	1	1	1	0	1	0	1	0	0	0	1	7	5,7,9,10,11
PKHy-2 (H)	1	1	1	1	0	1	0	1	0	1	0	0	7	5,7,9,11,12
AK-32 (F)	1	1	1	1	0	1	0	1	0	1	1	1	9	5,7,9
DHY-286 (M)	1	0	1	1	1	1	0	1	0	0	0	0	6	2,7,9,10,11,12
NHH-44 (H)	1	1	1	1	1	1	1	1	1	1	1	1	12	----
BN-1 (F)	1	1	1	1	1	1	1	1	1	1	1	1	12	----
ACC-738 (M)	1	1	1	1	1	1	0	1	1	0	0	1	9	7,10,11
H-10 (H)	1	1	1	1	1	1	1	1	1	0	1	1	11	10
BC-68/2 (F)	1	0	1	1	1	1	1	1	0	1	1	1	10	2,9
LRA 5166 (M)	1	1	1	1	0	1	1	1	0	1	1	1	10	5,9
PHH-316 (H)	1	1	1	1	1	1	1	1	1	1	1	1	12	---
PH-093 (F)	1	1	1	1	1	1	1	1	1	1	1	1	12	---
PKV-081 (M)	1	1	1	1	0	1	0	1	1	0	0	0	7	5,7,10,11,12

"1" represents presence "0" and represents absence of a band

Distinction of genotypes

NHH-44, BN-1,
PHH-316 & PH093
(All bands present)

Rest of the genotypes
are distinct

4.1.3.5 Alcohol dehydrogenase (ADH)

Four of the hybrids *i.e.*, Savitha, PKHy-2, NHH-44 and PHH-316 were characterized by the presence of two light to medium intensity bands having Rm values of 0.57 and 0.60, which were absent in the parental lines (Fig 8, Table14). However, hybrids Savitha, PKHy-2 and NHH-44 were not distinguishable from each other, though PHH-316 was distinguishable due to the low intensity of all isoenzyme bands in it.

Thus, ADH isoenzyme pattern could distinguish four hybrids from their female parental lines, but due to a low degree of polymorphism, it was not very much useful for the purpose of establishing the identity of individual genotypes (hybrids and parental lines).

4.1.3.6 Superoxide dismutase (SOD)

Negative staining of the gel revealed a total of 7 bands for the SOD isoenzymes, exhibiting very little polymorphism amongst different genotypes (Fig 9, Table15). Of these, three bands having Rm values of 0.60, 0.62 and 0.66 were common to all genotypes. Except for hybrid H-10, in which band no. 2 (Rm. value: 0.58) and band no. 6 (Rm value: 0.68) were contributed by its male and female parents, respectively, the isoenzyme patterns exhibited by the hybrids were similar to one or both parents.

Thus, SOD isoenzymes were of limited use for characterization of cotton genotypes.

Fig. 8: **Alcohol dehydrogenase isozyme pattern in five hybrids and parental lines of cotton**

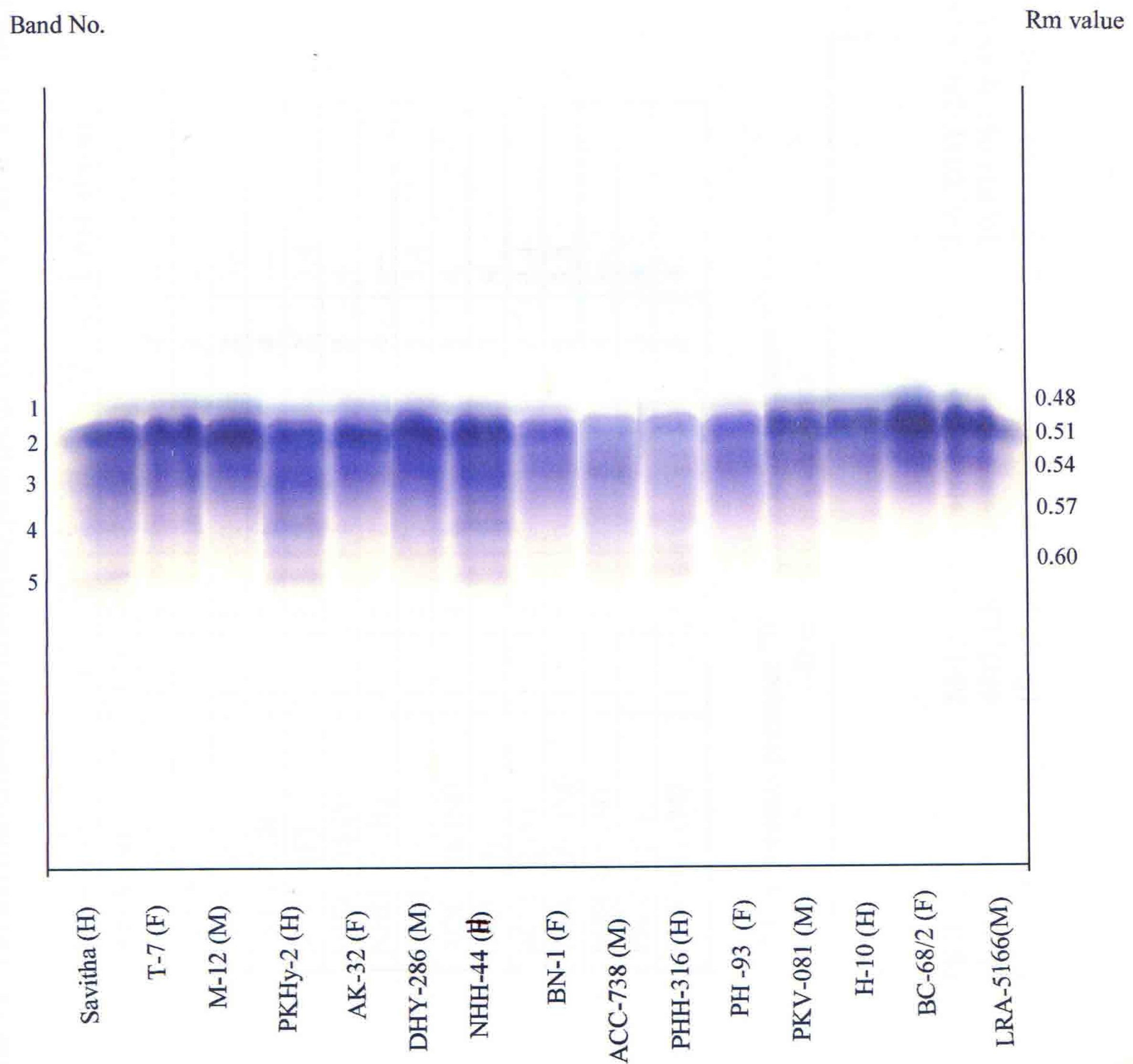
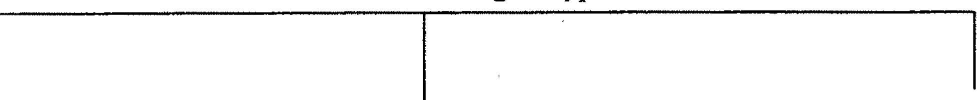


Table 14: Alcohol dehydrogenase isoenzyme profiles of five cotton hybrids and their parents

Band No.:	1	2	3	4	Total	Band absent
Rm Value	0.48	0.51	0.57	0.60		
Savitha (H)	1	1	1	1	**4**	----
T-7 (F)	1	1	1	0	**3**	4
M-12 (M)	1	1	0	0	**2**	3,4
PKHy-2 (H)	1	1	1	1	**4**	---
AK-32 (F)	1	1	0	0	**2**	3,4
DHY-286 (M)	1	1	1	0	**3**	4
NHH-44 (H)	1	1	1	1	**4**	---
BN-1 (F)	1	1	0	0	**2**	3,4
ACC-738 (M)	1	1	1	0	**3**	4
H-10 (H)	1	1	1	0	**3**	4
BC-68/2 (F)	1	1	0	0	**2**	3,4
LRA 5166 (M)	1	1	0	0	**2**	3,4
PHH-316 (H)	1	1	1	1	**4**	---
PH-093 (F)	1	1	1	0	**3**	4
PKV-081 (M)	1	1	1	0	**3**	4

"1" represents presence "0" and represents absence of a band

Distinction of genotypes



Savitha, PKHy-2, NHH-44, PHH-316 (All bands present)

M-12, AK-32, BN-1, BC-68/2, LRA-5166 (Band nos. 3 & 4 absent)

T-7, DHY-286, ACC_738, H-10, PH-093 & PKV-081 (Band no. 4 absent)

Fig. 9: Superoxide dismutase isozyme profile in five hybrids and parental lines of cotton

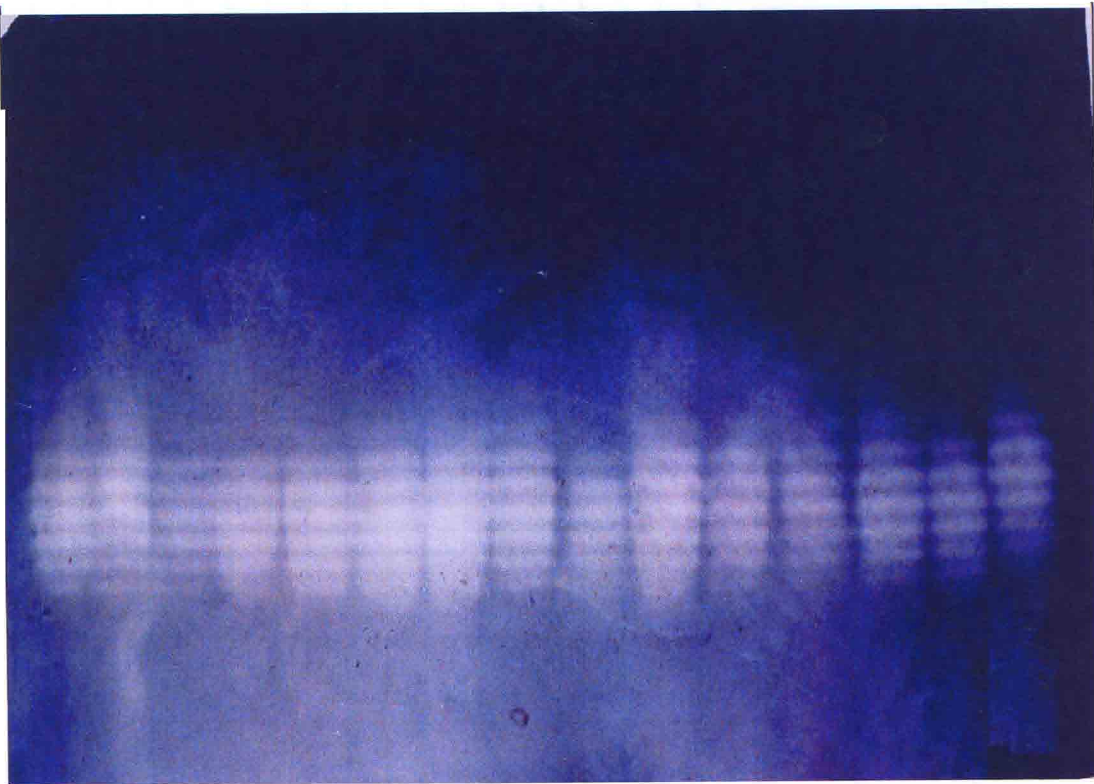
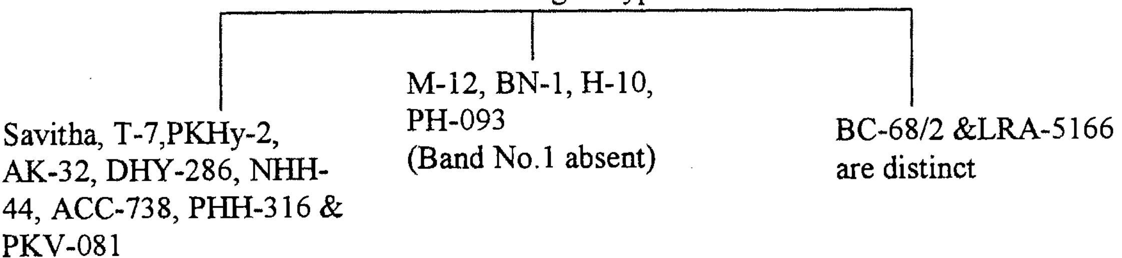


Table 15: Superoxide dismutase isoenzyme profiles of five cotton hybrids and their parents

Band No.:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**	**Band absent**
Rm Value	0.53	0.58	0.60	0.62	0.66	0.68	0.71		
Savitha (H)	1	1	1	1	1	1	1	**7**	----
T-7 (F)	1	1	1	1	1	1	1	**7**	----
M-12 (M)	0	1	1	1	1	1	1	**6**	1
PKHy-2 (H)	1	1	1	1	1	1	1	**7**	----
AK-32 (F)	1	1	1	1	1	1	1	**7**	----
DHY-286 (M)	1	1	1	1	1	1	1	**7**	----
NHH-44 (H)	1	1	1	1	1	1	1	**7**	----
BN-1 (F)	0	1	1	1	1	1	1	**6**	1
ACC-738 (M)	1	1	1	1	1	1	1	**7**	----
H-10 (H)	0	1	1	1	1	1	1	**6**	1
BC-68/2 (F)	0	1	1	1	1	1	0	**5**	1,7
LRA 5166 (M)	1	0	1	1	1	0	1	**5**	2,6
PHH-316 (H)	1	1	1	1	1	1	1	**7**	----
PH-093 (F)	0	1	1	1	1	1	1	**6**	1
PKV-081 (M)	1	1	1	1	1	1	1	**7**	----

"1" represents presence "0" and represents absence of a band



4.1.3.7 Catalase (CAT)

The resolution of CAT isoenzyme bands was poor. Only two activity zones having Rm values 0.47 and 0.51 were seen for catalase isoenzymes, of which, the first one was more active than the second (Fig 10, Table16). All hybrids and the male parental lines exhibited a brightly stained band no.1 (Rm value: 0.47), and less prominent band no.2 (Rm value: 0.51), whereas, only a weakly stained band no. 1 was seen in the female parents, except PH-093 which is the only male sterile line (female parent of PHH-316).

Thus, in spite of a poor level of polymorphism among the CAT isoenzymes, these were useful in distinguishing hybrids from their female parental lines, except in case of PHH-316.

4.1.3.8 Peroxidase (POX)

Little polymorphism was seen with respect to the peroxidase isoenzymes among the genotypes as shown in fig 11. The patterns were identical in all hybrids except NHH-44 and all male parental lines except ACC-738. The hybrid NHH-44 and the female parents of PKHy-2, H-10 and NHH-44 were distinct by the absence or very low intensity of band no. 2 having a Rm value of 0.05 (Table17).

Thus, POX isoenzyme pattern was not much useful for the purpose of characterization of cotton genotypes, even though it could distinguish hybrids PKHy-2 and H-10 from their respective female parents.

T-6717

Fig. 10: Catalase isozyme profile in five hybrids and parental lines of cotton

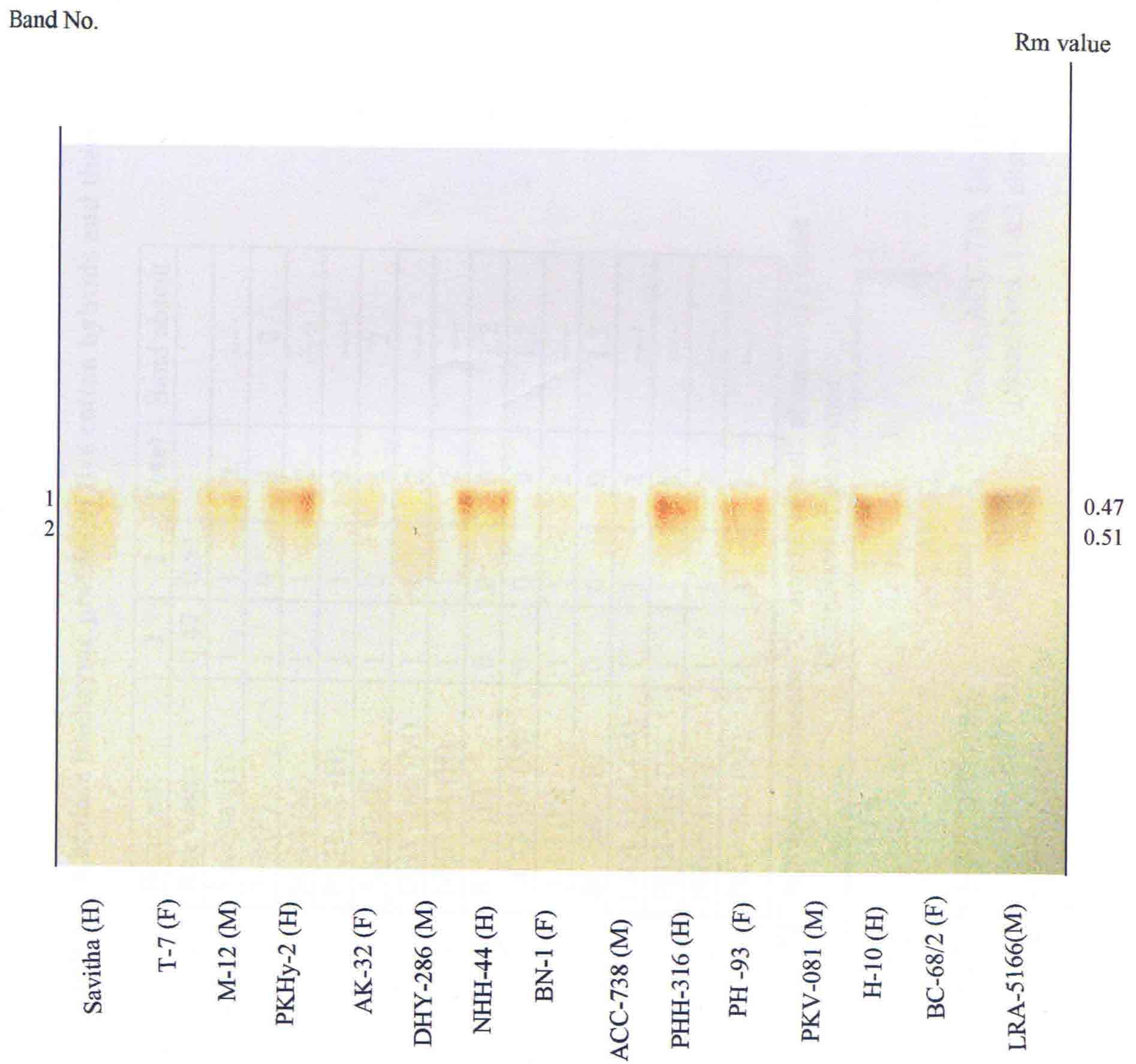
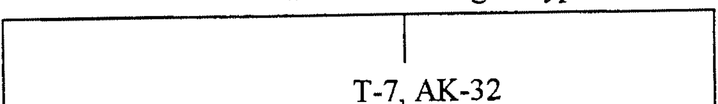


Table 16: Catalase isoenzyme profiles of five cotton hybrids and their parents

Band no:	**1**	**2**	**Total**	Band absent
Rm Value	0.47	0.51		
Savitha (H)	1	1	**2**	----
T-7 (F)	1	0	**1**	2
M-12 (M)	1	1	**2**	----
PKHy-2 (H)	1	1	**2**	----
AK-32 (F)	1	0	**1**	2
DHY-286 (M)	1	1	**2**	----
NHH-44 (H)	1	1	**2**	----
BN-1 (F)	0	0	**0**	1,2
ACC-738 (M)	0	0	**0**	1,2
H-10 (H)	1	1	**2**	----
BC-68/2 (F)	0	0	**0**	1,2
LRA 5166 (M)	1	1	**2**	----
PHH-316 (H)	1	1	**2**	----
PH-093 (F)	1	1	**2**	----
PKV-081 (M)	1	1	**2**	----

"1" represents presence "0" and represents absence of a band

Distinction of genotypes



Savitha, M-12, PKHy-2, DHY-286, NHH-44, H-10, LRA-5166, PHH-316, PH-093 & PKV-081
(All bands present)

T-7, AK-32
(Band no.2 absent)

BN-1, ACC-738, BC-68/2
(Band Nos. 1 &2 absent

Fig. 11: Peroxidase isozyme pattern in five hybrids and parental lines of cotton

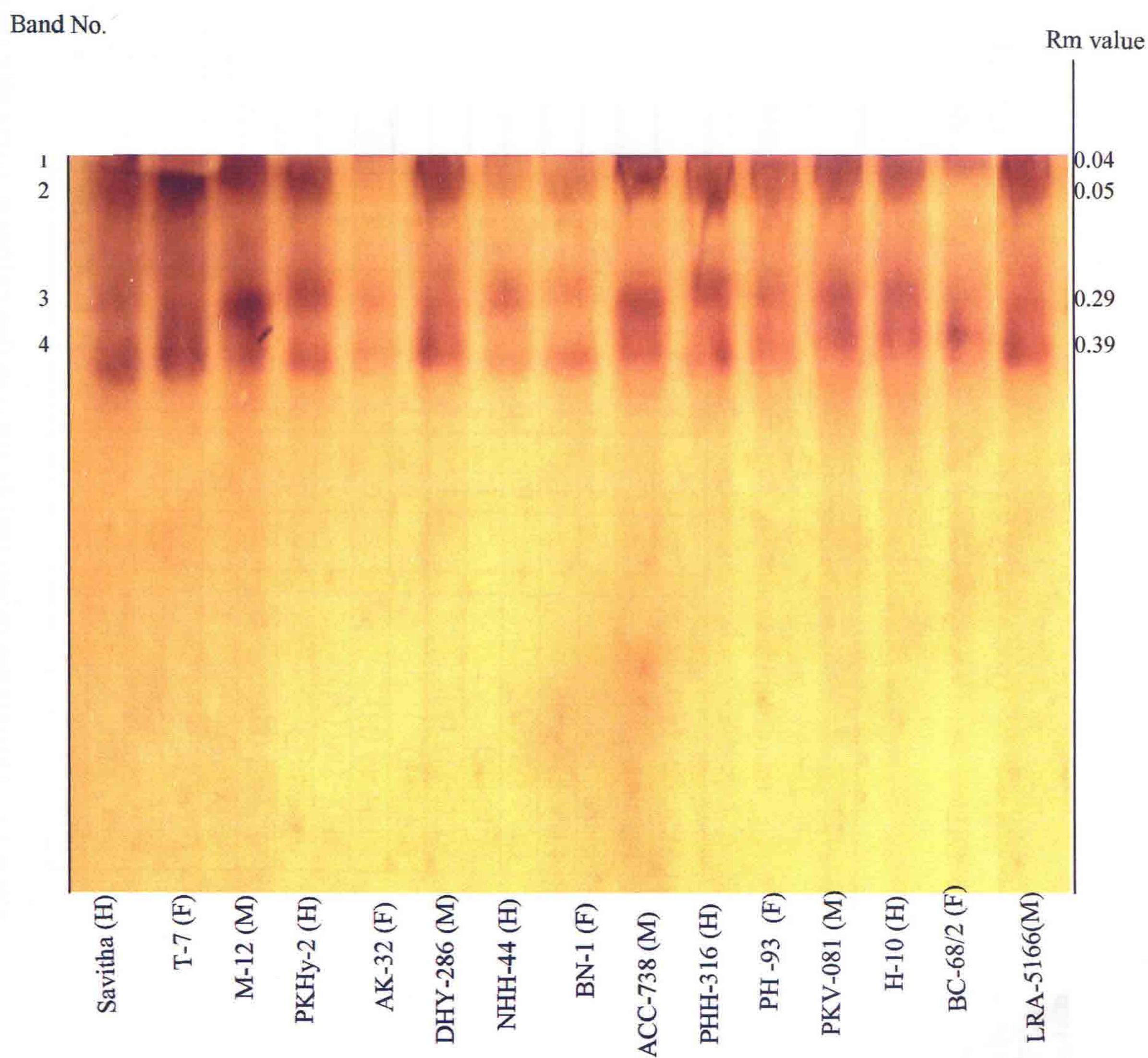
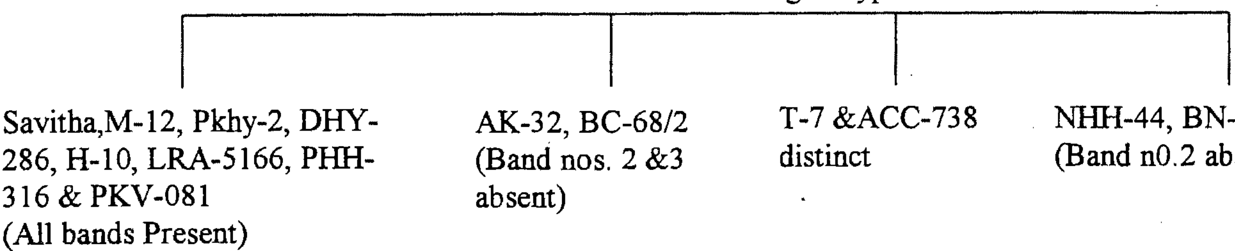


Table 17: Peroxidase isoenzyme profiles of five cotton hybrids and their parents

Band no:	**1**	**2**	**3**	**4**	**Total**	Band absent
Rm Value	0.04	0.05	0.29	0.39		
Savitha (H)	1	1	1	1	**4**	-----
T-7 (F)	0	1	1	1	**3**	1
M-12 (M)	1	1	1	1	**4**	----
PKHy-2 (H)	1	1	1	1	**4**	----
AK-32 (F)	1	0	0	1	**2**	2,3
DHY-286 (M)	1	1	1	1	**4**	----
NHH-44 (H)	1	0	1	1	**4**	2
BN-1 (F)	1	0	1	1	**3**	2
ACC-738 (M)	1	1	1	0	**3**	4
H-10 (H)	1	1	1	1	**4**	----
BC-68/2 (F)	1	0	0	1	**2**	2,3
LRA 5166 (M)	1	1	1	1	**4**	----
PHH-316 (H)	1	1	1	1	**4**	----
PH-093 (F)	1	0	1	1	**3**	2
PKV-081 (M)	1	1	1	1	**4**	----

"1" represents presence "0" and represents absence of a band



4.1.3.9 Malate dehydrogenase (MDH)

The electrophoretic profile of MDH isoenzymes represented in fig.12, table 18 showed a total of 8 bands with one combination of trimeric isoenzymes at Rm values of 0.37, 0.39 and 0.42. Except for the variation in intensities, little polymorphism was observed among the genotypes with respect to MDH isoenzyme patterns, and hence this isoenzyme system primarily exhibited monomorphic loci.

Thus, electrophoresis banding profiles of globulins, soluble proteins, acid phosphatase (ACP) and esterase (EST) isoenzymes, singly, or in combination, could distinguish each of the 15 genotypes studied. Globulins were particularly useful in establishing the hybridity in four of the parental combinations, whereas, soluble proteins, ACP, EST and ADH profiles could distinguish hybrids from their female parents and established hybridity in one or more hybrids.

4.1. .4 Test of homogeneity with respect to isoenzyme patterns

As the parental lines of the cotton hybrids are open pollinated and not strictly inbred lines, which are maintained on the basis of morphological homogeneity, an attempt was made to randomly assess the level of homogeneity in different parental lines with respect to two of the isoenzyme systems studied.

Homogeneity of the parental lines of hybrid H-10 *i.e.*, BC-68/2 and LRA-5166 (Fig 13) was tested by 30 single seedling analysis for esterase isoenzyme pattern and that of the parental line of Savitha, T-7 and M-12 (Fig 14) were tested by 30 single seed analysis of ADH isoenzyme. In addition, homogeneity was also checked with respect to globulin patterns of the parental lines of all hybrids (further discussed

Fig.12: Malate dehydrogenase isozyme profile in five hybrids and parental lines of cotton

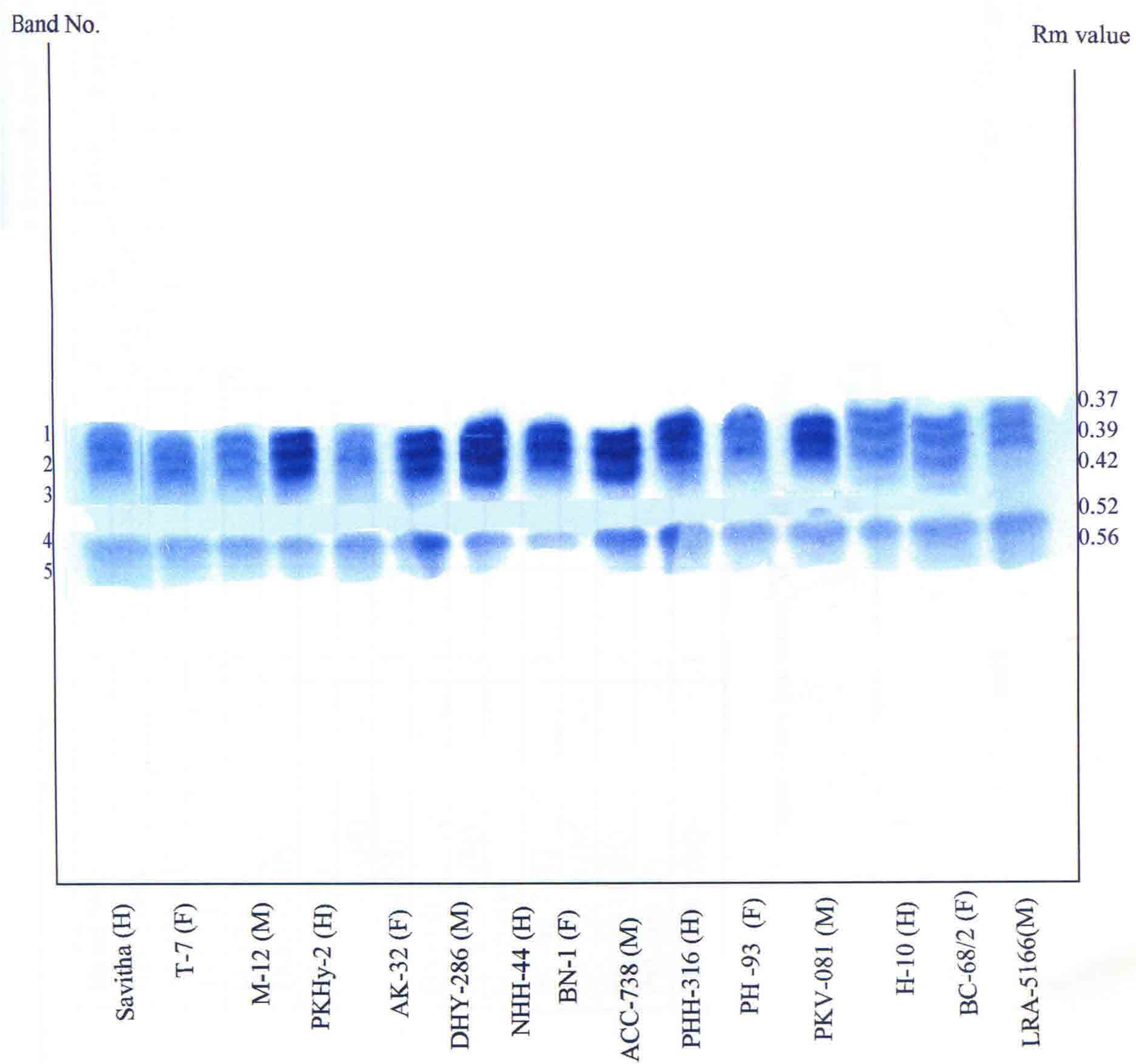


Table 18: Malate dehydrogenase isoenzyme profiles of five cotton hybrids and their parents

Band No.:	**1**	**2**	**3**	**4**	**5**	**Total**	**Band absent**
Rm Value	0.37	0.39	0.42	0.52	0.56		
Savitha (H)	1	1	1	1	1	**5**	---
T-7 (F)	1	1	1	1	1	**5**	---
M-12 (M)	1	1	0	1	1	**4**	3
PKHy-2 (H)	1	1	1	1	1	**5**	---
AK-32 (F)	1	1	1	1	1	**5**	---
DHY-286 (M)	1	1	1	1	1	**5**	---
NHH-44 (H)	1	1	1	1	1	**5**	---
BN-1 (F)	1	1	1	0	1	**4**	4
ACC-738 (M)	1	1	1	1	1	**5**	----
H-10 (H)	1	1	1	1	1	**5**	---
BC-68/2 (F)	1	1	1	1	1	**5**	---
LRA 5166 (M)	1	1	1	1	1	**5**	---
PHH-316 (H)	1	1	1	1	1	**5**	----
PH-093 (F)	1	1	1	1	1	**5**	---
PKV-081 (M)	1	1	1	1	1	**5**	---

"1" represents presence "0" and represents absence of a band

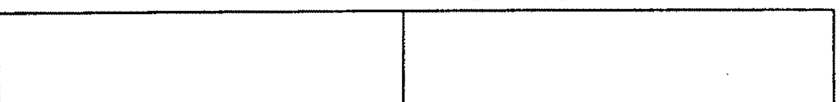
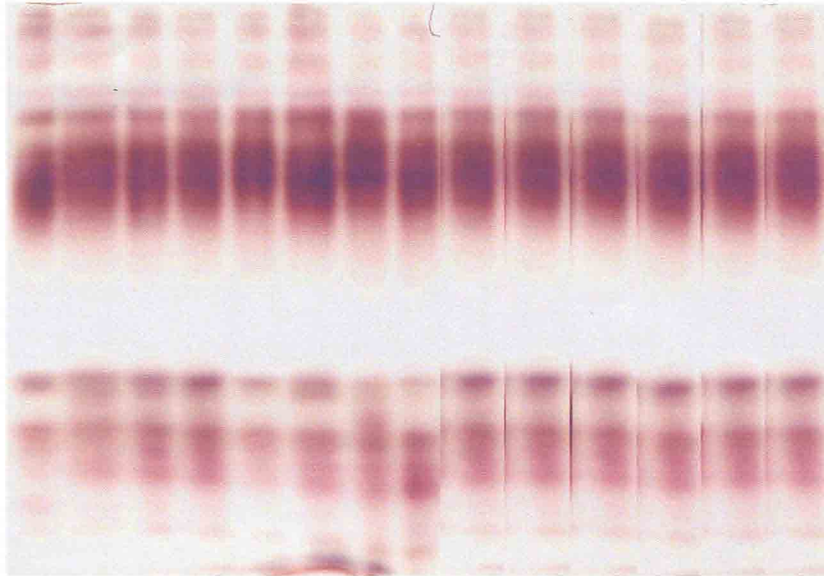
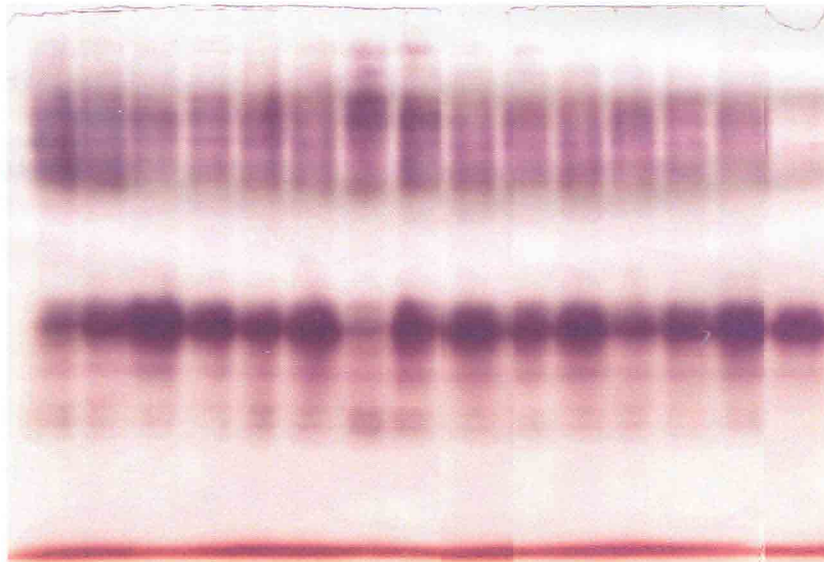


Fig.13: Homogeneity analysis using esterase isoenzyme in parental lines of H-10

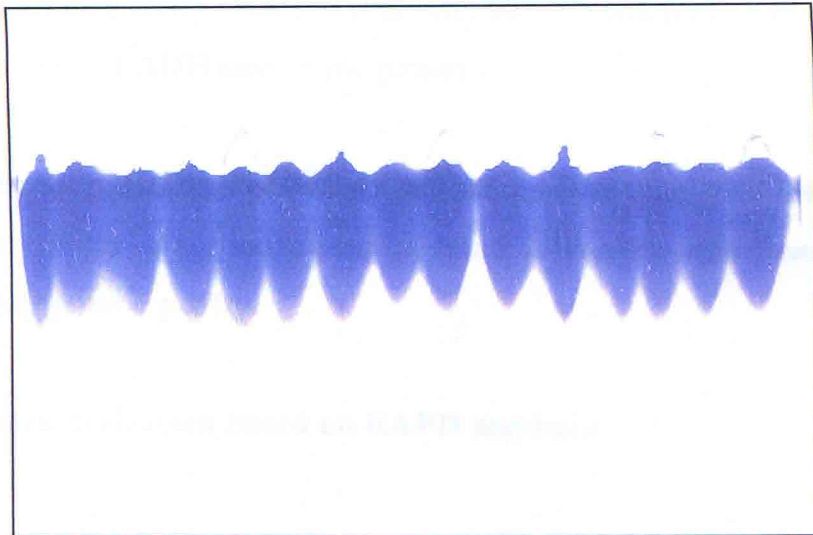


BC-68/2 (F)

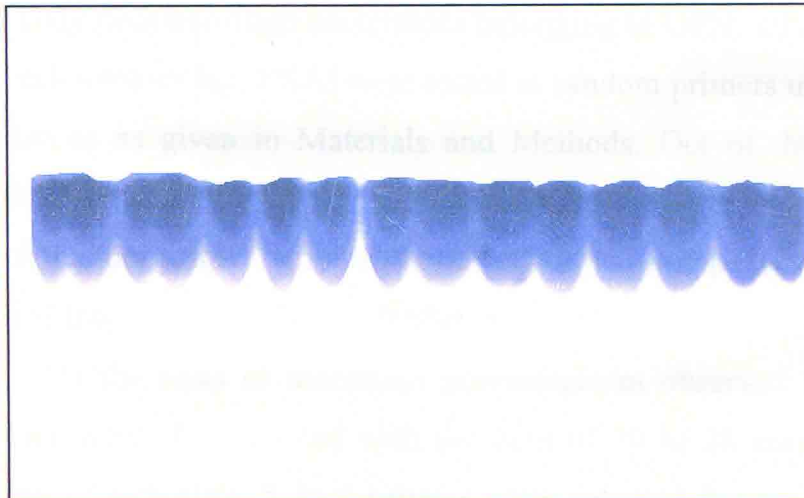


LRA-5166 (M)

Fig.14: **Homogeneity analysis using ADH isoenzyme in parental lines of Savitha**



T-7



M-12

under section4.2.2). Homogeneity test revealed only one clear off type with respect to esterase isoenzyme (lane 7) in BC-68/ 2, whereas, T-7 and M-12 were homogeneous for ADH isoenzyme patterns.

The globulin patterns were fairly uniform within the seed lots of the parental lines, except for some variations in band intensities and hence, were suitable for testing genetic purity.

4.1.5 Characterization based on RAPD markers

The fifteen genotypes were characterized on the basis of polymorphism obtained with random primers using RAPD analysis. The analysis was done on pooled samples of 10 individual seeds each.

A total of sixty decamer oligo nucleotides belonging to OPN, OPO and OPR series (Operon Technologies Inc, USA) were tested as random primers under the specified PCR conditions as given in Materials and Methods. Out of these three primers (OPN-5, OPO-17 and OPR-18) did not amplify the template DNA. The number of bands amplified per primer varied from one (OPR-13) to ten (OPN-10). The size of the amplified fragment varied from 300bp to 3000bp, with an average fragment size of 1000bp. On the basis of maximum polymorphism observed hybrids and their parental lines were characterized with the help of 20 to 28 random primers. For confirmation of hybridity, 5 best primers were selected for each hybrid and the analysis was repeated twice. The discrimination ability of selected primers are discussed below.

4.1.5.1 Savitha and its parental lines

The results of RAPD analysis with different primers in fig.15. Banding scores with using five random primers OPN-09, OPN-10, OPO-6, OPR-1 and OPR-20, which resulted in maximum polymorphism for this hybrid combination, are presented in table19.

OPR- 20

A total of four fragments were amplified with this primer, of which two were common to all. The parental lines T-7 and M-12 were distinct by the amplification of 400 bp and 900 bp size fragments, respectively. The hybrid Savitha exhibited a combination of all four fragments, as complimented by the two parental lines.

OPR-01

Primer OPR-01 produced only three amplified fragments of 800 bp, 900 bp and 1200 bp sizes. Of these, one fragment (900 bp) was common to all. Amplification of fragments of size 1200bp and 800 bp distinguished parents M-12 and T-7 from each other. Hybrid Savitha exhibited a combination of all three bands, complimented by the parental lines.

OPN-09

Primer OPN-09 amplified four polymorphic fragments (fig.15) of 1200bp, 1000bp, 900bp, 700bp and 500bp sizes as presented in the table19. Savitha was represented with the complimentary fragments of 1200bp and 500 bp sizes from T-7 and M-12,

Fig. 15: RAPD profile of hybrid Savitha and its parents using random primers

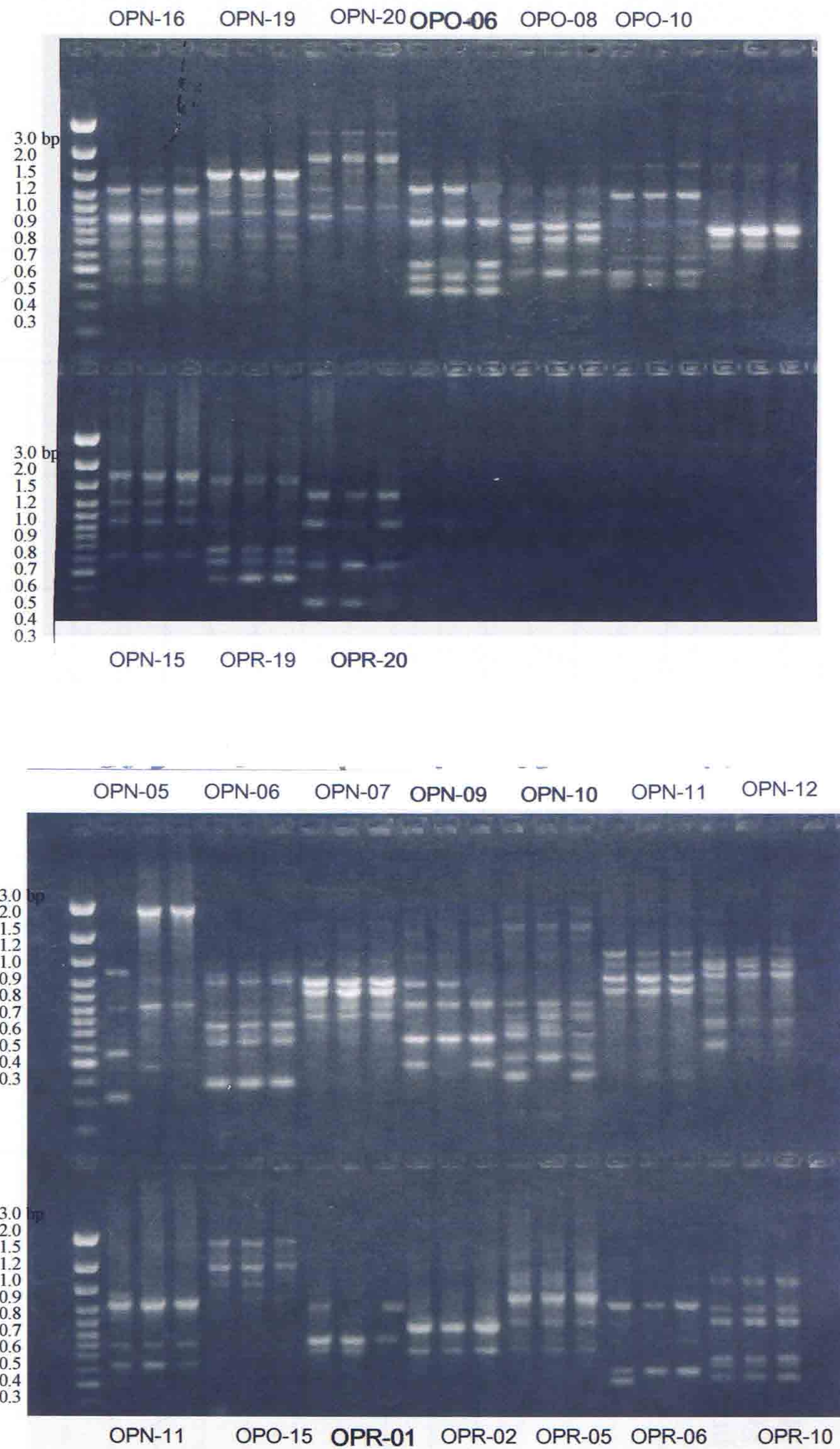


Table 19: RAPD profiles of hybrid Savitha and its parents using random Operon primers

Primer	OPR-20			OPR-01			OPN-09			OPN-10			OPO-06		
Frag (bp)	Savitha (H)	T-7 (F)	M12 (M)	Savitha (H)	T-7 (F)	M12 (M)	Savitha (H)	T-7 (F)	M12 (M)	Savitha (H)	T-7 (F)	M12 (M)	Savitha (H)	T-7 (F)	M12 (M)
3000															
2500															
2200															
2000										1	1	1			
1800										1	1	1			
1500										1	1	1			
1200	1	1	1	1	0	1	1	1	0				1	1	0
1000													1	1	1
900	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
800				1	1	0				1	1	1			
700							1	1	1	1	1	0			
600	1	1	1												
500							1	0	1	1	1	1	1	0	1
400	1	1	0							1	0	1	1	1	1
300													1	1	1

"1" represents presence "0" represents absence of a fragment

T-7 : Female parent

M-12 : Male parent

respectively. Rest of the fragments are monomorphic and common among the genotypes.

OPN-10

A total of eight fragments (fig.15) were amplified, ranging from 400bp to 2000 bp sizes. Hybrid Savitha exhibited a complimentary pattern represented by fragments of size 700bp contributed by T-7, and 400 bp contributed by M-12. The rest of the fragments were common to all.

OPO-06

A total of six fragments were amplified with this primer. Of these, four fragments of 1000 bp, 900 bp, 400 bp and 300 bp were common to all. The fragments of size 1200 bp and 500 bp characteristics of T-7 and M-12, respectively were complimented in hybrid Savitha.

4.1.3.2 PKHy-2 and its parental lines

The RAPD analysis of PKHy-2 and its parental lines with five random primers, which established the hybridity are shown in fig.16 and table20.

OPR-12

A total of four fragments of size ranging from 1000bp to 2500 bp were amplified with OPR-!2. Two fragments of 1000 bp and 1200 bp were common to all. Fragments of size 1500 bp and 800 bp were distinct for the male (DHY-286) and

Fig.16: RAPD profile of hybrid PKHy-2 and its parents using random primers

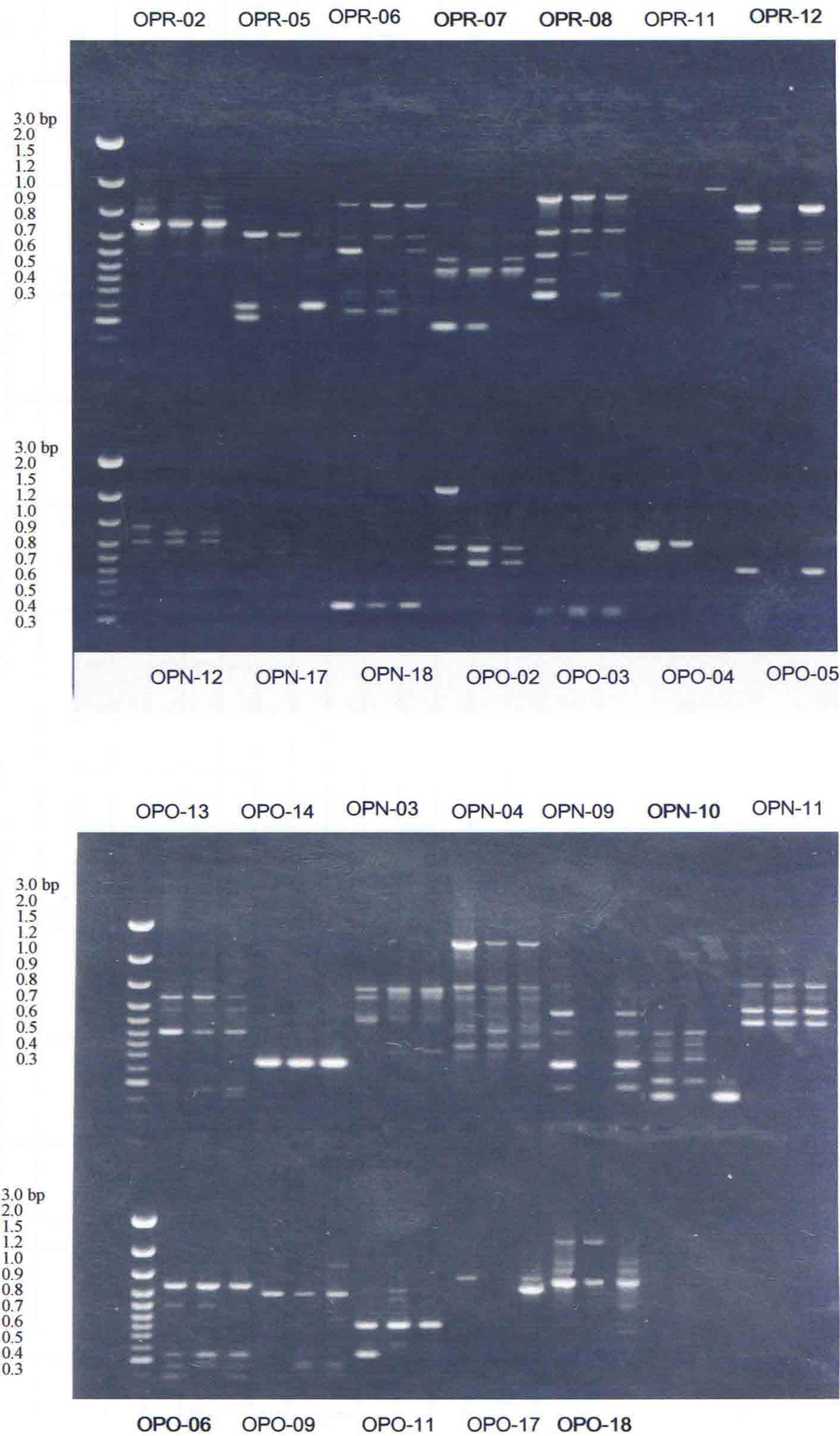


Table 20 : RAPD profiles of hybrid PKHy-2 and its parents using random Operon primers

Primer	OPR-12			OPO-06			OPO-18			OPR-07			OPR-08		
Frag (bp)	PKHy-2 (H)	AK-32 (F)	DHY-286 (M)	PKHy-2 (H)	AK-32 (F)	DHY-286 (M)	PKHy-2 (H)	AK-32 (F)	DHY-286 (M)	PKHy-2 (H)	AK-32 (F)	DHY-286 (M)	PKHy-2 (H)	AK-32 (F)	DHY-286 (M)
3000															
2500															
2200							1	1	0						
2000															
1800							1	0	1						
1500	1	0	1				1	0	1				1	1	1
1200	1	1	1	1	1	1	1	1	1				1	1	1
1000	1	1	1	1	1	0				1	0	1	1	1	0
900													1	0	0
800	1	1	0							1	1	1			
700													1	0	1
600				1	1	1									
500				1	1	1				1	1	0			
400				1	0	1									
300															

"1" represents presence "0" and represents absence of a fragment

AK-32 : Female parent

DHY-286 : Male paren

female (AK-32) parents, respectively, which were represented in combination in hybrid PKHy-2.

OPO-06

Five fragments were amplified by the primer OPO-06, of which three fragments of size 1200, 600 and 500 bp were common to all. Parental lines DHY-286 and AK-32 were characterized by one fragment each of size 400 bp and 1000 bp, respectively. PKHy-2 was distinct by the presence of fragments of size 400bp and 1000bp contributed by DHY-286 and AK-32, respectively.

OPO-18

The primer OPO-18 amplified four fragments in PKHy-2. Of these, two fragments of size 1800 bp and 1500 bp were complimented from the male parent (DHY-286), and the fragment of size 2200 bp was from the female parent (AK-32).One fragment was common to all.

OPR-07

This primer amplified a total of three fragments. Hybrid PKHy-2 was complimented by one fragmentof size 1000 bp from the male parent DHY-286 500 bp from the female parent AK-32 . One fragment was common to all.

OPR-08

The primer OPR-08 amplified five fragments. Hybrid PKHy-2 was complimented by one fragment from each of the parents *i.e.*, fragment of size 700 bp from the

male parent (DHY-286) and fragment of size 1000 bp from the female parent AK-32. Rest of the fragments are common to all.

4.1.5.3 Hybrid NHH-44 and its parental lines

The results of RAPD analysis with five random primers, which distinguished NHH-44 and its parental lines and established the hybridity, are shown in fig.17 and table21.

OPO-02

The primer OPO-02 produced six amplified fragments of size ranging from 300 bp to 1200 bp, of which one was common to all. Hybrid NHH-44 was complimented by two bands from each of the parental lines *i.e.*, fragments of size 300 bp, 700 bp and 800 bp from the male parent ACC-738 and fragments of 1200 and 1000 bp from the female parental line BN-1.

OPO-08

The primer OPO-08 amplified three fragments of size ranging from 500bp to 1200bp. Of these one band is common. Hybrid NHH-44 was complimented by one fragment of size 1200bp from ACC-738 and one fragment of size 500 bp from BN-1.

Fig. 17: RAPD profile of hybrid NHH-44 and its parents using random primers

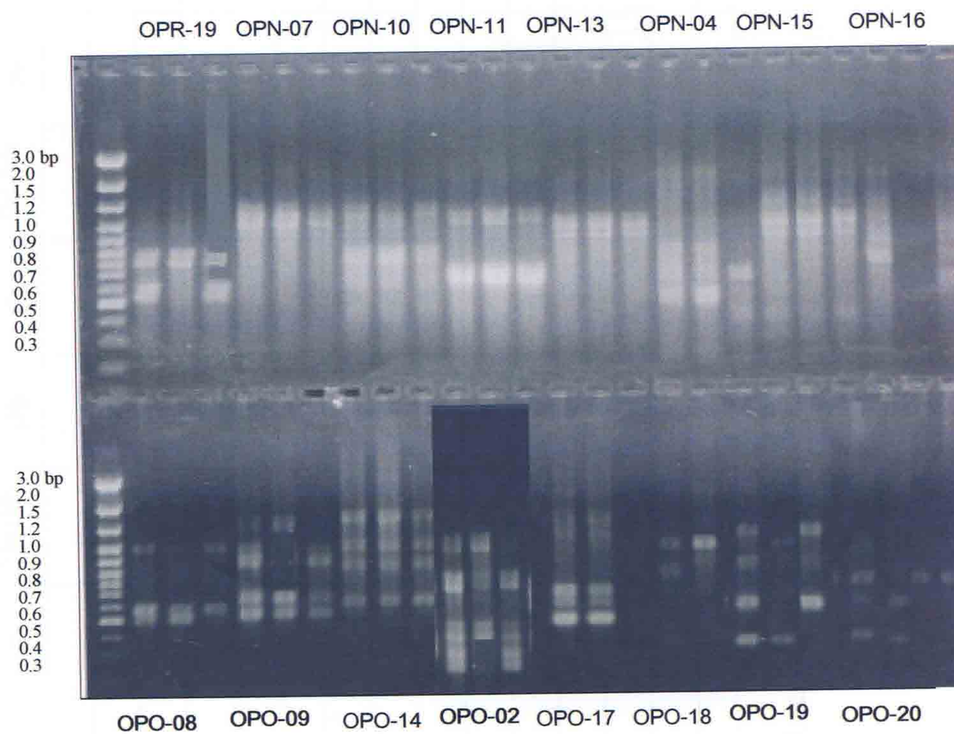


Table 21: RAPD profiles of hybrid NHH-44 and its parents using random Operon primers

Primer	OPO-02			OPO-08			OPO-09			OPO-19			OP0-20		
Frag (bp)	NHH-44 (H)	BN-1 (F)	ACC-738 (M)	NHH-44 (H)	BN-1 (F)	ACC-738 (M)	NHH-44 (H)	BN-1 (F)	ACC-738 (M)	NHH-44 (H)	BN-1 (F)	ACC-738 (M)	NHH-44 (H)	BN-1 (F)	ACC-738 (M)
3000															
2500															
2200															
2000															
1800															
1500							1	1	0	1	0	1			
1200	1	1	0	1	0	1	1	0	1						
1000	1	1	0				1	0	1						
900										1	0	1			
800	1	0	1												
700	1	0	1				1	1	1	1	0	1	1	0	1
600				1	1	1	1	1	1				1	1	0
500				1	1	0									
400	1	1	1												
300	1	0	1							1	1	0	1	1	1

"1" represents presence "0" and represents absence of a fragment

BN-1 : Female parent

ACC-738 : Male parent

OPO-09

This primer amplified a total of five fragments. Hybrid NHH- 44 was complimented by one fragment of size 1500 bp from female parent BN-1, and two fragments of size 1200 bp and 1000 bp from male parent ACC-738.

OPO-19

A total of four fragments were amplified with the primer OPO-19. Hybrid NHH-44 was complimented by three fragments of sizes 1200 bp, 900 bp and 700bp from the male parent ACC-738 and with a single fragment of size 300bp from the female parent BN-1

OPO-20

The primer OPO-20amplified a total of three fragments, of these one fragment 300 was common to all. Hybrid NHH- 44 was complimented by one fragment of size 700 bp from male parent ACC-738, and a single fragment of size 600 bp from female parent BN-1.

4.1.5.4 H-10 and its parental lines

The results of RAPD analysis using six primers, which distinguished the H-10 and its parents, establishing the hybridity, are given in fig.18 and table 22.

Fig.18: RAPD profile of hybrid H-10 and its parents using random primers

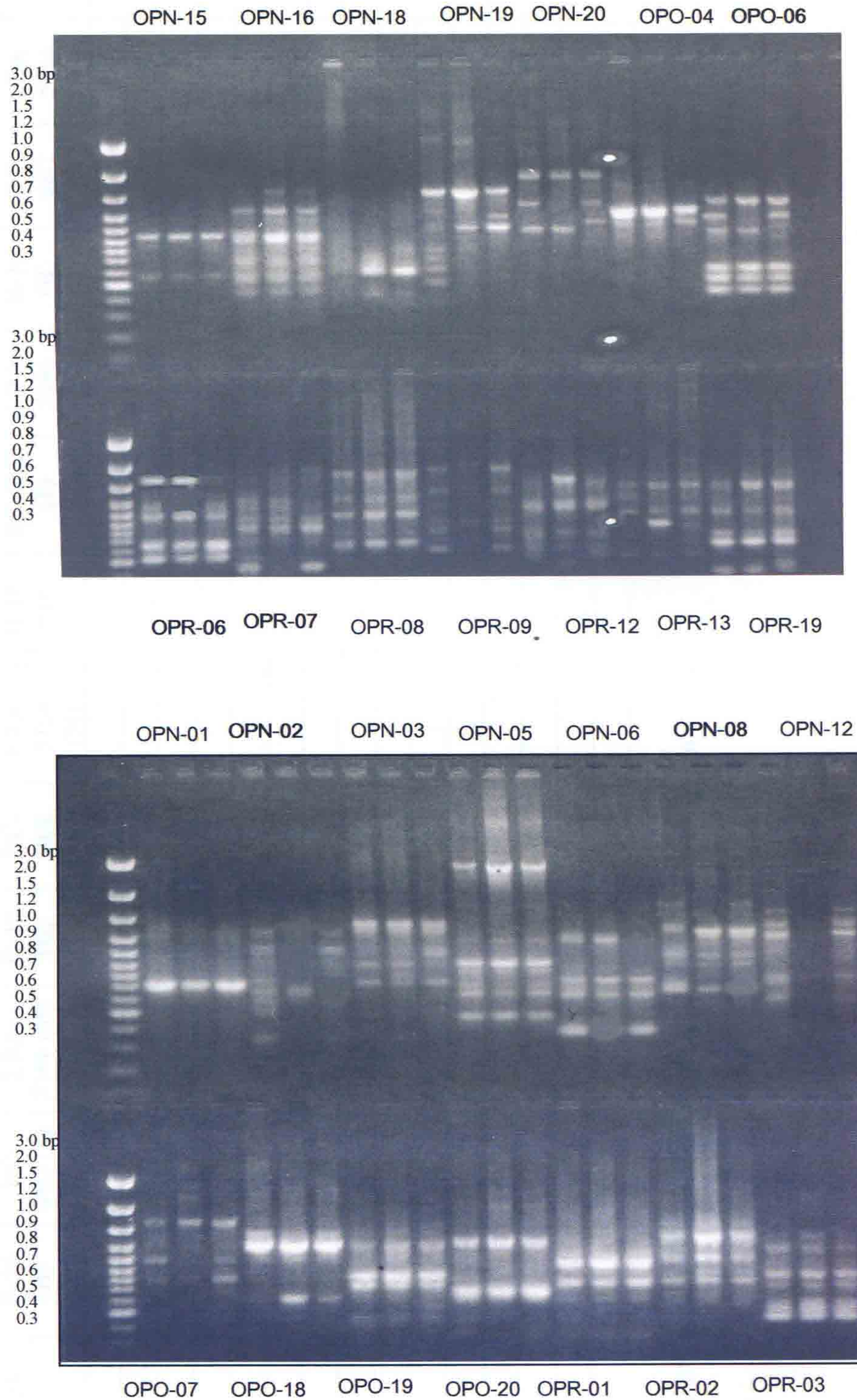


Table 22: RAPD profiles of hybrid H-10 and its parents using random Operon primers

Primer	OPO-06			OPN-08			OPR-07			OPN-06			OPN-02		
Frag (bp)	H-10 (H)	BC-68/2 (F)	LRA-5166 (M)	H-10 (H)	BC-68/2 (F)	LRA-5166 (M)	H-10 (H)	BC-68/2 (F)	LRA-5166 (M)	H-10 (H)	BC-68/2 (F)	LRA-5166 (M)	H-10 (H)	BC-68/2 (F)	LRA-5166 (M)
3000															
2500															
2200															
2000															
1800															
1500	1	1	1	1	0	1									
1200	1	0	1				1	1	0						
1000	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1
900				1	1	1							1	0	1
800				1	1	1	1	1	1	1	1	1	1	0	1
700				1	1	0	1	1	1	1	1	1	1	1	0
600										1	0	1			
500															
400							1	0	1						
300															

"1" represents presence "0" and represents absence of a fragment

BC-68/2 : Female parent

LRA-5166 : Male parent

OPO-06

The primer OPO-06 amplified a total of three fragments. Of these one fragment of size 1500 was common to all. H-10 was complimented by fragments of size 1000 bp and 1200 from BC-68/2 and LRA-5166, respectively.

OPN-08

A total of five fragments were amplified with the primer OPN-08, of these three fragments of sizes 1000, 900 and 800 bp were common to all and the parents are distinguished by one fragment each. H-10 had been complimented by fragments of 1500 bp from LRA-5166 and 700 bp from BC-68/2.

OPR-07

A total of five fragments were also amplified with the primer OPR-07. Of these, fragments of size 400 bp from LRA-5166 had complimented H-10 whereas two fragments of size 1000 bp and 1200 bp were from BC-68/2. Two fragments of size 700 and 800 bp were common to all genotypes.

OPN-06

A total of four fragments were amplified with the primer OPN-06, of these two fragments of sizes 800 and 700 bp were common to all. Fragments of size 600 bp from LRA-5166 and 1000 bp from BC-68/2 had complimented H-10.

OPN-02

A total of four fragments were amplified with the primer OPN-02. Three fragments of 1000, 900 and 800 bp from LRA-5166 and one fragment of 700 bp from BC-68/2, respectively were represented in hybrid H-10.

4.1.[illegible].5 PHH-316 and its parental lines

The RAPD profiles obtained by using five random primers, which established the hybridity showing maximum polymorphism, was shown in fig.19 and table 23.

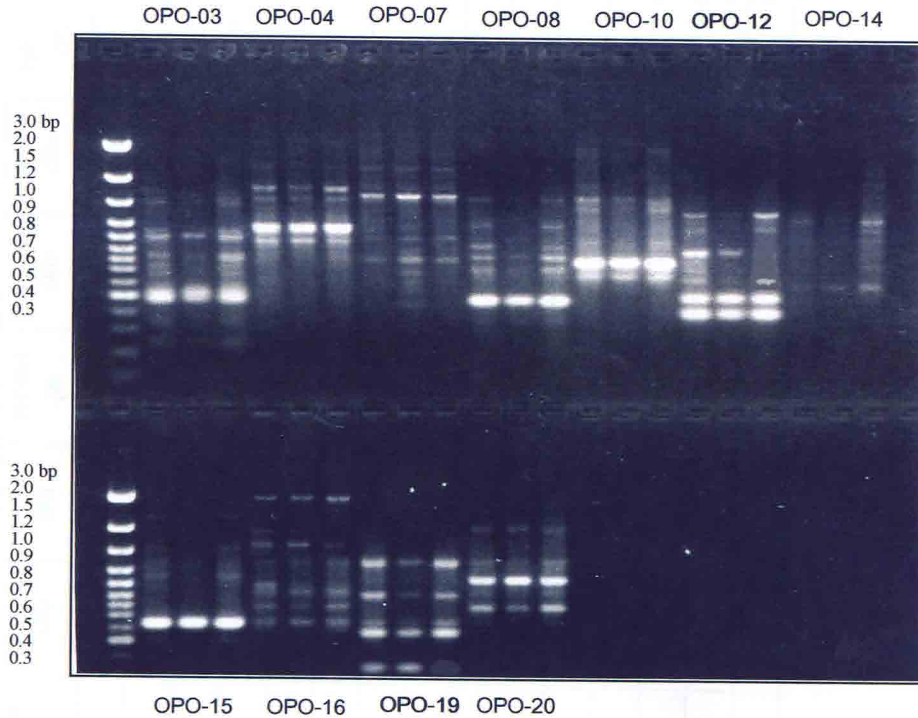
OPN-16

A total of four fragments were amplified with the primer OPN-09. Of these, two fragments of sizes 1500 and 1200 bp were common to all and PHH-316 was characterized by the presence of all four fragments. Fragments of size 3000 bp and 2000 bp were characteristic of PH-093 and PKV-081, respectively.

OPO-12

A total of seven fragments of size ranging from 400 bp to 1200 bp were amplified with this primer. Of this, two bands were common. Three fragments of size 1200, 1000 and 700 were characteristic of PKV-081 and one fragment of size 900 bp of the female parental line PH-093. Hybrid PHH-316 was characterized by the presence of all six fragments, contributed by the two parental lines.

Fig. 19: RAPD profile of hybrid PHH-316 and its parents using random primers



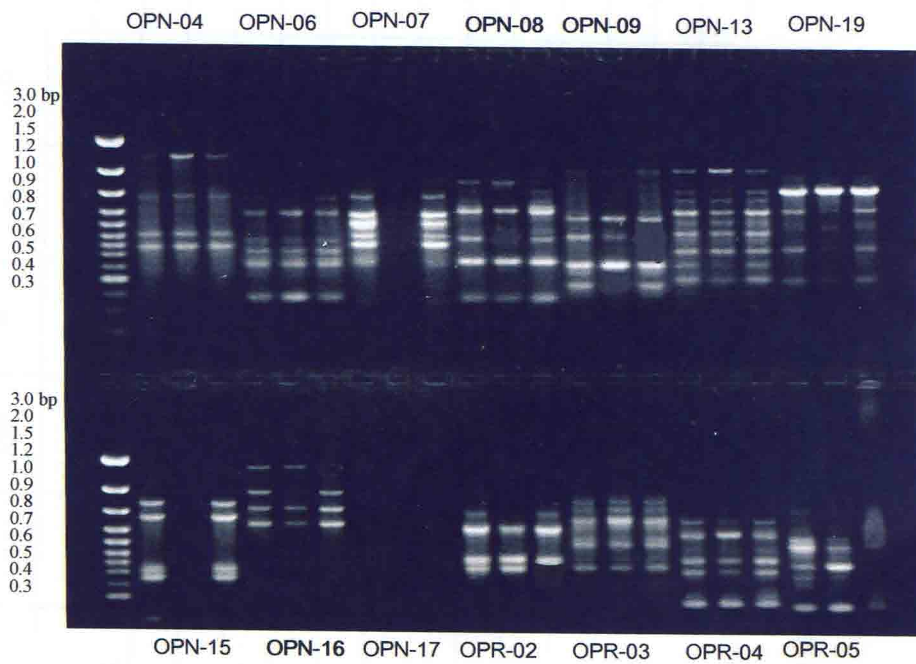


Table 23: RAPD profiles of hybrid PHH-316 and its parents using random Operon primers

Primer	OPN-16			OP0-12			OP0-19			OPN-08			OPN-09		
Frag (bp)	PHH-316 (H)	PH-093 (F)	PKV-081 (M)	PHH-316 (H)	PH-093 (F)	PKV-081 (M)	PHH-316 (H)	PH-093 (F)	PKV-081 (M)	PHH-316 (H)	PH-093 (F)	PKV-081 (M)	PHH-316 (H)	PH-093 (F)	PKV-081 (M)
3000															
2500	1	1	0												
2200															
2000															
1800	1	0	1												
1500	1	1	1							1	1	0			
1200	1	1	1	1	1	1	1	0	1	1	1	1			
1000							1	0	1				1	1	1
900				1	0	1	1	1	0				1	1	0
800							1	0	0	1	0	1			
700							1	0	1						
600				1	0	1				1	1	1	1	1	1
500				1	1	1	1	1	1				1	0	1
400							1	1	1						
300				1	1	0									

"1" represents presence "0" and represents absence of a fragment

PKV-081 : Male parent

PH-093 : female parent

OPN-08

The primer OPN-08 amplified four fragments of size ranging from 600 bp to 1500 bp. Of these, two fragments of 1200 and 600 bp sizes were common to all while parental lines were distinguished by the presence of one additional fragment each. PHH-316 was complimented with the fragment of size 800 bp from the male parental line PKV-081 and fragment of size 1500 bp was from the female parental line PH-093.

OPN-09

This primer amplified a total of four fragments. Of these two fragments of sizes 1000 bp and 700 bp were common to all whereas one fragment of size 900 and 400 bp respectively was characteristic of the parental lines PH-093 and PKV-081. Fragments of sizes 900 bp and 600 bp from PH-093 and PKV-081, respectively complimented hybrid PHH-316.

OPN-08

The primer OPN-08 amplified four fragments, of these two fragments of size 1200and 600 bp were common to all. One fragment of size 800 and 1500 bp was characteristic of PKV-081 and PH-093, respectively. Banding pattern of the hybrid PHH-316 was complimented by the marker fragments of both the parents.

Thus, based on RAPD analysis each of the five hybrids and their parental lines could be distinguished reliably. Each hybrid combination could be identified and hybridity established with the help of at least five primers independently.

Three primers (out of 22) were common for two sets of hybrids and parental lines, though the sizes of the amplified fragments differed among these. Hence, with the help of four to five random primers (one for each set), it is possible to distinguish each of the five hybrids and their parents.

4.2. Genetic purity testing

4.2.1 Field Grow-Out trial

For the purpose of testing genetic purity of four commercial lots of three hybrids, field grow out trial was conducted at IARI farm, New Delhi. The grow-out test for the above lots was also conducted by the APSSCA at Amaravati farm, Andhra Pradesh. A total of 300-400 individual plants per sample at each of the locations were evaluated as per standard descriptors to calculate the percent off types and selfed females (Table24). The purity level of four lots of each hybrid recorded at New Delhi varied from 86 to 95.5 per cent in Savitha (Tables 25 to 28), 91.5 to 95.0 per cent in NHH-44 (Tables 29 to 32) and 95.5 to 99.0 per cent in H-10 (Tables 33 to 36). The corresponding values recorded by the APSSCA at Amaravati ranged from 89.0 to 96.0 per cent, 91.0 to 96.0 per cent and 93.0 to 98.5 percent, respectively. Only one lot of hybrid Savitha was found below the prescribed standard of genetic purity (90 per cent), whereas other eleven lots showed fairly high levels of purity. The occurrence of off types was nil in eight lots and ranged from one to two in the remaining.

4.2.2. Electrophoresis analysis

SDS-PAGE profile of globulin fractions from 200 single seeds from the above 12 lots were examined for the purpose of testing the genetic purity (Fig. 20 to 22). The

Table: 24 Results of genetic purity tests

Hybrid	Lot no.	Grow-out test, Amaravati	Grow-out test, New Delhi.	Lab. Test, New Delhi
Savitha	1	89.0	86.0	88.0
Savitha	2	92.0	94.5	92.0
Savitha	3	90.0	93.5	93.0
Savitha	4	96.0	95.5	95.5
H-10	5	98.5	99.0	98.0
H-10	6	93.0	95.5	95.0
H-10	7	96.5	98.5	98.0
H-10	8	95.5	97.0	97.5
NHH-44	9	96.0	95.0	93.5
NHH-44	10	91.0	91.5	92.0
NHH-44	11	93.0	92.0	92.0
NHH-44	12	96.0	95.0	91.0

Fig.20: Genetic purity analysis of Savitha using salt soluble globulin pattern

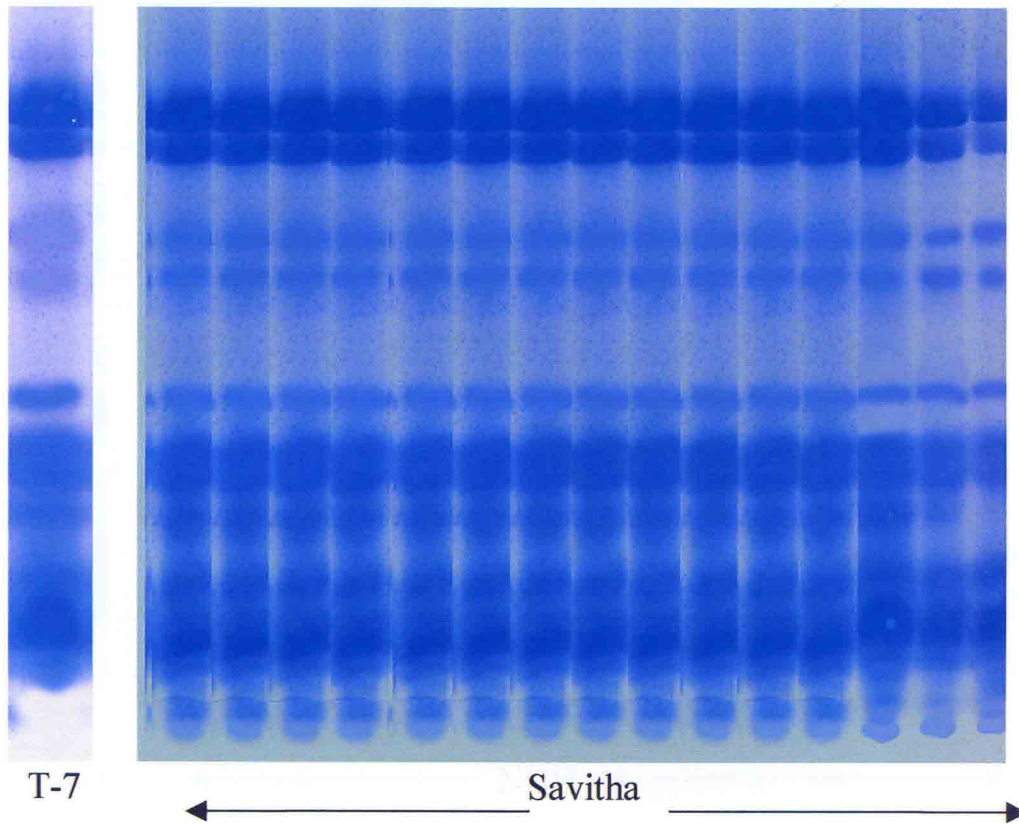


Fig.21: Genetic purity analysis of NHH-44 using salt soluble globulin pattern

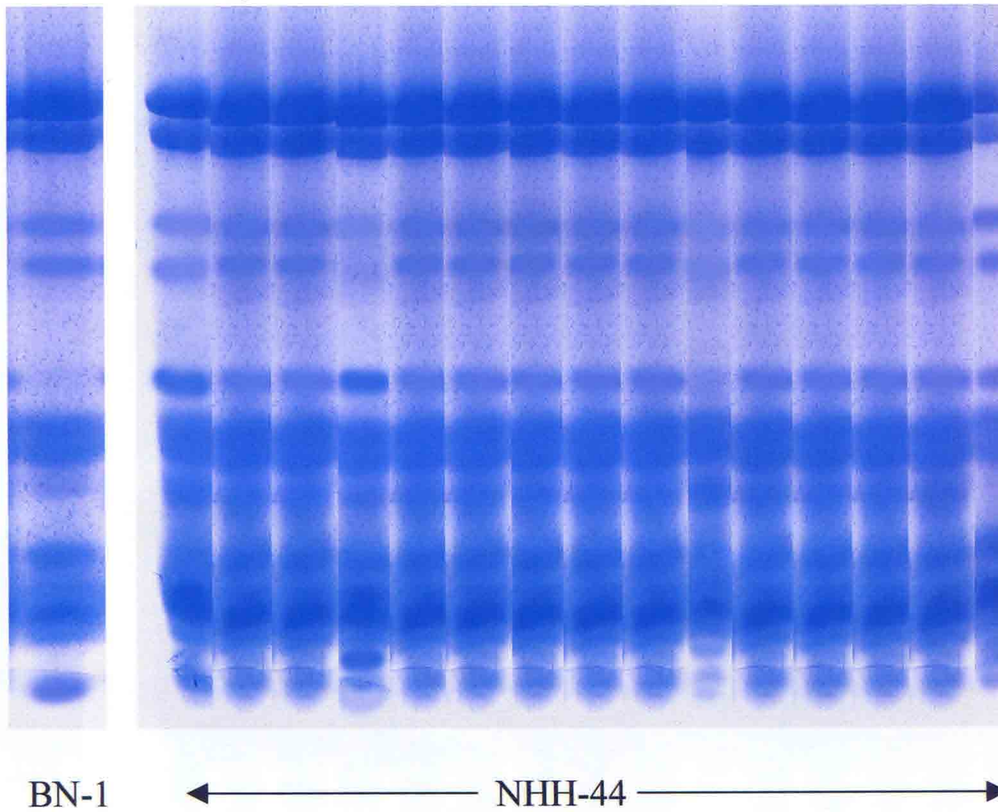
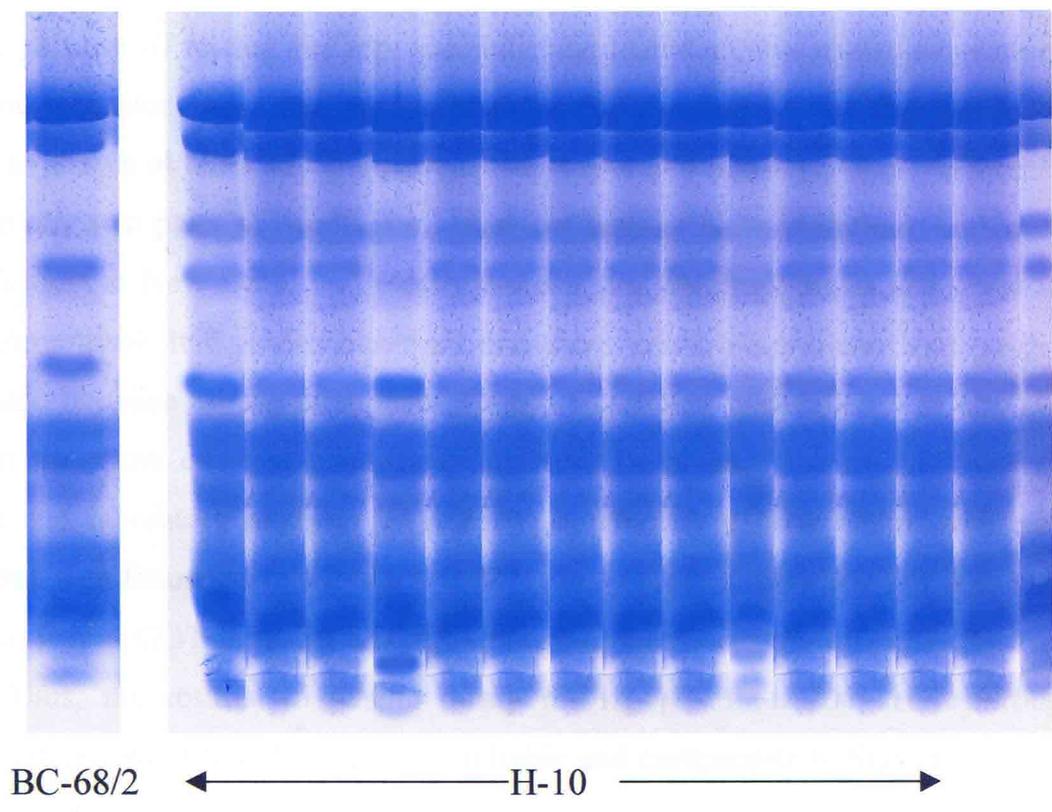


Fig. 22 Genetic purity analysis of H-10 using salt soluble globulin pattern



purity level ranged from 88.0 to 95.5 per cent in Savitha, from 91.0 to 93.0 per cent in NHH-44 and 95.0 to 99.0 per cent in H-10 (Table 24). The number of off types was nil in 8 samples and one out of 200 in the rest of the four. Thus, rejection was solely on the occurrence of higher number of selfed seeds.

4.2.3 Correlation of results

For the purpose of testing the reproducibility and relating the results of grow out trials and laboratory test, the data were subjected to paired 't' test and correlation studies as shown in table 37. The paired 't' test revealed non-significant differences between any two pairs of results, i.e., grow out tests at New Delhi and Amaravati, grow out test at New Delhi and electrophoresis and electrophoresis and grow out test at Amaravati. It was also observed that there exists a significant and positive correlation between all the three tests. Highly significant correlation was observed between the grow out test conducted at New Delhi and that of APSSCA (r= 0.81**) and laboratory analysis (r= 0.85**) conducted at New Delhi, whereas a lower but significant correlation (r= 0.72*) was obtained between the results of grow-out test at GOT farm, APSSCA, Amaravati and laboratory test at IARI, New Delhi. Thus, the results of genetic purity testing based on 200 seeds through electrophoresis were found to be fairly reliable and comparable to that of grow-out test.

4.2.4. Sequential sampling (SS)

For the purpose of reducing the sample size to an optimum level, observations on selfed and off types recorded in batches of 20individual plants / seeds in a sequence in the field grow out trial at New Delhi and laboratory test were analyzed using the sequential sampling technique. Samples of 20 individual plants were evaluated for

Table 25: Sequential sampling analysis for testing genetic purity

Hybrid : Savitha Lot no.:1														
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
ıpto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	1	0	1	c	c	c	4	0	4	c	c	c
59	**4**	**7**	4	1	5	c	c	c	6	0	6	c	c	c
79	**6**	**9**	5	1	6	c	c	c	8	0	8	c	c	c
99	**8**	**11**	10	1	11	c	c	c	11	0	11	c	c	c
119	**10**	**13**	11	1	12	c	c	c	11	1	12	c	c	c
139	**12**	**15**	14	1	15	r	c	r	13	1	14	c	c	r
159	**14**	**17**	16	1	17	r	c	r	16	1	17	c	c	r
179	**16**	**19**	18	1	19	r	c	r	19	1	20	r	c	r
199	**18**	**21**	20	1	21	r	c	r	23	1	24	r	c	r
219	**20**	**23**	21	1	22	r	c	r						
239	**22**	**25**	24	1	25	r	c	r						
259	**24**	**27**	26	1	27	r	c	r						
279	**26**	**29**	28	1	29	r	c	r						
299	**28**	**31**												
319	**30**	**33**												
339	**32**	**35**												
359	**34**	**37**												
369	**36**	**39**												
399	**38**	**41**												
Total no. of plants:278					Genetic purity %: 86.0				Total no. of seeds:200			Genetic purity %:88.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 26: Sequential sampling analysis for testing genetic purity

Hybrid : Savitha Lot no.:2															
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE						
CSS	WTT		Observations			Decision			Observations			Decision			
	A	**R**	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c	
39	**2**	**5**	0	0	0	c	c	c	4	0	4	c	c	c	
59	**4**	**7**	4	0	4	c	c	c	5	0	5	c	c	c	
79	**6**	**9**	8	0	8	c	c	c	7	0	7	c	c	c	
99	**8**	**11**	7	1	8	c	c	c	8	0	8	c	c	c	
119	**10**	**13**	7	1	8	c	c	c	9	0	9	c	c	c	
139	**12**	**15**	8	1	9	a	c	a	12	0	12	a	a	a	
159	**14**	**17**	8	1	9	a	c	a	13	0	13	a	a	a	
179	**16**	**19**	9	1	10	a	c	a	16	0	16	a	a	a	
199	**18**	**21**	10	1	11	a	c	a	16	0	16	a	a	a	
219	**20**	**23**	11	1	12	a	c	a							
239	**22**	**25**	11	1	12	a	c	a							
259	**24**	**27**	11	2	13	a	c	a							
279	**26**	**29**	11	2	13	a	c	a							
299	**28**	**31**	12	2	14	a	c	a							
319	**30**	**33**	12	2	14	a	c	a							
339	**32**	**35**	12	2	14	a	c	a							
359	**34**	**37**	16	2	18	a	c	a							
369	**36**	**39**	16	2	18	a	c	a							
399	**38**	**41**													
Total no. of plants:364					Genetic purity %:94.5				Total no of seeds: 200			Genetic purity %:92.0			

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 27: Sequential sampling analysis for testing genetic purity

Hybrid : Savitha			Lot no.:3											
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	0	0	0	c	c	c	4	0	4	c	c	c
59	**4**	**7**	2	0	2	c	c	c	5	0	5	c	c	c
79	**6**	**9**	5	0	5	c	c	c	7	0	7	c	c	c
99	**8**	**11**	6	0	6	c	c	c	8	0	8	c	c	c
119	**10**	**13**	6	0	6	c	c	c	11	0	11	c	c	c
139	**12**	**15**	7	0	7	a	a	a	12	0	12	a	a	a
159	**14**	**17**	8	0	8	a	a	a	13	0	13	a	a	a
179	**16**	**19**	9	0	9	a	a	a	13	0	13	a	a	a
199	**18**	**21**	11	0	11	a	a	a	14	0	14	a	a	a
219	**20**	**23**	12	0	12	a	a	a						
239	**22**	**25**	13	0	13	a	a	a						
259	**24**	**27**	14	0	14	a	a	a						
279	**26**	**29**	16	0	16	a	a	a						
299	**28**	**31**	17	0	17	a	a	a						
319	**30**	**33**	18	0	18	a	a	a						
339	**32**	**35**	19	0	19	a	a	a						
359	**34**	**37**	21	0	21	a	a	a						
369	**36**	**39**	21	0	21	a	a	a						
399	**38**	**41**	22	0	22	a	a	a						
Total no. of plants:378					Genetic purity %:93.5				Total noof seeds: 200			Genetic purity %:93.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 28: Sequential sampling analysis for testing genetic purity

Hybrid : Savitha			Lot no.:4											
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	4	0	4	c	c	c	2	0	2	c	c	c
59	**4**	**7**	5	0	5	c	c	c	2	0	2	c	c	c
79	**6**	**9**	5	1	6	c	c	c	4	0	4	c	c	c
99	**8**	**11**	5	1	6	c	c	c	4	0	4	c	c	c
119	**10**	**13**	7	1	8	c	c	c	4	0	4	c	c	c
139	**12**	**15**	9	1	10	a	c	a	6	0	6	a	a	a
159	**14**	**17**	9	1	10	a	c	a	8	0	8	a	a	a
179	**16**	**19**	9	1	10	a	c	a	8	0	8	a	a	a
199	**18**	**21**	12	1	13	a	c	a	9	0	9	a	a	a
219	**20**	**23**	13	1	14	a	c	a						
239	**22**	**25**	13	1	14	a	c	a						
259	**24**	**27**	13	1	14	a	c	a						
279	**26**	**29**	15	1	16	a	c	a						
299	**28**	**31**	16	1	17	a	c	a						
319	**30**	**33**	16	1	17	a	c	a						
339	**32**	**35**	16	2	18	a	c	a						
359	**34**	**37**	18	2	20	a	c	a						
369	**36**	**39**	20	2	22	a	c	a						
399	**38**	**41**	20	2	22	a	c	a						
Total no. of plants:397					Genetic purity %:95.5				Total no of seeds: 200			Genetic purity %:95.5		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 29: Sequential sampling analysis for testing genetic purity

Hybrid NHH-44 Lot no.:1															
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE						
CSS	WTT		Observations			Decision			Observations			Decision			
	A	**R**	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	1	0	1	c	c	c	3	1	4	c	c	c
59	**4**	**7**	2	0	2	c	c	c	5	1	6	c	c	c
79	**6**	**9**	4	0	4	c	c	c	5	1	6	c	c	c
99	**8**	**11**	5	0	5	c	c	c	8	1	9	c	c	c
119	**10**	**13**	6	1	7	c	c	c	8	1	9	c	c	c
139	**12**	**15**	6	1	7	a	c	a	10	1	11	a	a	a
159	**14**	**17**	7	1	8	a	c	a	11	1	12	a	a	a
179	**16**	**19**	7	1	8	a	c	a	13	1	14	a	a	a
199	**18**	**21**	8	1	9	a	c	a	14	1	15	a	a	a
219	**20**	**23**	8	1	9	a	c	a						
239	**22**	**25**	8	1	9	a	c	a						
259	**24**	**27**	9	1	10	a	c	a						
279	**26**	**29**	9	1	10	a	c	a						
299	**28**	**31**	11	1	12	a	c	a						
319	**30**	**33**	13	1	14	a	c	a						
339	**32**	**35**	13	1	14	a	c	a						
359	**34**	**37**	15	1	16	a	c	a						
369	**36**	**39**	15	1	16	a	c	a						
399	**38**	**41**												
Total no. of plants:360					Genetic purity %:95.0				Total no of seeds: 200			Genetic purity %:92.5		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 30:Sequential sampling analysis for testing genetic purity

Hybrid : NHH-44			Lot no.:2											
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	**R**	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	1	0	1	c	c	c
39	**2**	**5**	0	0	0	c	c	c	4	0	4	c	c	c
59	**4**	**7**	4	0	4	c	c	c	5	0	5	c	c	c
79	**6**	**9**	6	0	6	c	c	c	7	0	7	c	c	c
99	**8**	**11**	8	0	8	c	c	c	8	0	8	c	c	c
119	**10**	**13**	12	0	12	c	c	c	9	0	9	c	c	c
139	**12**	**15**	13	0	13	a	a	a	10	0	10	a	a	a
159	**14**	**17**	14	0	14	a	a	a	12	0	12	a	a	a
179	**16**	**19**	14	0	14	a	a	a	13	0	13	a	a	a
199	**18**	**21**	15	0	15	a	a	a	16	0	16	a	a	a
219	**20**	**23**	16	0	16	a	a	a						
239	**22**	**25**	16	0	16	a	a	a						
259	**24**	**27**	19	0	19	a	a	a						
279	**26**	**29**	20	0	20	a	a	a						
299	**28**	**31**	21	0	21	a	a	a						
319	**30**	**33**	23	0	23	a	a	a						
339	**32**	**35**	25	0	25	a	a	a						
359	**34**	**37**	26	0	26	a	a	a						
369	**36**	**39**												
399	**38**	**41**												
Total no. of plants:341					Genetic purity %:91.5				Total no of seeds: 200			Genetic purity %:92.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 31: Sequential sampling analysis for testing genetic purity

Hybrid : NHH-44 Lot no.:3																
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE							
CSS	WTT		Observations			Decision			Observations			Decision				
	A	**R**	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total		
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c		
39	**2**	**5**	0	0	0	c	c	c	5	0	5	c	c	c		
59	**4**	**7**	6	0	6	c	c	c	6	0	6	c	c	c		
79	**6**	**9**	8	0	8	c	c	c	6	0	6	c	c	c		
99	**8**	**11**	10	0	10	c	c	c	8	0	8	c	c	c		
119	**10**	**13**	12	0	12	c	c	c	10	0	10	c	c	c		
139	**12**	**15**	12	0	12	a	a	a	13	0	13	a	a	a		
159	**14**	**17**	14	0	14	a	a	a	13	0	13	a	a	a		
179	**16**	**19**	14	0	14	a	a	a	16	0	16	a	a	a		
199	**18**	**21**	15	0	15	a	a	a	16	0	16	a	a	a		
219	**20**	**23**	16	0	16	a	a	a								
239	**22**	**25**	16	0	16	a	a	a								
259	**24**	**27**	18	0	18	a	a	a								
279	**26**	**29**	21	0	21	a	a	a								
299	**28**	**31**	23	0	23	a	a	a								
319	**30**	**33**	23	0	23	a	a	a								
339	**32**	**35**	26	0	26	a	a	a								
359	**34**	**37**	28	0	28	a	a	a								
369	**36**	**39**	28	0	28	a	a	a								
399	**38**	**41**	31	0	31	a	a	a								
Total no. of plants:387					Genetic purity %:92.0				Total no of seeds: 200			Genetic purity %:92.0				

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 32: Sequential sampling analysis for testing genetic purity

Hybrid : NHH-44 Lot no.:4														
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	0	0	0	c	c	c	5	0	5	c	c	c
59	**4**	**7**	1	0	1	c	c	c	6	0	6	c	c	c
79	**6**	**9**	2	0	2	c	c	c	8	0	8	c	c	c
99	**8**	**11**	4	0	4	c	c	c	10	0	10	c	c	c
119	**10**	**13**	6	0	6	c	c	c	12	0	12	c	c	c
139	**12**	**15**	7	0	7	a	a	a	14	0	14	a	a	a
159	**14**	**17**	8	0	8	a	a	a	16	0	16	a	a	a
179	**16**	**19**	8	0	8	a	a	a	17	0	17	a	a	a
199	**18**	**21**	9	0	9	a	a	a	18	0	18	a	a	a
219	**20**	**23**	10	0	10	a	a	a						
239	**22**	**25**	10	0	10	a	a	a						
259	**24**	**27**	12	0	12	a	a	a						
279	**26**	**29**	12	0	12	a	a	a						
299	**28**	**31**	14	0	14	a	a	a						
319	**30**	**33**	14	0	14	a	a	a						
339	**32**	**35**	15	0	15	a	a	a						
359	**34**	**37**	15	0	15	a	a	a						
369	**36**	**39**	16	0	16	a	a	a						
399	**38**	**41**												
Total no. of plants:360					Genetic purity %:95.0				Total no. of seeds:200			Genetic purity %:91.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 33: Sequential sampling analysis for testing genetic purity

Hybrid : H-10			Lot no.:1											
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	0	0	0	c	c	c	0	0	0	c	c	c
59	**4**	**7**	0	0	0	c	c	c	1	0	1	c	c	c
79	**6**	**9**	1	0	1	c	c	c	1	0	1	c	c	c
99	**8**	**11**	1	0	1	c	c	c	1	1	2	c	c	c
119	**10**	**13**	1	0	1	c	c	c	1	1	2	c	c	c
139	**12**	**15**	2	0	2	a	a	a	1	1	2	a	c	a
159	**14**	**17**	2	0	2	a	a	a	1	1	2	a	c	a
179	**16**	**19**	2	0	2	a	a	a	1	1	2	a	c	a
199	**18**	**21**	2	0	2	a	a	a	1	1	2	a	c	a
219	**20**	**23**	3	0	3	a	a	a						
239	**22**	**25**	3	0	3	a	a	a						
259	**24**	**27**	3	0	3	a	a	a						
279	**26**	**29**	4	0	4	a	a	a						
299	**28**	**31**	4	0	4	a	a	a						
319	**30**	**33**	4	0	4	a	a	a						
339	**32**	**35**	4	0	4	a	a	a						
359	**34**	**37**	4	0	4	a	a	a						
369	**36**	**39**	4	0	4	a	a	a						
399	**38**	**41**												
Total no. of plants:364					Genetic purity %:98.5				Total no.of seeds: 200			Genetic purity %:99.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 34: Sequential sampling analysis for testing genetic purity

Hybrid : H-10 Lot no.:2															
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE						
CSS	WTT		Observations			Decision			Observations			Decision			
	A	**R**	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c	
39	**2**	**5**	0	0	0	c	c	c	1	0	1	c	c	c	
59	**4**	**7**	2	0	2	c	c	c	2	0	2	c	c	c	
79	**6**	**9**	2	0	2	c	c	c	2	0	2	c	c	c	
99	**8**	**11**	3	0	3	c	c	c	3	0	3	c	c	c	
119	**10**	**13**	5	0	5	c	c	c	4	0	4	c	c	c	
139	**12**	**15**	5	0	5	a	a	a	5	1	6	a	a	a	
159	**14**	**17**	6	0	6	a	a	a	7	1	8	a	a	a	
179	**16**	**19**	8	0	8	a	a	a	8	1	9	a	a	a	
199	**18**	**21**	8	0	8	a	a	a	8	1	9	a	a	a	
219	**20**	**23**	8	0	8	a	a	a							
239	**22**	**25**	9	0	9	a	a	a							
259	**24**	**27**	10	0	10	a	a	a							
279	**26**	**29**	12	0	12	a	a	a							
299	**28**	**31**	12	0	12	a	a	a							
319	**30**	**33**	13	0	13	a	a	a							
339	**32**	**35**	14	0	14	a	a	a							
359	**34**	**37**	14	0	14	a	a	a							
369	**36**	**39**													
399	**38**	**41**													
Total no. of plants:358					Genetic purity %:95.5				Total no. of seeds:200			Genetic purity %:95.0			

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 35: Sequential sampling analysis for testing genetic purity

Hybrid :H-10 Lot no.:3														
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	1	0	1	c	c	c	0	0	0	c	c	c
59	**4**	**7**	1	0	1	c	c	c	0	0	0	c	c	c
79	**6**	**9**	1	0	1	c	c	c	0	0	0	c	c	c
99	**8**	**11**	1	0	1	c	c	c	1	0	1	c	c	c
119	**10**	**13**	1	0	1	c	c	c	2	0	2	c	c	c
139	**12**	**15**	1	0	1	a	a	a	2	0	2	a	a	a
159	**14**	**17**	1	0	1	a	a	a	3	0	3	a	a	a
179	**16**	**19**	1	0	1	a	a	a	3	0	3	a	a	a
199	**18**	**21**	3	0	3	a	a	a	3	0	3	a	a	a
219	**20**	**23**	3	0	3	a	a	a						
239	**22**	**25**	4	0	4	a	a	a						
259	**24**	**27**	4	0	4	a	a	a						
279	**26**	**29**	5	0	5	a	a	a						
299	**28**	**31**	5	0	5	a	a	a						
319	**30**	**33**	5	0	5	a	a	a						
339	**32**	**35**	5	0	5	a	a	a						
359	**34**	**37**	5	0	5	a	a	a						
369	**36**	**39**	5	0	5	a	a	a						
399	**38**	**41**												
Total no. of plants:362					Genetic purity %:98.6				Total no. of seeds:200			Genetic purity %:98.0		

A: Maximum permissible number for accepting the sample
R: Minimum permissible number for rejecting the sample
c: Continue the sampling procedure
a: Accept the sample
r: Reject the sample

Table 36: Sequential sampling analysis for testing genetic purity

Hybrid: H-10 Lot no.: 4														
Genetic purity based on field grow out at IARI farm, New Delhi									Genetic purity based on Globulin-PAGE					
CSS	WTT		Observations			Decision			Observations			Decision		
	A	R	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total	Selfed	Offtypes	Total
upto 19	**0**	**3**	0	0	0	c	c	c	0	0	0	c	c	c
39	**2**	**5**	1	0	1	c	c	c	1	0	1	c	c	c
59	**4**	**7**	1	0	1	c	c	c	1	0	1	c	c	c
79	**6**	**9**	2	0	2	c	c	c	1	0	1	c	c	c
99	**8**	**11**	2	0	2	c	c	c	3	0	3	c	c	c
119	**10**	**13**	4	0	4	c	c	c	3	0	3	c	c	c
139	**12**	**15**	4	0	4	a	a	a	4	0	4	a	a	a
159	**14**	**17**	5	0	5	a	a	a	4	0	4	a	a	a
179	**16**	**19**	5	0	5	a	a	a	4	0	4	a	a	a
199	**18**	**21**	5	0	5	a	a	a	4	0	4	a	a	a
219	**20**	**23**	5	0	5	a	a	a						
239	**22**	**25**	6	0	6	a	a	a						
259	**24**	**27**	7	0	7	a	a	a						
279	**26**	**29**	8	0	8	a	a	a						
299	**28**	**31**	9	0	9	a	a	a						
319	**30**	**33**	9	0	9	a	a	a						
339	**32**	**35**	10	0	10	a	a	a						
359	**34**	**37**												
369	**36**	**39**												
399	**38**	**41**												
Total no. of plants:324					Genetic purity %:97.0				Total noof seeds: 200			Genetic purity %:97.5		

A: Maximum permissible number for accepting the sample

R: Minimum permissible number for rejecting the sample

c: Continue the sampling procedure

a: Accept the sample

r: Reject the sample

the presence of selfed females and off types, separately. Data on the number of female and off types was compared with the working tolerance table (WTT). Further, samples of same size were analyzed subsequently. Data were compared to WTT till a decision is arrived at *i.e.,* whether to continue or terminate the test procedure. For the one lot not meeting the purity standard, for a decision to reject the lot a minimum of 109 individual seeds / plants were required to be tested as per the WTT (Table 25). Whereas, an acceptance decision for eight lots having 91.0 to 99.0 per cent could be taken by sampling 139 plants / seeds by this procedure. In three cases, i.e., lot no.2 and 4 of Savitha and lot no, 1 of NHH-44, of 2, 2 and 1 off-type plants would require observations to be continued since as per the sequential sampling procedure, acceptance can be given only at zero (0) off-types in upto 208 and one off-type in 399 plants. However, as per fixed sampling 3 off-types in 200 plants (1.5%) are permissible. Hence, an acceptance decision was taken.

For the purpose of fixing an optimum sample size, correlation coefficients were calculated between observations in accumulated sub- sample sizes of $\sum$100, $\sum$200, $\sum$300 and $\sum$400 seeds on the basis of genetic purity percentage as shown in table 38. The high correlation between the purity results calculated on 100 (0.73*), 200 (0.99**), 300 (0.99**) and 400 (or above 350) plants revealed that the observations recorded on 200 or 300 plants were as reliable as that on 400 plants. Hence, there is a possibility to reduce the sample size for grow-out test from 400 to 200, without significantly affecting the accuracy of result.

To test the possibility of reducing the sample size for laboratory analysis, for detecting selfed female and off type seeds through electrophoresis, correlation coefficients were calculated between accumulated sub-sample sizes in 50, 100, 150 and 200 seeds (Table 39) The correlation coefficients were positive and highly significant for all the four sample sizes. However, the highest correlation (0.957**)

Table:37 Correlation coefficients between the genetic purity tests based on grow-out trials and laboratory test

	APPSCA,Amaravati	IARI, New Delhi	Lab. PAGE test
APPSCA, Amaravati	1.00		
IARI, New Delhi	0.81**	1.00	
Lab. PAGE test	0.72*	0.85**	1.00

n= 12

Table:38 Correlation coefficients between various sub-sample sizes in field grow-out trial, IARI, New Delhi

	Σ400	Σ300	Σ200	Σ100
Σ400	1.00			
Σ300	0.99**	1.00		
Σ200	0.99**	0.986**	1.00	
Σ100	0.73*	0.75*	0.87**	1.00

n= 12

Σ= 100, 200, 300 and 400 (more than 350) plants

Table: 39 Correlation coefficients between various sub-sample sizes in laboratory electrophoresis technique, IARI, New Delhi.

	Σ200	Σ150	Σ100	Σ50
Σ200	1.00			
Σ150	0.957**	1.00		
Σ100	0.895**	0.912**	1.00	
Σ50	0.885**	0.928**	0.974**	1.00

n= 12

* = Significant linear relationship

** =Highly Significant linear relationship

Σ= 50, 100, 150 and 200 (more than 350) plants

was obtained between $\sum 200$ and $\sum 150$ seeds, which decreased progressively to 0.895** and 0.885** with $\sum 100$ and $\sum 50$ seeds. Thus, for a quick assessment of seed lot purity even a sample size of 50 seeds may provide reliable result.

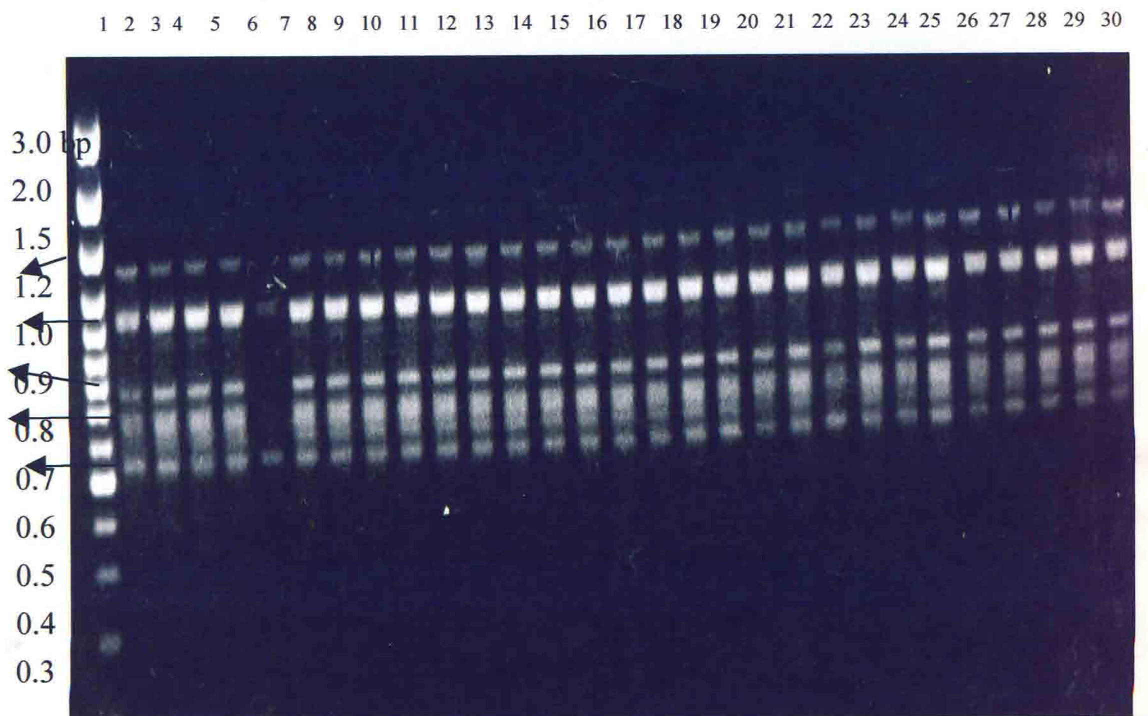
Thus, the results revealed that assessment of genetic purity of cotton hybrids seeds based on single seed electrophoresis analysis was reliable as grow-out test. It also indicated that the sample size may be reduced to 200 plants or 100 / 150 seeds for grow out test and electrophoresis analysis for testing genetic purity of hybrid cotton, without any significant effect on accuracy of results.

2.5 RAPD analysis

An attempt was made to test the possibility of applying RAPD analysis for testing genetic purity of the hybrid seed. DNA extracted from 30 individual seeds of one lot of H-10 (showing 95.5 per cent genetic purity in grow-out test) was subjected to RAPD analysis with a random primer OPN-02 (Fig 23). On the basis of similar RAPD profile as that obtained from pooled sample of 10 seeds for the purpose of identification, out of 30 individual seeds, only one seed (fifth lane) was identified as selfed female, whereas the rest confirmed to the amplification pattern of the hybrid.

Thus, the results clearly indicated that it is possible to use single seed RAPD analysis to test the genetic purity of hybrid seed lots.

Fig.23: Single seed analysis of hybrid H-10 using OPN-02 by RAPD polymorphism



DISCUSSION

Chapter-V
Discussion

Characterization of cultivars, establishment of identity and genetic purity of the seed lot are crucial to varietal improvement, protection of varieties and seed production aspects. UPOV (The International Union for protection of new varieties of plants) has developed detailed guidelines for DUS testing of about 160 important crops (including cotton) based primarily on phenotypic characters for appropriate characterization of varieties. Though morphological distinctness of the varieties are simple to record, generation of data are time consuming and laborious. Being liable to be influenced by environmental factors and limited in number, the discriminating ability of these descriptors is limited, particularly for related varieties or genotypes *viz.*, the parental lines of hybrids.

Being produced through manual emasculation and pollination technique, the hybrid seed of cotton is likely to be contaminated by some proportion of selfed female seeds other than the usual off types. To ensure the standard of genetic purity, field grow out test is mandatory for the certification of hybrid cotton seed (Tunwar and Singh, 1988). Hence, a reliable technique to differentiate female selfed seeds from the hybrid seed is needed.

Keeping these two considerations in mind, the present investigation was conducted to standardize rapid and reliable laboratory techniques for characterization of five commercial inter- *hirsutum* cotton hybrids and their parental lines and to test their application in testing genetic purity of hybrid seeds.

5.1 Seedling response tests

Examinations of seedling morphology under controlled conditions of growth are found useful and cost effective for describing and distinguishing cultivars in certain species (ISTA, 1993). In the present study, the differential response of seedlings grown in nutrient media containing higher concentrations of sodium or calcium and application of 2,4-D could clearly distinguish all the five hybrids from their female parental lines.

Seedling height, length and width of the first leaf in 14day old seedlings were used to characterize 29 cultivars of maize into discrete groups (Chesneaux and Kabilinsky, 1982). Characters such as pigmentation, trichome density, hairiness of the first leaf of the seedlings grown under controlled conditions were found to be useful in characterization of wheat, sorghum and pearl millet genotypes (Niemyski and Grzelak, 1978; Payne *et al.*, 1980; Halim and Saxena, 1985, and Nagaraja, 2000). Anthocyanin pigmentation of the seedling under controlled conditions of growth is recommended by UPOV as a character of DUS testing for sorghum genotypes. Payne *et al.*, (1980) found that a medium completely devoid of the nutrients was more suitable than growing seedlings in nutrient medium *viz.*, Hoagland solution, for characterizing sorghum varieties based on anthocyanin pigmentation. Nagaraja *et al.*, (2000) following the method prescribed by the ISTA (1993) described 23 sorghum varieties into several groups based on seedling anthocyanin pigmentation while four of the inbred lines could be identified individually. Sahoo *et al.*, (2000) distinguished sunflower hybrids and their parental lines based on hypocotyl pigmentation of seedlings grown in phosphorous deficient Hoagland medium.

Simple characters *viz.*, leaf area and shoot length of the seedling grown under controlled conditions are useful in characterization of cotton genotypes in the present study. Three of the hybrids, Savitha, PKHy-2 and H-10 were distinguishable from their female parents, which were characterized by stunted growth of the seedlings grown in calcium deficient nutrient medium. Hybrid NHH-44 and its female parent were found to be sensitive of high concentration of sodium. However, response to sodium deficiency was not much pronounced. Though, relatively a salt tolerant species, cotton varieties are reported to exhibit varying degrees of salinity sensitivity at seedling stage (Abus-Naas & Omran, 1974: Kent & Lauchli, 1985). High concentrations of Na^{+} and / or Ca^{++} influence size as well as multiplication of root cells in cotton (Kurth et al., 1986). As seen in the present study, this character is useful in distinguishing varieties. Marked reduction of seedling growth and wrinkling of leaves of the female parents in the presence of 2,4-D particularly at 10 ppm differentiated these from the respective hybrids, which were marginally affected. Thus, these seedling response tests showed promise for the purpose of distinguishing cotton hybrids from their female parents and grouping of genotypes on the basis of sensitivity of modified media.

5.2 Colour of the seed extracts

During the process of defatting the seed material for electrophoresis, the colour of the organic solvent mixture was found to change differentially in different varieties. This indicated that genotypes differed with respect to certain coloured substances, extractable by organic solvents. Since cotton seeds and seedlings are known to contain appreciable amounts of anthocyanin and gossypol, which exhibit genotypic variability, it was considered worth an attempt to determine the qualitative and/ or quantitative variability among cotton genotypes with respect to these two classes of substances and test its application for characterization of varieties. Colour of the

seed extracts in organic solvents specific for anthocyanin and gossypol were determined to differentiate cotton hybrids and their female parents.

5.2.1 Anthocyanins

Anthocyanins (glycosylated anthocyanidins) belong to a class of flavanoids ubiquitous in higher plants, which differ genetically with regard to their potential to synthesize anthocyanins in different plant parts (Drumm-Herrel and Mohr, 1985). In dicotyledonous seedlings, anthocynains are reported to be localized in epidermis of the cotyledons and the sub epidermal layer of the hypocotyl. Yunoskhanov and Ibragimov (1984) demonstrated a relationship between the protein markers and anthocyanin colouration of cotton plants in different varieties of two cotton species *i.e.*, G. *hirsutum* and G. *barbadense*. Anthocyanin synthesis in mustard cotyledon is reported to take place at approximately 27 hours of the sowing (Steinitz *et al.*, 1976). Hence, in the present study, seeds imbibed for 72 hours in light and devoid of seed coat (to eliminate the seed coat of maternal origin) were used. Acidic methanolic extracts of the hydrated seeds, which largely contain anthocyanin, besides certain other phenolic compounds, exhibited a range of qualitative and quantitative variability of colouration among the genotypes ranging from wood to olive yellow. This was found useful both in characterization of cotton genotypes and in differentiating three of the hybrids from their female parents.

5.2.2 Gossypol

Gossypol, yellowish phenolic pigment is reported to be distributed throughout the cotton plant and is concentrated in seed kernels, flowers and fruits. It's content varies widely depending upon the species, variety and agronomic characters (Sadasivam and Manickam, 1996). It occurs both in free and bound forms in cotton

seeds. Intensity of gossypol glands on leaf, stem and bolls are used as a varietal character in cotton (Krishna and Saxena, 1996; Anonymous, 1999). Gossypol content of the decorticated seeds exhibited a range of qualitative and quantitative variations which characterized the 15 cotton genotypes studied, and differentiated three of the hybrids from their female parental lines.

Simple estimation of anthocyanin and gossypol contents of dry or hydrated (and decorticated) seeds, thus, could provide a rapid method of characterization distinguishing cotton genotypes. However, accuracy of such tests for evaluation of genetic purity may require fine tuning of the technique, as the estimation of gossypol content of twenty individual seeds of hybrid PKHy-2 and its female parent AK-32 showed (Table 9) that the O.D. values range from 0.31 to 0.41 (for PKHy-2) and from 0.53 to 0.66 (from AK-32). This could be for several factors *viz.*, variability of the seed size, maturation level etc. Also, there is a need to test the variability in gossypol content due to location effect, environmental conditions and biotic stresses at maturity.

5.3 Protein and isozyme markers

High degree of polymorphism exhibited by the SDS-PAGE of tris soluble proteins and salt soluble globulins extracted from seeds differentiated each of the five hybrids and their parental lines studied. SDS-PAGE is a simple yet powerful technique, which has been used in the past for identification and characterization of varieties of crop species *viz.*, cotton (Kapse and Nerkar, 1985; Agrawal *et al.*, 1988; Rao *et al.*, 1990), sunflower (Varier *et al.*, 1992; Sahoo *et al.*, 2000), maize (Parra *et al.*, 1983; Smith and Wynch, 1986) and other crop species (Wrigley *et al.*, 1982; Cooke 1984). Of the protein fractions i.e., total tris soluble proteins and salt soluble globulins, the later provided a better marker system, which not only distinguished

each of the hybrid from their female parents but also confirmed the hybridity in four of the five hybrids which could be an useful marker for testing genetic purity of hybrid cotton seed. For the purpose of characterization, however the tris soluble proteins profiles were equally useful. Rao *et al.*, (1990) has observed that globulins were more useful than tris soluble protein profiles in distinguishing G. *arboreum* and G. *hirsutum* varieties and three hybrids belonging to inter *hirsutum* and G. *hirsutum* X G. *barbadense*. Globulin fractions were also preferred over tris-soluble proteins, being physiologically more stable. The direct multilocus approach by studying tris soluble protein profile has been advocated for testing distinctness in self-pollinated crops (Cooke, 1989). It's usefulness as a supplementary character for establishment of varietal distinctness has been accepted by UPOV in case of wheat and barley. Electrophoresis banding patterns of tris soluble proteins and globulins, have amply established the usefulness of this technique for the purpose of establishing identity and distinctness of cotton varieties (Kapse and Nerkar, 1985; Agrawal *et al.*, 1988; Rao *et al.*, 1990; Nerkar and Rao, 1993 and Zang *et al.*, 1998). Thus, SDS-PAGE analysis of tris soluble proteins or of salt soluble globulins is advocated for identification and genetic purity testing of cotton hybrids. However, genetic control of marker protein bands need to be worked out before this can be recommended by the UPOV as a supplementary test for distinctness.

Isoenzymes, which are structural variants of the same enzyme having identical functions present in the same organism (Markert and Moller, 1959), provide an efficient marker system for varietal characterization. Specific enzyme bands migrating to different distances are indicative of altered molecular structure resulting from a mutational event or addition of new genetic loci. This property has also been used in several species to establish varietal identity on the basis of distinct isoenzyme patterns (Cooke, 1995) and recommended by UPOV for maize.

Of the seven isoenzymes studied in the present case, highest polymorphism was observed with respect to acid phosphatase isoenzymes followed by esterase and superoxide dismutase, respectively.

Nerkar and Rao (1993) failed to observe polymorphism with respect to acid phosphatase (ACP) isoenzymes among 20 cotton cultivars belonging to *Gossypium* spp. whereas a good degree of polymorphism was noted with respect to esterase and malate dehydrogenase. It may be noted that isoenzyme analyses by Nerkar and Rao (1993) were made in soaked seeds, whereas 5day old seedlings were used in the present study. Expression of isoenzymes being highly stage specific, it is important that the analysis be done at the right stage of growth and in the tissues where the expression is maximum. Our observations have also shown that activity of both acid phosphatase and esterase isoenzymes is higher in cotyledonary leaves than the whole seedling.

Isoenzyme polymorphisms have been useful in studying genetic diversity in genus *Gossypium* and inter and intra species relationships among cotton genotypes (Cherry *et al.*, 1972; Percy and Wendel, 1990; Wendel *et al.*, 1992 and De Joode and Wendel, 1992). Percy and Wendel (1990) scored 59 isozyme loci in *Gossypium barbadense*, of which 24 were polymorphic with a mean number of 1.69 alleles per locus and an average panmictic heterozygosity of 0.062.

Inter-species polymorphism of esterases was observed among 29 species with in the genus *Gossypium* by Cherry *et al.*, (1972). Wendel *et al.*, (1992) studying the genetic diversity in *Gossypium hirsutum* analyzed 14 enzyme systems, which revealed 30 polymorphic allozyme loci, however, acid phosphatase isoenzymes have not been analyzed in any of the above mentioned studies.

This indicates the need to explore the degree of polymorphism existing in isoenzyme systems other than those reported for the purpose of variety characterization. For testing the genetic purity of hybrid cotton seeds, even a limited degree of polymorphism between the hybrid and female parent could be extremely relevant. As revealed in the present study, four of the five hybrids were distinguishable from their respective female parents with respect to one or more discrete bands of acid phosphatase isoenzyme. Hybridity of two of the hybrids could also be established on the basis of the bands contributed by their respective parents. Similarly, the polymorphism with respect to esterase isoenzymes was useful for characterization of 15 genotypes studied, though only two of the hybrids Savitha and H-10 were distinguishable from their female parents. Alcohol dehydrogenase isoenzymes, which are encoded by two loci (Percy and Wendel, 1990), showed limited, but useful polymorphism among the hybrids and their parental lines studied, which could distinguish four hybrids from their female parental lines. As Malate dehydrogenase isoenzymes are reported to show a fair degree of polymorphism and its genetic control is known (De Joode and Wendel, 1992) in the Hawaiian islands cotton *Gossypium tomentosum*, this enzyme system was also analyzed. However, very little polymorphism was seen among the genotypes studied.

To establish the distinctness of a cultivar, the marker is required to be fairly uniform within the population. This is the basis of testing uniformity under DUS test system. Though, the uniformity of the enzyme / protein markers was not tested as per the UPOV guidelines (UPOV, 1994), the homogeneity of these was tested randomly in limited number of individual seeds (30), and was found to be fairly high. The reproducibility of banding profiles was also verified by replicating and comparing each of the electrophoresis analyses three times or more. This indicates the

possibility of using such markers for the purpose of establishing varietal distinctness in cotton.

Cooke (1989) suggested three possible uses of electrophoresis in DUS testing, which included

1. Use of electrophoresis variance as a routine additional character for all the candidate varieties.
2. Use of electrophoresis as a special or additional character to be used only when distinctness cannot be ascertained between a group of varieties by other means and,
3. Integration of electrophoresis and small number of highly discriminating morphological characters in a revised scheme of DUS testing.

He suggested the last option to be most logical in economizing time and cost. The results of the present investigation, though restricted only to inter-*hirsutum* hybrids and parental lines, clearly affirmed the potential of electrophoresis as an additional test distinguishing cotton genotypes.

5.4 RAPD markers

Technological refinements have helped in developing accurate, rapid and easy to perform analysis of plant DNA that can be useful to test distinctness, uniformity and stability of a plant variety.

The evaluation of DNA profiling as a tool for describing distinguishable characters under UPOV system is being taken up extensively by a working group on biochemical and molecular techniques (BMT) (Morrell *et al.,* 1995). The polymerase chain reaction (PCR), which was invented by Karry B. Mullis in 1985,

has revolutionized many areas of biological science. Of the PCR based techniques, Random Amplified Polymorphic DNA (RAPD) markers have been successfully used for cultivar identification in a number of plant species (Morrell *et al.*, 1995 and Lee *et al.*, 1998).

Notwithstanding doubts about the reproducibility of RAPD, Lee *et al.*, (1998) found this technique useful for the plant identification as it is highly discriminative, convenient, relatively rapid, suitable for site to site comparison of samples and can be carried out at any time of the year. However, they observed considerable intra varietal heterozygosity in RAPD profiles of *Brassica napus* and *Hordeum vulgare* varieties and suggested a number of ways for approaching this problem. These included determining the level of expected non-conformity within a variety. Thus, if the variability within a variety remains stable over generations, that should be accepted as sufficiently uniform.

The application of RAPD marker technology to distinguish cotton genotypes belonging to different species has been demonstrated by several workers (Multani and Lyon, 1995; Matsinkinovskaya *et al.*, 1996; Tatineni *et al.*, 1996; Iqbal *et al.*, 1997). In the present study, 15 cotton genotypes were characterized on the basis of RAPD profiles. A set of 5-6 primers each was selected from a total of 60 random primers (Operon Technologies Inc, USA) for each of the hybrid combination examined, which could clearly establish the hybridity.

The results demonstrated high discriminating power of this technique not only to identify different hybrids but also to distinguish these from their respective parental lines. Reproducibility of results was verified at least twice. A method suitable for extraction of DNA from individual seeds (Krishna and Jawali, 1997) was followed

and found appropriate, which would favour single seed analysis for genetic purity testing.

Multani and Lyon (1995) have reported fairly reproducible results of RAPD analysis in cotton and found cultivar specific markers, which were consistently stable and could be used for varietal identification and classification. The advantage of RAPD markers, which can differentiate very closely related genotypes showing strong phenotypic homology, are particularly useful in distinguishing various parental lines of cotton and the resultant hybrids. Multani and Lyon (1995) were able to confer the pedigree relationship of the cultivars using RAPD data. A high correlation of 0.63 was reported between the results of genetic distance calculated on the basis of RAPD analysis and taxonomic classification among cotton genotypes. (Tatineni *et al.*, 1996).

Obtaining meaningful and reproducible amplification of DNA using random primers is one of the primary concerns in RAPD analysis. Choice of the random primers, therefore, is of critical importance. Tatineni *et al.*, (1996) reported that 8 out of 27 primers used for classification of cotton genotypes, did not produce any polymorphism, while Multani and Lyon (1995) observed 98 per cent of 50 primers to be polymorphic for cotton varieties. In the present study, 57 out of 60 random primers belonging to OPO, OPN and OPR series of Operon Technologies Inc, USA were capable of amplification, producing 1 to 10 bands in each case. Most of these primers (20-30) exhibited polymorphism with respect to amplified fragments. However, since the emphasis of the present study was more towards distinguishing the parental lines and their hybrids, 5-6 primers were selected for each combination showing maximum meaningful polymorphism. The results established that RAPD analysis is a reliable technique for establishing the identity of commercial hybrids of Indian cotton and their parental lines.

5.5 Genetic purity

Testing the genetic purity of cotton hybrid seed was one of the major objectives of this study. A rapid and reliable method to determine genetic purity of hybrid seed is crucial to commercial production of cotton hybrids. For this reason, an In-house quality control by the seed producing agencies is as important as certification requirements. Electrophoresis analysis of hybrid seeds, particularly for the identification of female selfs, is known to provide a reliable assessment in testing the genetic purity of hybrid maize seed Electrophoresis results were found consistently more accurate than that of field grow- out test. (Smith and Wych, 1986).

Similar studies have demonstrated the reliability and potential of electrophoresis analysis of seed proteins or isoenzymes for assessing genetic purity of hybrids and inbred lines of maize (Orman *et al.*, 1991; Wang *et al.*, 1994 and Drezewiecki *et al.*, 1996), tomato (Vodenicharova *et al.*, 1996); cotton (Dadlani *et al.*, 1994) and several other crops. However, only in few of these studies results were compared with that of field grow -out test (Smith and Wych, 1986; Orman *et al.*, 1991; Dadlani *et al.*, 1994).

Comparisons of results of field grow out tests and electrophoresis analysis of individual seeds in the present study exhibited a highly significant correlation indicating the reliability of the latter. This clearly established the reliability of electrophoresis analysis for testing genetic purity of hybrid cotton seeds.

The major constraints in applying electrophoresis technique for the purpose of genetic purity testing in cotton lies in

a. requirement of a large sample size comprising 400 individual seeds as per Indian Minimum Seed Certification Standards (Tunwar and Singh, 1988).
b. complexity of the technique and
c. cost of testing.

Singh and Singhal (1999) have advocated a reduction in sample size from 400 to 300 plants or less for grow out test of hybrid cotton based on the observation that highly significant correlation exists between results based on 200, 300 and 400 plants data. They also observed that if the seed quality status is very high (less than 9 percent contaminants) or very low (more than 11 per cent contaminants) there is little variation (non-significant) in results based on a total population of 100, 200, 300 and 400 plants. The sequential sampling procedure suggested by Singh and Agrawal (1995) advocated analyzing a minimum of 109 plants to satisfy the criterion of N=100 for detecting 1.5 per cent off types provided the number of selfed female plants does not exceed 9 out 109 plants and so on. A permissible level of 1.5 per cent off types in hybrid cotton seed lot allows a maximum of one off-type in 67 plants and maintaining a minimum of three replications would mean observing a minimum of 3 X 67 =201 plants or seeds for genetic purity testing. Though, modification of certification requirements on this basis may require generation of more data, the results of 200 individual seed analysis of 12 seed lots of hybrid cotton and its correlation with that of field grow-out trials in the present study firmly established the authenticity of this test as a quick, reliable alternative for in-house quality control. It may further be seen that a highly significant correlation exists between the results of 50, 100, 150 and 200 individual seed analysis indicating that a sample size as small as 50 seeds may suffice the requirement of a rapid purity assessment. This would be both cost effective and considerably quicker than the routine procedures. SDS-PAGE analysis of seed

globulins or total tris- soluble proteins offered a robust technique which was relatively simpler and cost-effective than isozyme or RAPD analysis, but equally effective in verifying the hybridity.

In most of the studies in past, comparing field and laboratory methods testing genetic purity, were made on seed samples with known genetic purity levels (Smith and Wych, 1986; Orman *et al.,* 1991), whereas the analysis were made simultaneously in the laboratory and field in the present study. While this helped in eliminating any bias factor in laboratory analysis it also resulted in a narrow range of purity. It was observed that 11 out of 12 lots were well above the genetic purity standards (91.0% to 99.0%) with only one lot below the standard due to more than of 10 per cent selfed seeds. Though one may argue that the conclusions of this study are drawn from samples, which predominantly were of high genetic purity standards, it can be seen that the interpretations are fairly consistent and hence, can be recommended at least for the lots, which are clearly above or below the prescribed standards. The results of this investigation emphasize the need to adopt a supplementary technique for making rapid decisions for rejecting or accepting hybrid seed lots. However, before suggesting this as an alternative to field grow out test, analysis of a larger number of samples, fairly distributed in a range of sub-standard to highest genetic purity level, need to be taken.

Determination of genetic purity of F_1 hybrid and parental line seeds by RAPD markers has been attempted in a number of field crops, vegetables and flowers. Number of individual seed / seedling for RAPD analysis varied from 10 to 20 in pepper (Ballester and Vicente, 1998), 30 in Chinese cabbage (Meng *et al.,* 1998), 40 in tomato (Rom *et al.,* 1995), 120 in canola (Marshall *et al.,* 1994) and 400 seeds in chicory (Bellamy *et al.,* 1998) to assess the genetic purity of the hybrid seeds. Though hybrid seed purity was not determined on the basis of RAPD analysis in the

present study an evaluation of amplification patterns in 30 individual seeds of one hybrid (H-10) exhibited a high degree of intra-varietal homogeneity suggesting its potential use in rapid assessment of genetic purity.

The present study, thus, showed that cotton varieties can be characterized by seed and seedling response tests, electrophoresis of seed proteins and isoenzymes and RAPD analysis, each of which exhibited varying degrees of discriminating ability in identifying individual varieties.

Of these, SDS-PAGE of seed globulins was found to be a useful technique that is also rapid, reliable and simple for assessment of genetic purity of hybrid cotton seed.

SUMMARY

Chapter VI
Summary

Characterization of plant varieties to establish and verify their identity is crucial to variety release, its protection and production of genetically pure seed. Cotton is a commercially important field crop of India, which is cultivated in about 9.0 million hectares producing 16.8 million bales of cotton fibre. Of this, hybrids constitute 30 per cent of the total area contributing 48 per cent of the total production. Hence, there is a great demand for high quality seed of cotton hybrids. To maintain the genetic purity of hybrid cotton seed, which is produced through manual emasculation and pollination technique, a grow-out test is conducted prior to its certification. The grow-out test relies on identifying the selfed female and off type plants from the hybrid on the basis of morphological descriptors recorded till maturity. Though reliable in testing the purity of the seed lot, this method is time consuming (requiring about 80days or so) and labour intensive. Further, there is a need to develop rapid and reliable methods to assess purity of hybrid seed lots to facilitate quick business decisions to be taken by the seed producers. Keeping these considerations in view, the present investigation was undertaken on "Biochemical methods for identification and genetic purity testing of cotton (*Gossypium* spp.) hybrids".

The materials constituted of five inter-*hirsutum* cotton hybrids *viz.*, Savitha, PKHy-2, NHH-44, H-10 and PHH-316 and their parental lines. Genotypes were characterized on the basis of

a) Seedling response to chemicals
b) Colour of seed extracts in organic solvents
c) Protein and isoenzyme electrophoresis profiles and
d) Random Amplified Polymorphic DNA markers.

Based on the precision to establish hybridity, reproducibility and ease to perform, SDS-PAGE of salt soluble globulins of single seeds was chosen for testing genetic purity of twelve commercial lots of three hybrids (four lots of each hybrid) obtained from the Andhra Pradesh State Seed Certification Agency (APSSCA). The results of genetic purity testing based on field grow out test (*kharif* 98) and electrophoresis analysis of single seeds were compared with those recorded by the APSSCA for the respective lots using paired 't' and correlation tests.

The major findings of this study are given below:

1. Cotton genotypes exhibited variable response to high and low levels of calcium and sodium, with respect to change in foliage colour, reduction in leaf area, stem diameter and shoot length. Reduction in leaf area and altered foliage colour were particularly useful characters to differentiate hybrids from their female parents.
2. Sensitivity to 2,4-D application of hybrids *vis-à-vis* their female parents was also a useful character for differentiating these. In general, hybrids were more tolerant than the female parents, which showed highly stunted seedling growth, with dark green and wrinkled foliage both at 5 and 10 ppm of 2,4-D, whereas, reduction in seedling growth was less severe and seen only at 10 ppm level of 2,4-D in hybrids.
3. A simple test based on colour variation of organic solvent extracts of decorticated seeds was developed for characterization of cotton genotypes. Anthocyanin and gossypol extracts were useful in characterizing hybrids and their parental lines into 4-5 broad classes and differentiating hybrids from their respective female parents. The colour variation was detectable both visually (qualitative) and colorimetrically (quantitative). However, single seed extracts showed varying colour intensity, though the difference

between hybrid and its female parent could be detected. Thus, to use this for quick estimation of genetic purity, the test needs to be refined further.

4. Electrophoresis profiles of seed proteins and isoenzymes (seed / seedling) exhibited varying degrees of polymorphism which was useful for identification of cotton hybrids and parental lines.
5. SDS-PAGE profiles of salt-soluble globulins showed polymorphism with respect to one to five bands, which distinguished eleven of fifteen genotypes. More importantly, hybridity could be established in all but PHH-316, by the presence of bands contributed by the male and female parents. All hybrids were distinguishable from their female parents, which is a useful marker for testing the genetic purity.
6. Tris soluble protein profiles were useful in characterization of cotton genotypes. Thirteen of the genotypes studied could be distinguished on the basis of soluble protein profile. Hybridity could be established in hybrid H-10 by the presence of bands contributed by each of the parents, though all hybrids were distinguishable from their respective female parents.
7. Of the seven isoenzymes systems analyzed *i.e.*, acid phosphatase (ACP), esterase (EST), alcohol dehydrogenase (ADH), superoxide dismutase (SOD), malate dehydrogenase (MDH), catalase (CAT) and peroxidase (POX), highest degree of polymorphism was seen in ACP isoenzymes, followed by EST, ADH and SOD isoenzymes. MDH, CAT and POX did not exhibit much polymorphism
8. ACP isoenzyme was useful in determining hybridity in Savitha and PKHy-2 and in distinguishing hybrids from their female parents except in PHH-316.
9. EST isoenzyme patterns could distinguish three hybrids *i.e.*, Savitha, H-10 and NHH-44 from their female parents.

10. ADH isoenzyme patterns could differentiate four the hybrids studied from their female parents.
11. SOD isoenzyme showed limited polymorphism among the genotypes studied, but was useful in establishing the hybridity in H-10 and differentiating from its female parent.
12. Intra-varietal homogeneity with respect to globulin profile, EST and ADH isoenzymes was found to be fairly high. Due to clarity of banding patterns establishing the hybridity, ease to perform and reproducibility of results, SDS-PAGE of soluble seed globulins was found most suitable for genetic purity testing.
13. Random Amplified Polymorphic DNA analysis was performed using 60 random decamer oligonucleotides. Based on the level of inter varietal polymorphism with respect to fragments amplified, and reproducibility of amplification, each of the genotype was analyzed with 20-28 random primers, of which 5-6 exhibited amplification patterns establishing the hybridity in case of each hybrid and its parental lines.
14. Intra- varietal homogeneity observed with respect to RAPD profile suggested the possibility of its use for testing genetic purity.
15. Genetic purity of 12 commercial lots of cotton hybrids was tested by grow-out test and electrophoresis analysis of globulins from 200 single seeds to test the reliability of the latter.
16. Eleven of twelve lots showed purity above the standard (91 to 99 per cent), with only one lot showing 86 per cent purity. Results showed that assessment of genetic purity based on electrophoresis analysis of 200 single seeds was as reliable as field grow-out test ($r = 0.85^{**}$).
17. Following the sequential sampling (SS) procedure, it was possible to accept/ reject 10 lots on the basis of 139 seeds / plants. In two lots though

the decision could not be finalized with respect to off types as per SS, following the fixed sampling criterion, lots were accepted.

18. Highly significant correlation was seen between field grow-out tests based on 400 and 200 plants (r= 0.99**).

Thus, the laboratory based techniques described above were very useful in identification of cotton hybrids and their parental lines. Electrophoresis analysis of 200 single seeds or less (upto 50) can provide a reliable and quick method for estimation of genetic purity of hybrid cotton seed, which could be highly beneficial for in- house quality control and taking quick decisions regarding acceptance of commercial lots by the seed producers.

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APPENDICES

Composition of Hoagland solution used for seedling growth test

Compound	Mol.wt	Conc of stock (M)	Macronutrient conc. of stock g/L	Vol. of stock per litre of final solution (mL)	Element	Final conc. of element (µM)	Final conc of element
KNO_3	101.0	1.00	101.0	6.0	N	16000	224
$Ca(NO_3)_2.4H_2O$	236.16	1.00	236.16	4.0	K	6000	235
				2.0	Ca	4000	160
$NaH_2PO_4.2H_2O$	156.01	1.00	156.01	2.0	Na		
				1.0	P	2000	62
$MgSO_4.7H_2O$	246.49	1.00	246.49	1.0	S	1000	32
				1.0	Mg	1000	24
Micronutrients		(µM)					
KCl	74.55	50	3.728	1.0	Cl	50	1.77
H_3BO_4	61.84	25	1.546	1.0	B	25	0.27
$MnSO_4.H_2O$	169.01	2.0	0.338	1.0	Mn	2.0	0.11
$ZnSO_4.7H_2O$	287.55	2.0	0.575	1.0	Zn	2.0	0.131
$CuSO_4.5H_2O$	249.71	0.5	0.125	1.0	Cu	0.5	0.032
H_2MoO_4	161.97	0.5	0.081	1.0	Mo	0.5	0.05
Fe.EDTA	346.08	20	0.922	1.0	Fe	20	1.12
2,4-D		100ppm	0.1gm	10	2,4-D	10 ppm	10ppm
2,4-D		100ppm	0.1gm	50	2,4-D	5 ppm	5ppm

Modifications to Hoagland solution:

For high sodium nutrient medium: Add double the quantity of $NaH_2PO_4.2H_2O$ to get a sodium concentration of 440 ppm.
For sodium deficient medium: Mix all the nutrients except the $NaH_2PO_4.2H_2O$ to get a sodium concentration of 0 ppm.
For high calcium nutrient medium: Add double the quantity of Ca $(NO_3)_2$.$4H_2O$ to get a calcium concentration of 320 ppm.
For calcium deficient medium: Mix all the nutrients except the Ca $(NO_3)_2$.$4H_2O$ to get a calcium concentration of 0 ppm.
For 5ppm 2,4-D solution: Add 50 ml of 100ppm 2,4-D stock
For 10ppm 2,4-D solution: Add 10 ml of 100ppm 2,4-D stock

Appendix-2

Tris soluble seed proteins

1. Defatting solvent mixture:
   Chloroform, Methanol & Acetone mixed in a 2:1:1 ratio.

2. Stock protein extraction solution:
   2 g SDS and 10 mg Pyronin G dissolved in 10.4 ml of 0.6 M Tris-HCl buffer (pH 6.6) and 7.9 ml distilled water and 10 ml glycerol, warmed gently and mixed well.

3. Working protein extraction solution.

   This was prepared by mixing 4.25 ml stock protein extraction solution, 0.75 ml
   β-Mercaptoethanol and make upto 10 ml by adding distilled water.

Reagents for Gel electrophoresis

1. 30% acrylamide for running gel
   75 g acrylamide and 1 g bis acrylamide dissolved in distilled water and make upto 250 ml.

2. 30% acrylamide for stacking gel
   75 g acrylamide and 2 g bis-acrylamide dissolved in distilled water and make upto 250 ml.

3. Stock buffer for running gel
   1.875 M Tris-HCl buffer (pH 8.8): 22.69 g Tris dissolved in 50 ml distilled water and pH adjusted to 8.8 by adding conc. HCl drop by drop. The volume was made upto 100 ml with distilled water.

4. Stock buffer for stacking gel
   0.6 M Tris-HCl buffer (pH 6.8): 7.26 g Tris dissolved in 50 ml distilled water and pH adjusted to 6.8 by adding conc. HCl drop by drop. The volume was made upto 100 ml with distilled water.

5. Stock SDS solution (10%)
   10 g SDS dissolved in distilled water with constant stirring and gentle heating. The volume was made upto 100 ml with distilled water.

6. Ammonium persulphate (5%) (Freshly prepared)
   0.5 g ammonium per sulfate dissolved in 10 ml distilled water.

SDS-Tris Glycine (pH 8.3) 9.0 g Tris, 42.3 g glycine and 3 g SDS dissolved in distilled water and made upto 3 litres.

8. Fixing solution (15% TCA)
   150 g trichloroacetic acid dissolved in distilled water to make upto 1 litre.

9. Staining solution
   One gram comassie blue was dissolved in 100 ml of methanol and 10 ml of it was added to 100 ml 15 percent TCA solution to make a final staining solution.

Preparation of gel:

1. Separating gel / running gel (15%)

Tris buffer (pH 8.8)	12.0 ml
Water	7.4 ml
30% running gel acrylamide	20.0 ml
10% SDS	0.4 ml
5% APS	0.4 ml

0.04 ml of TEMED was added just before pouring the gel mixture.

All the reagents were mixed well and poured between the plates of the cassette. Care was taken to avoid air bubbles to be trapped in the gel solution. Cassette was filled 3/4th and the gel was allowed to set.

2. Stacking gel (4%)
   After the running gel gets polymerized the following solutions were mixed and poured above it carefully.

Tris buffer (pH 6.8)	1.5 ml
Water	6.0 ml
30% stacking gel acrylamide	2.0 ml
10% SDS	0.10 ml
5% APS	0.40 ml

0.04 ml TEMED was added just before pouring solution.

After pouring the stacking gel solution, an acrylic comb having required number of wells was set, without trapping any bubble and gel was allowed to polymerize. The comb was removed and the wells were washed with tank buffer.

Appendix-3

Globulins

1. Extraction buffer

   5% NaCl in 50 mM Tris HCl (pH 8.0)
   Dissolve 0.605 g Tris in 50 ml distilled water, adjust the pH to 8.0 with conc. HCl and made up the volume to 100 ml with distilled water.

Tris HCl buffer (pH 8.0) 50 mM	25 ml
NaCl (sodium chloride)	1.25 g

   The above mentioned components are mixed thoroughly.

2. 25 mM Tris glycine buffer (pH 8.3)

   Dissolve 3 g Tris in 50 ml of distilled water, adjust the pH to 8.3 with conc. HCl. Dissolve 2.075 gm glycine in Tris HCl (pH 8.3) and make up the volume to 100 ml with distilled water.

3. Stock working sample buffer, 62.5 mM Tris-HCl buffer (pH 6.8)

   Dissolve 0.756 g of Tris in 50 ml distilled water, adjust the pH to 6.8 with conc. HCl and the volume made upto 100 ml with distilled water.

4. Working sample buffer

Tris-HCl buffer (pH 6.8) 62.5 mM	25 ml
Sodium dodecyl sulfate (SDS)	0.5 g
β-Mercaptoethanol	1.25 ml
Urea	9.00 g
Sucrose	2.5 g

   All the above components were mixed thoroughly.

Reagents for Gel electrophoresis

All the gel reagents (solutions) prepared were same as mentioned for total tris-soluble seed protein.

1. 30% acrylamide for running gel
   75 g acrylamide and 1 g bis acrylamide dissolved in distilled water and make upto 250 ml.

2. 30% acrylamide for stacking gel
   75 g acrylamide and 2 g bis-acrylamide dissolved in distilled water and make upto 250 ml.

3. Stock buffer for running gel
   1.875 M Tris-HCl buffer (pH 8.8): 22.69 g Tris dissolved in 50 ml distilled water and pH adjusted to 8.8 by adding conc. HCl drop by drop. The volume was made upto 100 ml with distilled water.

4. Stock buffer for stacking gel
   0.6 M Tris-HCl buffer (pH 6.8): 7.26 g Tris dissolved in 50 ml distilled water and pH adjusted to 6.8 by adding conc. HCl drop by drop. The volume was made upto 100 ml with distilled water.

5. Stock SDS solution (10%)
   10 g SDS dissolved in distilled water with constant stirring and gentle heating. The volume was made upto 100 ml with distilled water.

6. Ammonium persulphate (5%) (Freshly prepared)
   0.5 g ammonium per sulfate dissolved in 10 ml distilled water.

7. Electrode (tank) buffer
   SDS-Tris Glycine (pH 8.3): 9.0 g Tris, 42.3 g glycine and 3 g SDS dissolved in distilled water and made upto 3 litres.

8. Fixing solution (15% TCA)
   150 g trichloroacetic acid dissolved in distilled water to make upto 1 litre.

9. Staining solution
   One gram comassie blue was dissolved in 100 ml of methanol and 10 ml of it was added to 100 ml 15 percent TCA solution to make a final staining solution.

Preparation of gel:

1. Separating gel / running gel (15%)

Tris buffer (pH 8.8)	12.0 ml
Water	7.4 ml
30% running gel acrylamide	20.0 ml
10% SDS	0.4 ml
5% APS	0.4 ml

0.04 ml of TEMED was added just before pouring the gel mixture.

All the reagents were mixed well and poured between the plates of the cassette. Care was taken to avoid air-bubbles to be trapped in the gel solution. Cassette was filled 3/4th and the gel was allowed to set.

2. Stacking gel (4%)

After the running gel gets polymerized the following solutions were mixed and poured above it carefully.

Tris buffer (pH 6.8)	1.5 ml
Water	6.0 ml
30% stacking gel acrylamide	2.0 ml
10% SDS	0.10 ml
5% APS	0.40 ml

0.04 ml TEMED was added just before pouring solution.

After pouring the stacking gel solution, a comb was set having required number of wells, without trapping any bubble and gel was allowed to polymerize. The comb was removed and the wells were washed with tank buffer.

Appendix-4

Reagents for isoenzymes

1. Extraction buffer (0.1M Tris HCl, pH 7.5)
   1.21 g Tris dissolved in 50 ml distilled water, pH adjusted to 7.5 by adding conc. HCl drop by drop and make up the volume to 100 ml.

2. Extraction buffer (Percy *et al.*, 1990) for Alcohol Dehydrogenase (ADH)

i)	75 mM Sodium phosphate (pH 7.5)	100 ml
ii)	Bovine serum albumin (BSA)	0.5 g
iii)	Sucrose	5 g
iv)	Polyvinyl Pyruvate (PVP)	10 g
v)	β-Mercaptoethanol	1.22 ml
vi)	Ascorbic acid	0.176 g
vii)	Dithioerythritol (DTT)	0.144 g
viii)	Diethyl dithiocarbamate	0.225 g

   All the above components were mixed thoroughly.

3. Stock acrylamide gel solution

Acrylamide	30.08 g
Bisacrylamide	0.8 g

   Dissolved in distilled water and volume made upto 100 ml.

4. 1.875 M Tris HCl buffer (pH 8.8)
   22.69 g Tris was dissolved in 50 ml distilled water. The pH was adjusted to 8.8 with conc. HCl and volume was made upto 100 ml with distilled water.

5. 0.6M Tris HCl buffer (pH 6.7)
   6 g Tris was dissolved in 50 ml distilled water, pH adjusted to 6.7 with conc. HCl and the volume was made upto 100 ml with distilled water.

6. 10% APS (ammonium persulphate)
   One g APS was dissolved in 10 ml of distilled water. This solution was prepared freshly each time.

7. Electrode buffer

Tris base	1.8 g
Glycine	8.64 g

   Dissolved in distilled water and pH adjusted to 8.3. The volume was made upto 3 litres.

Preparation of gel:

1. Running gel (8%)

Stock gel solution (B)	12.0 ml
Tris-HCl buffer (pH 8.8) (C)	5.6 ml
Distilled water	27.4 ml
10% APS (E)	0.4 ml

0.04 ml TEMED was added just before pouring the gel.
The solutions were mixed thoroughly and poured into a cassette till it filled three-fourth.

2. Stacking gel (4%)

Stock gel solution (B)	1.5 ml
Tris-HCl buffer (pH 6.7) (D)	3.0 ml
Distilled water	10.5 ml
10% APS	0.4 ml

0.04 ml TEMED was added just before pouring the gel. The solutions were mixed thoroughly and poured slowly over the separating gel. An acrylic comb with required number of wells was placed immediately inside the cassette to form wells. Once the gel was polymerized, the comb was removed and wells filled with tank buffer.

Appendix-5

Staining procedures for isoenzymes

1) Esterase (Glaszman, 1985)

1M Phosphate buffer solution (pH 6.0):

Solution-A
2.789 g sodium dihydrogen phosphate (NaH_2PO_4) was dissolved in distilled water and volume made upto 100 ml.

Solution-B
5.363 g disodium hydrogen phosphate (Na_2HPO_4) was dissolved in distilled water and volume made upto 100 ml.

To obtain a pH 6.0 phosphate buffer, 85 ml of solution-A and 15 ml of solution-B were mixed. To this 50 mg fast blue RR salt and 3 ml solution of 50 mg α-naphthyl acetate and β-naphthyl acetate dissolved in 3:2 acetone & water mixture was added. The mixture was stirred on magnetic stirrer. The staining solution was protected from light by covering the container with black paper.

The gel was incubated in dark for 30 min at a temperature of 35°C. The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

2) Alcohol dehydrogenase (Shaw and Prasad, 1970).

0.1M Tris (pH 5.8)	50 ml
β-NAD (Nicotinamide adenine dinucleotide)	20 mg
Nitro Blue Tetrazolium (NBT)	20 mg
Phenazine Metho Sulphate (PMS)	5 mg
25% ethanol	6 ml
(Added just before staining)	

The above reagents were mixed and the gel was stained in dark at 30°C till blue bands appeared. The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

3) Superoxide Dismutase (Shaw and Prasad, 1970).

Solution A

50 mM Sodium phosphate buffer (pH 7.5)	100 ml
NBT	200 mg

Solution B

50 mM Sodium phosphate (pH 7.5)	100 ml
TEMED	0.4 ml
Riboflavin	1 mg

Gel was first incubated in solution A for 20 minutes in dark at $30^{O}C$. Then, solution A was drained off and solution B was poured and gel incubated under illumination for 30 minutes or until achromatic bands are easily distinguishable.
The gels were photographed immediately.

4) Acid Phosphatase (Tanskley and Orton, 1983)

Sodium acetate 50mM (pH 5.5)	100mL
$MgCl_2$ (1M)	1mL
Fast Black K Salt	100mg
β- Naphthyl acid phosphate (1%)	3mL

Procedure:

Dissolve the fast black K salt in the sodium acetate buffer, and then add the β-Naphthyl acid phosphate. At this point, suspension of particles will appear. If the solution is to be divided among the trays, it should be stirred vigorously just before pouring. Incubate the gel in darkness at 30 ^{o}C for 1 to 5 hours or until purple or red bands appear. Discard the solution and rinse with tap water and fix.

The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

5) Catalase (Tanskley and Orton, 1983)

A	Sodium thiosulphate (60mM)	30ml
	H_2O_2 3%	70ml

B	Potassium iodide (90Mm)	100ml
	Glacial acetic acid	0.5ml

Procedure:

Solution 'A' was mixed very quickly just before pouring on to the gel. Gel was incubated for 30 seconds, and then solution "A" was poured off and solution "B" was added. The chromophore forms slowly. The gel was documented by scanning immediately as soon as the achromatic bands become evident otherwise bands vanish very soon.

The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

6) Malate dehydrogenase (Shaw and Prasad, 1970)

Tris 0.1M	(7.5pH)	100ml
DL-malate	(1M)	3ml
NAD+		30mg
MTT		20mg
PMS		4mg

Procedure:

Staining solution was thoroughly mixed. Gel was incubated in the dark at 30°C for 15 to 60 minutes or until the blue bans appear. Reaction was stopped and fixed. The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

7) Peroxidase:

Staining procedure (Graham *et al.*, 1964)

Reagents:

A) Sodium acetate 50mM (4.5pH)		100mL
B) N, N Dimethyl formamide	(DMF)	3mL
Benzidine		50mg
C) H_2O_2 (3%)		0.75mL

Procedure:

Benzidine was dissolved in the DMF solution (B) then, poured into solution (A) and mixed thoroughly. Hydrogen peroxide solution (C) was added just before incubation. Gels were incubated at room temperature until faint bands appeared in about 5 minutes, Reaction was stopped by draining off the solution and washed repeatedly with tap water and fixed. The gels were scanned in Epson GT-950 scanner and prints were taken. The electrophoregrams were prepared by observing gels over a trans-illuminator measuring the distance of each band from the point of loading.

Appendix-6

PCR analysis

Preparation of buffers

1. Extraction buffer

100 mM Tris-HCl (pH 8.0)	1.214 g / l
50 mM EDTA (pH 8.0)	2.80 g / l
500 mM NaCl	5.848 g / l

All the above were mixed and autoclaved.

2. 20% Sodium dodecyl Sulphate (SDS) — 20 g /100 ml

3. 5M Potassium acétate — 4.9 g /100 ml

4. Isopropanol : Ammonium acetate (10 M)
Isopropanol and 10M Ammonium acetate were mixed in 3:1 (v/v) ratio

5. 70% ethanol
70 ml of ethanol was mixed with 30 ml of distilled water.

6. Tris-EDTA ($T_{10}E_1$) buffer
TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) was prepared by mixing 1.21 g Tris (10 mM) and 0.372 g EDTA (1 mM), pH adjusted to 8.0 with 1N HCl and volume made upto 1 litre with double distilled water and autoclaved.

Preparation of RNAase

Tris-NaCl buffer (10 mM Tris, 15 mM NaCl) was prepared by mixing 10 µl of 1 M Tris (pH 7.5) and 3.75 µl of 4 M NaCl in an Eppendorf tube. The final volume was made upto 1 ml with distilled water. 10 mg of RNAase was added to this and it was heated for 15 min at 100^0C. It was cooled slowly at room temperature. Aliquot of 50 µl of this cooled RNAase was stored in Eppendorf tubes for later use. Finally it was stored at -20^0C.

Preparation of electrode buffer (Tris-acetic Acid EDTA, TAE buffer)

TAE (50X) stock buffer was prepared by mixing 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA. Final volume was made upto 1 litre with single distilled water.

From this stock of 50 X TAE, working buffer of 1 X TAE was prepared by mixing 20 ml of stock buffer with 980 ml of distilled water to get a final volume of 1 litre. This was poured into the electrophoresis tank so that the gel gets completely immersed in it.

Preparation of tracking dye

Tracking dye was prepared as given below:

Glycerol	50 ml
Double distilled water	50 ml
Bromophenol blue	250 mg
Xylene cyanol	250 mg

These chemicals were mixed thoroughly by magnetic stirrer for 4-5 hrs transferred into 1.5 ml Eppendorf tubes and stored at 4^0C.

Appendix-7

Primers used for RAPD analysis

KIT R		KIT O		KIT N	
Code	**5' to 3'**	**Code**	**5' to 3'**	**Code**	**5' to 3'**
OPR-01	TGCGGGTCCT	OPO-01	GGCACGTAAG	OPN-01	CTCACGTTGG
OPR-02	CACAGCTGCC	OPO-02	ACGTAGCGTC	OPN-02	ACCAGGGGCA
OPR-03	ACACAGAGGG	OPO-03	CTGTTGCTAC	OPN-03	GGTACTCCCC
OPR-04	CCCGTAGCAC	OPO-04	AAGTCCGCTC	OPN-04	GACCGACCCA
OPR-05	GACCTAGTGG	OPO-05	CCCAGTCACT	OPN-05	ACTGAACGCC
OPR-06	GTCTACGGCA	OPO-06	CCACGGGAAG	OPN-06	GAGACGCACA
OPR-07	ACTGGCCTGA	OPO-07	CAGCACTGAC	OPN-07	CAGCCCAGAG
OPR-08	CCCGTTGCCT	OPO-08	CCTCCAGTGT	OPN-08	ACCTCAGCTC
OPR-09	TGAGCACGAG	OPO-09	TCCCACGCAA	OPN-09	TGCCGGCTTG
OPR-10	CCATTCCCCA	OPO-10	TCAGAGCGCC	OPN-10	ACAACTGGGG
OPR-11	GTAGCCGTCT	OPO-11	GACAGGAGGT	OPN-11	TCGCCGCAAA
OPR-12	ACAGGTGCGT	OPO-12	CAGTGCTGTG	OPN-12	CACAGACACC
OPR-13	GGACGACAAG	OPO-13	GTCAGAGTCC	OPN-13	AGCGTCACTC
OPR-14	CAGGATTCCC	OPO-14	AGCATGGCTC	OPN-14	TCGTGCGGGT
OPR-15	GGACAACGAG	OPO-15	TGGCGTCCTT	OPN-15	CAGCGACTGT
OPR-16	CTCTGCGCGT	OPO-16	TCGGCGGTTC	OPN-16	AAGCGACCTG
OPR-17	CCGTACGTAG	OPO-17	GGCTTATGCC	OPN-17	CATTGGGGAG
OPR-18	GGCTTTGCCA	OPO-18	CTCGCTATCC	OPN-18	GGTGAGGTCA
OPR-19	CCTCCTCATC	OPO-19	GGTGCACGTT	OPN-19	GTCCGTACTG
OPR-20	ACGGCAAGGA	OPO-20	ACACACGCTG	OPN-20	GGTGCTCCGT

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