BIOCHEMICAL AND MOLECULAR MARKERS IN COCONUT (*Cocos nucifera* L.)

Thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy in PLANT BREEDING AND GENETICS to the Tamil Nadu Agricultural University Coimbatore

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CERTIFICATE

This is to certify that the thesis entitled "**BIOCHEMICAL AND MOLECULAR MARKERS IN COCONUT (*Cocos nucifera* L.)**" submitted in part fulfilment of the requirements for the award of the degree of DOCTOR OF PHILOSOPHY (Agriculture) in PLANT BREEDING & GENETICS to the Tamil Nadu Agricultural University, Coimbatore is a record of **bonafide** research work carried out by **Mrs.Jayalekshmy,V.G.** under my supervision and guidance and that the part of this thesis has not been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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(JAYALEKSHMY.V.G.)

Abstract

ABSTRACT

STUDIES ON BIOCHEMICAL AND MOLECULAR MARKERS IN COCONUT (*COCOS NUCIFERA* L.)

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Coconut, **Cocos nucifera** L. is a monotypic species of tropical distribution, known as "Kalpa Vriksha", the tree of heaven due to its highly diversified utility. Because of its highly outcrossing nature, several types widely differing from each other exist. Only very little attempt has been made to differentiate these genotypes at the molecular and biochemical levels. Hence this study was undertaken to find out molecular and biochemical markers to assess the genetic diversity in coconut especially between the two size classes "Talls and Dwarfs" and their hybrids.

To get a critical awareness of the genetic variability existing in the species, morphological data on twenty characters in thirty genetically diverse genotypes were analysed statistically. Analysis revealed high variability existing between the different genotypes for the morphological

characters studied. The cluster analysis resolved them into six different groups.

Endosperm storage protein which is used for numerous edible purposes was found to vary among the coconut varieties studied. Total storage protein profile and the fractions, globulin, albumin and glutelin revealed variation among the varieties in the electrophoretic studies. Protein profile region between 29 KDa and 24 KDa was found to be polymorphic for the three fractions.

Isozyme studies revealed that peroxidase (PRX 7 and PRX 42) and polyphenol oxidase (PPO 43) could differentiate the two type classes (Talls and Dwarfs) and the hybrids in coconut. Esterase isozymes did not show much variation among the cultures.

RFLP analysis confirmed the use of the oil palm genomic clone CL 54 in combination with restriction enzymes EcoRI and Sau III powerful to develop hybridization in the coconut genomic DNA.

RAPD analysis was found to be the most efficient method for developing molecular markers to differentiate the coconut genotypes. The ten primers used could generate more than 50 per cent polymorphism in the amplification products from the DNA of six different genotypes of coconut studied. OPC-4 primer was chosen as the best primer to generate polymorphism and to differentiate the two type classes (Tall and Dwarf) and the hybrids. The cluster analysis and the dendrogram analysis following RAPD brought about a great deal of similarity.

Molecular markers can particularly assist coconut breeding and basic genetic research programmes and reveal genome organisation to a large context.

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LIST OF ABBREVIATIONS

CBB	-	Commassie Brilliant Blue
cDNA	-	Complementary Deoxy ribo Nucleic Acid
Cm	-	Centimetre
°C	-	Degree Centigrade
CV	-	Coefficient of variation
DNA	-	Deoxy ribo Nucleic Acid
EDTA	-	Ethylene Diamino Tetra Acetic acid
g	-	gram
h	-	hour
Kbp	-	Kilo base pairs
KDa	-	Kilo Daltons
LB	-	Liquid Broth
m	-	metre
m^2	-	Square metre
mg	-	milli gram
min	-	minutes
ml	-	milli litre
mM	-	milli molar
µg	-	Microgram
µl	-	Micro litre
nm	-	nanometre
OZ	-	Ounze
PCR	-	Polymerase Chain Reaction
PAGE	-	Poly Acrylamide Gel Electrophoresis
RAPD	-	Random Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
rRNA	-	ribosomal Ribo Nucleic Acid
Rm	-	Relative mobility
SDS	-	Sodium Doedecyl Sulphate
SE	-	Standard error
rpm	-	revolutions per minute
WCT	-	West Coast Tall
ECT	-	East Coast Tall
JT	-	Jamaican Tall
KGD	-	Kulasekharam Green Dwarf
COD	-	Chowghat Orange Dwarf

Introduction

CHAPTER I

INTRODUCTION

Among the world's approximate 2700 species of palms, coconut palm is the most versatile, providing edible and industrial oil, protein-rich milk and invigourating water and also valuable source of timber, fibre, roofing and matting material and a number of products from it's shell. The tree is rightly called the tree of heaven 'Kalpa Vriksha' as very few other cultivated plants have such highly diversified utility as the coconut. Coconut is grown in more than 90 countries of the tropics. India occupies the third position in terms of production among the countries next only to Philippines and Indonesia with an annual production of 7000 million nuts (Menon and Pandalai, 1960).

Though we do not know for certain the centre of origin of this palm, it has been known to occur for over 3000 years in India. The centre of diversity is in the south-east Asian and Melanesian regions. According to FAO statistics, 58.5 percent of the six million hectares under coconut in the world is in south-east Asia. Another 24.6 percent of the area occurs in South Asian countries. Therefore, the scientists of this region have a major responsibility to study critically the problems and potential of this wonderful palm.

Improvement of perennial crops in general and coconut in particular is a long drawn, slow and difficult process, because of its, (i) unique floral biology, (ii) long juvenile phase before flowering , (iii) prolonged interval between generations, (iv) heterozygous nature of the crop, (v) long period of experimentation to get results, (vi) lack of a reproducible asexual method of

rapid multiplication, (vii) low rate of sexual propagation and (viii) vast land area required for the experimentation because of low planting density. All these handicaps get compounded by the fact that the bulk of the coconut farming is in the small farmers holdings of 0.25 ha average size. Inspite of all these limitations, India has been in the forefront of coconut breeding and crop improvement researches.

Eventhough coconut is regarded by most taxonomists as monotypic, there is considerable variation in coconut. Till date no close relative that may introgress the genus has been reported. The high genetic variation observed in this palm is assumed due to highly outcrossing nature, spontaneous mutation and selection in the only species (Harland, 1957).

Basically in coconut there are only two varieties. They are the tall and the dwarf. In both these varieties, there are a number of cultivars/types existing because of the variation in size and colour of the nut, quantity of copra, oil content etc. Even with all these features, it is difficult to identify these cultivars because of the lack of detailed descriptor and the confusion is still persisting. A few examples to suggest are described hereunder. The Laccadive Dwarf and the Kenthali of Karnataka look similar to Chowghat Orange Dwarf. Similarly the Laccadive Green Dwarf resembles to a very great extent the Chowghat Green Dwarf. Laccadive Ordinary (Chandrakalpa) and the West Coast Tall are almost similar in most of the characters.

The first systematic classification of coconut varieties and forms was attempted by Narayana and John (1949). The coconut cultivars available in Srilanka have been classified by Liyanage (1958). The criteria used in the above classification were based on plant habit and geographical source. The

third classification was attempted by Fremond **et al.** (1966) on the basis of floral biology. Rao and Pillai (1982) also attempted to classify a few accessions based on the fruit components. One of the common criteria used in all these classifications was on the nut characters. For the purpose of classification, it is necessary to use data that are commonly available. It is also necessary to use parameters that can be assessed simply and accurately which may not be required to be repeatedly collected.

Characterization and the development of descriptors for coconut is apparent from the above references. Even today, there is a lot of confusion among experienced coconut breeders in classifying certain cultivars/types. Central Plantation Crops Research Institute, Kasargod Kerala, India developed two sets of descriptors, one for the collection of germplasm during survey and expedition which is also called the passport data and another for the evaluation. This descriptor was used by Pillai **et al.** (1991) for characterising 18 genotypes of coconut (14 tall and 4 dwarfs).

All these descriptors are morphology based in nature and many are quantitative in inheritance and have made unique identification difficult to achieve, due to the environmental influences. Advances in biochemistry, genetics, and molecular biology have provided descriptors based on proteins including isoenzymes and deoxyribo nucleic acid (DNA). These molecules which are universal in the animal and plant kingdoms can now be used as evidence of similarity and dissimilarity in numerous taxa including domesticated plant species. Beyond identification they can also be used in tests of parentage, genetic mapping of loci conditioning economic traits, measurement of genetic diversity, and in discerning patterns of genetic diversity. Usage of protein and DNA descriptors has propelled the science of

both human and cultivated plant identification into the mainstream of peer-reviewed and widely accepted science.

Most of the cultivated crops, cereals, pulses, vegetables, ornamentals and many fruit tree crops and forest trees had been subjected to intensive research in the field of molecular biology and molecular markers. But in coconut palm, the research done on this aspect is rather limited.

Hence this study was undertaken with the following objectives.

1. Assess the molecular level differences between the varieties, Tall and Dwarf.
2. Search for unique molecular markers to distinguish the Tall and Dwarf varieties by heterologous probing and oligonucleotide finger printing by RFLP.
3. PCR methodology to differentiate DNA fragments between Tall and Dwarf varieties by RAPD analysis.
4. Documenting the polymorphism among the two varieties and hybrids at biochemical level with protein data and isozyme analysis and statistical analysis of the data based on morphological descriptors.

Plate 1. "Kalpa Vriksha"

Kalpa Vriksha, the Coconut Palm is a traditional source of edible oil. Gainful achievements have been made through Genetic and Plant Breeding methods. Molecular markers can enhance the improvement in this "Tree of heaven".

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

The coconut belongs to the family, PALMAE included under the lower group of flowering plants known as monocotyledons. It occupies a conspicuous position in the vegetable kingdom owing to certain features characteristic of the palms, viz., comparatively slow growth; an unusual thickening at the base of the trunk giving mechanical rigidity; generally unbranched, erect, cylindrical, pillar like stem covered with heavy scars of old leaf bases; a compact magnificient crown of gigantic, feathery, glossy, thick cuticled leaves sheathed at the base providing firm attachment to the stem, orientated at the top of the trunk rendering a natural beauty and elegance; a branched inflorescence enclosed in a sheath collectively known as the spadix; the absence of tap root and the continually growing terminal bud commonly known as the cabbage. Genus **Cocos** is a monotypic genus containing only **Cocos nucifera** (Menon and Pandalai, 1960). The coconut is cultivated in the warm damp regions of the tropics and is particularly distributed on all tropical shores.

2.1. Origin

The origin of coconut was traced by Martius (1850) to the West Coast of Central America near Isthumus of Panama. Burkill (1935) presented three theories prevalent on the origin and distribution of the coconut. According to the first, the palm came from the stock which gave rise to the American members of the genus **Cocos** and originated in the northern end of the Andes in Tropical America from where it was taken into the Pacific. The second

theory is that from a place of origin on the coasts of Central America, the equatorial currents of the sea transported it to the Pacific Islands. According to the third, which is more generally accepted, coconut has originated in South Asia or in the pacific from where it reached America.

Debate on the origin and dispersal of **Cocos nucifera** has generally concluded that the palm has a South-East Asia Malenisian origin. The means of dispersal to the margins of it s pre-industrial range is in more dispute. A stochastic simulation model of the wind currents of islands of the Pacific ocean was developed by Ward and Brookfield (1992) and they tested the hypothesis of trans-pacific drift dispersal. The model suggested that the probability of coconuts drifting to the west coast of Panama while remaining viable is extremely low and dispersal to Panama by human seems to be more likely.

2.2. Coconut classification

Variety is generally a term designated to denote a single strain or a group of strains which distinctly differ in structural or functional characters from one another or a group of the same species which can be depended upon to reproduce itself true-to-type. Hunter and Leake (1933) pointed out that the number of varieties in coconut could not be determined because of the difficulties inherent in such determinations due to wide distribution and partly because of the lack of genetical purity due to cross fertilization. The cross-pollination prevailing in the coconut has given rise to a highly variable progeny of palms.

Many such varieties have been reported from the different coconut growing countries. Table 1 shows the number of varieties identified from each region and the authors who have reported them.

Table 1. Number of cultivars identified in coconut

Sl. No.	Country/Region	Number of cultivars	Authors
1.	Philippines	35	Wester (1918)
2.	Malayan region	50	Cook (1901)
3.	Singapore	14	Omar (1919)
4.	Kuala Kangson in Malaya	20	Burkill (1919)
5.	Philippines	40	Jumelle & Hubert (cited by Copeland, 1931)
6.	Travancore	30	Shortt (1885)
7.	Ceylon	19	Stockdale (1924)
8.	Laccadive islands	3	John & Satyabalan (1955)

Based on the study of the varieties made at the Coconut Research Station, Pilicode, Kerala, South India, a systematic classification of coconut has been made by Narayana and John (1949). According to them, the cosmopolitan species **Cocos nucifera** Linn., can be divided into five varieties. A key for the easy identification of coconut varieties prepared by them is given below :

Key for the identification of coconut varieties and forms.

VARIETIES

A. Inflorescence unbranched or rarely with one or two small spikes - variety **spicata**

A.A. Inflorescence normal and always branched

B. Tall late bearing palms

C. With both male and female flowers - variety **typica**

C.C. With only male flowers - variety **androgena**

B.B. Dwarf and early bearing palms

D. Vigorous palms bearing in four years - variety **javanica**

D.D. Delicate palms bearing in three years - variety **nana**

FORMS OF VARIETY TYPICA

A. Nuts very small (about 1,000 cc in volume) and very many in a bunch - even 100 or more **pusilla** (Laccadive small)

A.A. Nuts not small

B. Nuts very large (7,000 cc) majestic palms. Copra content low and quality inferior - **gigantea** (Andaman giant).

B.B. Nuts medium large (6,000 cc or less)

C. Nuts about 6,000 cc, copra superior and quantity high about 10 oz to 12 oz per nut.

D. Yield of nuts per tree per year, about 100, **ramona**.

D.D. Yield of nuts low (about 35 per tree per year) - **Kappadam**

C.C. Nuts about 4,000 cc or less in volume

E. Trunk robust - 90 to 100 cm in girth

F. Percentage of oil in copra high (74) - **siamea**

F.F. Percentage of oil in copra low (66-69)

G. Female flower production (annual) - very high (744) - **nova-guineana**

G.G. Female flower production (annual) low (220) - **malayensis**

E.E. Trunk of medium girth (73-83 cm)

H. Copra thin (0.82 cm) and percentage of oil in copra low (66) - **cochin chinensis**

H.H. Copra thicker (1.2 to 1.3 cm) and percentage of oil high (72) - **laccadive**

Liyanage (1958) discounted geographical variants, since he was concerned only with Ceylon, and reduced the coconut varieties to three-**typica**, **nana** and **aurantiaca**. **Androgena** and **spicata** were considered doubtful and were omitted. **Javanica** was also omitted, but, the description of **nana** was broadened sufficiently to include **Javanica**. The new variety, **aurantiaca**, was considered to be semi-tall and therefore distinct from either the tall or the dwarf groups. The terms semi-tall and medium-dwarf had already been used by Narayana and John (1949) when referring to the Malayan form of **javanica**, the 'Nyior Gading'. Liyanage (1958) gave quantitative data for some fruit components and economic characters, but the key for the identification of varieties in Ceylon was based on subjective estimates of fruit size and appearance. The forms corresponded with some of those in the Indian system, but had different names. Both classifications agree that the tall varieties are

predominantly cross-pollinated and out-breeding and the dwarf varieties are highly self-pollinated and in-breeding.

Pollination characteristics were made the basis for a third classification system by Fremond **et al.** (1966), who contrasted the flowering pattern of dwarf (autogamous or self-pollinating) and tall (allogamous or cross-pollinating). However, the dwarf is easily cross-pollinated, especially when surrounded by talls (as very often happens) and talls are known to be capable of self-pollination (Whitehead, 1965; Rognon, 1976). If the flowering pattern of coconut is considered as a function of evolution, then the ability, however slight, of the tall to self-pollinate ensures that the arrival of a single fruit at a new location will not prevent the next generation from being produced and disseminated, even if no more arrive. Thereafter, cross pollination between the heterozygous progenies would reduce the risk of inbreeding depression. The ability to self-pollinate also undermines the suggestion that dwarfs arose as a result of inbreeding among talls rather than by discontinuous mutation (Swaminathan and Nambiar, 1961), a proposal which has not been supported by subsequent cytological studies (Raveendranath and Ninan, 1974). Self-pollination is a function of selection under cultivation. The less vigorous dwarf survives because its colour markers may be recognized. Whatever the origin of the dwarf form, it is clear that it was selected for its precocity and different fruit colours.

It is generally considered that local forms from different countries have to be tested under controlled conditions before they can be classified. This has proven difficult to put into practice, most variety collections being too small and unrepresentative of the populations sampled. To allow different accessions to be referred to without pre-judgement, current terminology

corresponds tall with 'typica allogamous' and dwarf with 'nana autogamous', prefixed by the country of origin, e.g., 'Rennell Tall', 'Malayan Dwarf', etc. (Harries, 1978).

Fortunately, unlike almost all of the previous attempts, Whitehead's (1966) first tests of fruit component analysis in 1963 were made on the two extreme types that occur in Jamaica. He was able to standardize the procedure into one that can be carried out in the field on coconuts at a definable stage of maturity, with nothing more than a machete, a spring balance, a few plastic bags, paper and pencil and an intelligent approach to random sampling. This method has been used extensively since, with little need for modification. Rao and Pillai (1982) also attempted to classify a few accessions based on the fruit components. One of the common criteria used in all these classification was on the nut characters.

Since most of the phenotypic characters useful in the identification of the cultivars are polygenic, a clear cut demarcation is rather difficult in the identification. With the objective of proper documentation and evaluation of the coconut cultivars, Central Plantation Crop Research Institute, Kasargod developed a model descriptor characterisation of coconut cultivars. Pillai **et al**. (1991) used this descriptor for characterizing 18 genotypes of coconut comprising 14 talls and 4 dwarfs. Five major group of characters were taken into consideration. They were stem characters, leaf characters, inflorescence characters, breeding behaviour, nut characters and its ratios.

Nair and Ratnambal (1994) assessed the genetic resources of coconut and reported Tall and Dwarf are the two distinct varieties of coconut universally found. They described Tall and Dwarf palms as follows:

Tall palms

The tall palms, sometimes referred as var. **typica**, are the most commonly cultivated in all the coconut growing areas of the world. Tall palms generally grow to a height of 15 to 18 m or more and have a comparatively long pre-bearing age of 6-10 years. They are normally cross-pollinated as there is usually no overlapping of male and female phases. Fruit is generally medium to large in size and nuts mature within a period of 12 months. The copra content is usually over 150 g/nut and oil percentage varies from 66 to 70. West Coast Tall, Laccadive Ordinary, East Coast Tall and Andaman Ordinary are some of the distinct tall types present in India.

Dwarf palms

Dwarf palms, sometimes referred to as var. **nana** are characterised by their short stature. They are quicker to come to bearing (3-4 years), easier to harvest and short-lived. They have thin trunks without a swollen base or 'bole' and fully developed fronds rarely exceed 4 m. Though the dwarf palms yield heavily, they have a tendency to irregular bearing. Dwarfs are identified mainly by the colour of their nuts. They are presumed to have originated from tall palms either through mutation (Menon and Pandalai, 1958) or by inbreeding in talls (Swaminathan and Nambiar, 1961). In India, three important dwarf types found from Kerala are Chowghat Green Dwarf and Chowghat Orange Dwarf mainly described by the colour of their nuts and petiole and Gangabondam, a green dwarf from Andhra Pradesh. The copra content in dwarfs ranges from 90 to 120 g/nut and oil percentage is about 65.

Intermediate types

In India, in addition to these two groups, there are few other distinct tall types such as Laccadive Micro, Kappadam, Andaman Giant, Calangute, Nadora and Benaulim. Ramachandran **et al.** (1977) reported Ayiramkachi an intermediate type between tall and dwarf in Tamil Nadu.

2.3. Assessment of genetic variability in coconut

The study of variability in genetic stocks of coconut palms with regard to phenotypic and genotypic variability and genetic advance is a prerequisite for any breeding programme. But the estimation of these parameters in coconut is difficult due to its long pre-bearing period, a period of unstability in yield during the beginning years and a steady period of stabilised yield over a number of years followed by a decline due to senility. Senility normally sets in at the sixtieth year or a little earlier (Davis, 1958).

Louis and Chandrasekharan (1976) reported that the growth and yield characters of coconut palm are highly influenced by environment. Hence study of phenotypic variation may fail to indicate the genetic variability. Liyanage (1967) reported that palms of higher yield value could be identified within three years and six months.

Heritability estimates in different yield groups of coconut have indicated that the parameters were high for the number of female flowers, yield of nuts and percentage seed set (Nambiar and Nambiar, 1970). Analysis on the pattern of genetic variation for reproductive characters and its impact on yield potential have shown that selection of genotypes with low variance of distribution of female flowers, and greater number of spikes with one female flower tend to reduce instability in production and increase productivity

(Nambiar **et al.**, 1970; Nambiar and Ravindran, 1974). A study on the phenotypic and genotypic variability in 25 cultivars and hybrids has shown that the number of leaves per year, number of leaves on the crown, number of spathes per year, number of female flowers per palm, setting percentage and number of nuts have a high genetic advance and these characters were recommended for exercising selection (Louis, 1981). Sree Rangasamy and Sridharan (1993) suggested that by multistage selection during seedling, prebearing and at stable bearing stages of adult palms substantial improvement could be achieved. Path coefficient analysis for yield of nuts during stabilised period of yield showed that the major contributing characters which influenced yield directly or indirectly are the average number of female flowers, number of functional leaves at 19 years and internodal distance at a fixed mark. These characters influenced the yield indicating their value in selection (Sukumaran **et al.**, 1981).

Ramanathan (1984) carried out path coefficient analysis in a population of East Coast cultivar of coconut to identify characters which have direct and indirect influences on yield. Among the 12 characters studied, positive direct effects of a number of functional leaves, number of branches with nuts and number of leaflets on yield indicate the importance of these characters in the selection programme in East Coast Tall Coconut.

Narayanan Kutty and Gopalakrishnan (1991) studied the morphological and chemical components of yield in coconut palms and characterized different yield groups based on these components, with a view to utilize the information for identification of potential yielders. Balakrishnan **et al.** (1993) worked out selection indices for coconut. They selected coconut

hybrids considering all the characters simultaneously by using discriminant function analysis.

N'Cho , **et al.** (1993) assessed seventeen tall coconut ecotypes taking a biometric approach. Twenty four morphological descriptors were used. A discriminant analysis revealed the different relation existing between 10 ecotypes. These results which tally with those from the study of polyphenols, will be made precise with the addition of further characters of fruit. This approach for the assessment of ecotypes in a coconut collection, which brings out relations between ecotypes, can be used by breeders to forecast heterosis when choosing populations to be used in a breeding scheme.

Akpan (1994) evaluated tall coconut genotypes within the Nigerian coconut germplasm bank. He assessed the vegetative characteristics of the seedling, flowering time, floral biology and nut component analysis and found variability among genotypes for all characters studied.

Jay **et al.** (1991) studied the polymorphism of coconut leaf polyphenols for assessing genetic variability. They concluded that analysis of polyphenols by high performance liquid chromatography is an effective technique which confirms hypothesis put forward about dissemination of coconut. Variation for leaf polyphenols in coconut cultivars was also studied by Chempakam and Ratnambal (1993). They studied thirty six cultivars belonging to eight different geographical origins. Significant variations were noticed based on different geographical origins. They also reported that dwarf cultivars had higher phenolic levels as compared to the talls irrespective of the geographical origin.

2.4. Biochemical and molecular markers

The assessment of genetic variation is a major concern of plant

breeders and population geneticists. This is important at several levels. First, the ability to reliably distinguish members of different species is critical in controlling the material entering breeding programmes and in population genetic analyses. Second, the ability to reliably identify or "fingerprint" different genotypes is important in breeding programmes that rely on clonal propagation in testing and/or production and in population genetic analysis of naturally clonal species. Finally, an estimate of the amount of variation within a species is useful for predicting potential genetic gain in a breeding programme.

Traditionally, morphological and phenological characteristics have been used for these purposes. Since such characteristics are often controlled by multiple genes and subject to varying degrees of environmental modification and interaction, differences between clones or closely related species are not always absolute. Many of these traits are also difficult to analyse because they do not have the simple genetic control assumed by many population genetic models.

Biochemical and molecular markers are free of such association and have recently gained great attention in varietal identification, selection and genome mapping. In the following sections, the use of isozyme, protein and DNA markers (RAPD and RFLP) in major crop species with emphasis on perennials are dealt with.

2.4.1. Storage proteins as markers

The seed protein profile obtained by various extraction procedures is conspicuously species specific and is highly stable. Accessions among / cultivated plants from different geographical areas and adapted to diverse ecological zones still possess essentially the same profile (Larsen,1967;

Ladizinsky 1975; Ladizinsky and Adler,1975; Johnson 1972 and 1975). Furthermore the composition of seed protein is highly stable and is affected only slightly by environmental conditions or seasonal fluctuations (Dhunhill and Fowden, 1965; Lee and Ronald, 1967; Adriaanse **et al.** 1969).

Seed proteins are mainly storage proteins and are not likely to be changed in dry mature seed. Thus, mature seeds of different age still possess the same profile (Robinson and Megarrity, 1975).

2.4.1.1. Varietal profiles provided by storage proteins

Proteins can be used to provide varietal profiles. They are in popular usage because the variation for these markers is ubiquitous and this variation can be understood in genetic terms. These characters are in routine usage and are widely accepted as a source of reliable data in evolution, taxonomy and genetics (Tanksley and Orton, 1983; Crawford 1990). Proteins are molecules with net electrical charges that are affected by pH. Proteins can be separated by electrophoresis on the basis of their net electrical charge, molecular weight, isoelectric point or combinations of these criteria utilizing multidimensional separations. They can also be separated by chromatography, wherein the surface chemistry of the protein molecules determines relative retention lines on an absorption matrix (Bietz 1986). There is abundant evidence to show that protein profiles can be obtained for all crop species of major importance and that these profiles are independent of environmental or storage conditions. They are thus reflective of the genotype. It is imperative that data having a quantitative component, for example, those derived from High Performance Liquid Chromatography (HPLC) or two dimensional electrophoresis be carefully examined in order to be able to evaluate their

degree of significance in discriminating between qualitatively similar profiles (Smith and Smith 1992).

General protein detection either by stains or spectroscopic absorbance methods often reveal numerous bands or peaks per individual plant or variety bands, that may exhibit close or overlapping gel migration or column retention times. This level of complexity in banding patterns has prevented the establishment of genetic control for albumin and globulin proteins although these profiles appear to be strongly variety dependent (Koranyi,1989). Profiles of alcohol soluble seed proteins are frequently utilized for varietal description and the genetic control of these proteins is well established (Soare and Salamani 1983; Gupta and Shepherd, 1990).

Studies on storage proteins of palms was initiated by Chandrasekhar and Demason (1988a and b) by describing the electrophoretic characteristics of seed proteins from date- palm, **Phoenix dactylilfera** L. and **Washingtonia filifera**. The seed proteins from the two palm species were heterogenous in molecular mass ranging from 12 to 66 KDa and in charge ranging from pH 3 to 10 and no proteins were found with molecular masses above 100 KDa. Borroto and Dure (1987) have stated that very little is known about globulin protein in monocotyledons.

2.4.1.2. Endosperm protein in coconut

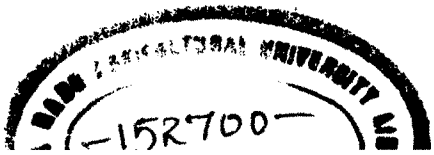
Sjogren and Spychalski (1930) first reported on seed storage proteins in coconut. They used high salt and phosphate buffer at various pH to analyse the storage proteins from coconut endosperm. They called this protein cocosin. Hagenmaeir **et al.** (1973) described the aminoacid composition of a water soluble protein fraction from coconut and found that it is deficient in

the essential amino acids isoleucine, methionine, threonine and tryptophan and rich in a number of charged amino acids (glutamic acid, arginine, aspartic acid and lysine) which explains its salt solubility.

Balasubramaniam and Sihotang (1979) studied coconut protein prepared from fresh coconut kernels and separated them into at least three protein peaks in column chromatography and six protein bands in electrophoresis. The protein solubilised maximally at pH 10.3 and precipitated at pH 5.8. It was found to be associated with lipid in a 10:1 ratio.

Electrophoretic characterisation and immunological localisation of coconut (**Cocos nucifera** L.) endosperm storage protein was dealt in detail by Demason and Chandrasekhar (1990). Proteins extracted from coconut endosperm were analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, tube gel isoelectric focussing, and two dimensional electrophoresis. Antisera to soybean 11s and 7s globulins were used with western blotting techniques to test for immunological similarities. The non-reduced proteins fractionated into four major bands whereas the reduced proteins fractionated into seven major bands ranging from 55 to 17 KDa. All major bands were glycosylated and each consisted of at least two polypeptides with same isoelectric points. A minor band at 67 KDa and two minor bands at 22 KDa were recognised by antibodies to 7s soybean globulin and a major and minor band at 35 and 32 KDa respectively were recognised by antibodies to 11s soybean globulins.

Balachandran and Arumughan (1992) studied solubility and electrophoretic characteristics of proteins in different regions of coconut endosperm. They observed a marked concentration gradient in the increasing



order for proteins from the inner to the outer regions of coconut endosperm. The testa and coconut water showed lower levels of protein concentration. Fractionation of proteins showed that more than 80 per cent of the proteins from the different regions were soluble in aqueous and salt media. Electrophoresis of the major albuminous and globular proteins showed the presence of 6 and 4 protein bands of molecular weights ranging from 14 to 52 and 17.5 to 45 KDa respectively. The various regions from the inner to the outer had the same electrophoretic profile but showed significant differences in their quantities with respect to the major and minor protein bands.

Eventhough electrophoresis of cocosin had been dealt in detail by many workers, the use of protein electrophoresis in developing biochemical marker is very little mentioned. Recently Canto **et al.** (1992) reported on the biochemical markers of variety in **Cocos nucifera** L. SDS-polyacrylamide gel electrophoresis revealed two polypeptides with relative mobilities of 18 and 38 KDa that were present in the three dwarf varieties, but greatly diminished in the tall variety. They have concluded that although dwarf and the tall varieties of coconut are closely related there are slight differences that permit a clear biochemical distinction between the Malayan Dwarf and Atlantic Tall trees.

2.4.2. Enzyme polymorphism as genetic markers

Multiple molecular forms of enzymes (isozymes) have been found by electrophoretic methods in nearly every organism studied (Brewer, 1970; Latner and Skillen, 1968). In broad sense, the term isozyme (Market and Moller,1959) refers to any two distinguishable proteins that catalyse the same biochemical reaction. The number of isozymes detectable depends on several factors such

as the number of genes coding for the enzyme, the number of alleles of each gene which specify electrophoretically distinct polypeptides, the quarternary structure of the enzyme and the extent to which the subunits of polymeric enzymes can cross multimerize as intergenic isozymes (Torres,1976) Isozymes have been widely used as markers in systematic, genetic and evolutionary studies. Most genetic studies have employed micro-organisms, short lived animals and annual plants. Long lived annuals and perennial plants have been subject of relatively few investigations.

In citrus isozyme studies started in 1974. Iglesias **et al.** (1974) developed isozyme markers for the identification of zygotic and nucellar seedlings. Enzymatic polymorphism in the root extract of citrus was studied by Button **et al.** (1976). They found the uses of peroxidase isozymes in citrus breeding and taxonomy.

Essen and Soost (1974) studied the peroxidase polymorphism in citrus. Essen and Scora (1977) found amylase polymorphism in citrus and some related genera. Leaf isozymes were used as genetic markers in citrus by Torres **et al.** (1978a). They determined the genetic control of isozymes from citrus and its near relatives for three genes · glutamate oxaloacetate transaminase, phosphoglucose isomerase and phosphoglucomutase. These enzymes were found to be controlled by four genes having 19 co-dominant alleles.

Torres **et al.** (1978b) studied enzyme polymorphism as genetic markers in the avocado. Mesocarp gene-enzyme systems were examined by starch gel electrophoresis and found to be highly polymorphic. Alcohol dehydrogenase, phosphoglucomutase, glutamic oxaloacetic transaminase and

leucine aminopeptidase were found to be coded by 10 genes with 20 codominant alleles.

Isozyme studies in date palm for generating genetic markers was reported by Torres and Tisserat in 1980. They studied the genetic control of isozymes of alcohol dehydrogenase, esterase, glutamate oxaloacetate transaminase, phosphoglucose isomerase and phosphoglucose mutase from leaf tissue. The five enzyme system was specified by seven polymorphic genes with 14 alleles. Wilaiwan and Phongdara (1990) reported the isozyme patterns in oil palm. In the study of isozymes they found that alcohol dehydrogenase (ADH) had two and three isozymes and glutamate dehydrogenase had one and two isozymes whereas 6- phosphogluconate dehydrogenase (6-PDH) and malate dehydrogenase (MDH) had only two and one isozymes. They also reported that difference in isozymic patterns of ADH and GDH in each tree may relate with the synthetic pathway of seed oil.

Canto **et al.** (1992) reported biochemical markers of varieties in **Cocos nucifera.** Isozyme patterns of acid phosphatases, esterases and malate dehydrogenase were detected in extracts from inflorescence by isoelectric focussing and observed to be monomorphic for the four varieties tested namely: Atlantic Tall, Malayan Green, Red and Yellow Dwarfs. Peroxidase isozyme patterns from both types of extracts were variable among the varieties but this was due to unknown factors other than the genotypic variation.

Shivashankar (1988) studied polyphenol oxidase isozymes in coconut genotypes under water stress. Coconut genotypes growing under rainfed and irrigated conditions were analysed for leaf polyphenol oxidase (PPO) activity

during the development of water stress. Comparison of PPO isozymes separated by polyacrylamide gel electrophoresis (PAGE) showed up two additional fast moving bands in the hybrids while there was no change in the West Coast Tall and Chowghat Dwarf Orange between the control and stressed leaves indicating the differential degree of tolerance to stress among the genotypes.

2.4.3. Restriction Fragment Length Polymorphism (RFLP)

Over the last ten years, DNA markers have become widely used in a variety of biological fields, including genetic fingerprinting and analysis of inherited diseases in humans as well as breeding and population studies in animals. However, one of their most powerful applications arises within plant breeding. This is reflected in the fact that complete DNA marker genetic maps have now been constructed for at least 10 major crop species and many more have been characterised at a less detailed level.

Broadly speaking DNA markers have been utilised in two distinct though related ways: the first being genotype characterization (for example genetic fingerprinting and germplasm evaluation) and the second being identification of markers tightly linked to the desirable breeding traits. The first application requires a relatively modest research effort, since all that is required for the crop of interest is the availability of probes capable of revealing differences between genotypes. Thus, over the last few years, a huge number of plant species have been examined. In some cases, heterologous probes have been used in DNA elements cloned from other plant, animal or viral species. Examples include the use of maize probes in sugarcane (Lu **et al.**, 1991) or wheat and rice ribosomal probes in banana (Lanaud, 1991) and pearlmillet (Hilu and Johnson, 1992). Thus, for example 80 per cent of maize

probes can be used to profile sorghum (**Sorghum bicolor**) inbred lines (Halbert **et al.**, 1990; Lee **et al.**,1990) ; and probes made from tomato (**Lycopersicon esculentum**) DNA can be used to profile potato (**Solanum tuberosum**) (Bonierbale **et al.**, 1988). Human DNA (Jeffrey's probe) has been utilized in ornamentals such as carnations and roses (Tzuri **et al.**, 1991) as well as rubber (Besse **et al.**, 1991) and rice (Dallas, 1988). Viral M13 DNA probes have been successfully used in apples (Nybom and Schaal, 1990). Even synthetic oligonucleotide DNA probes such as (GATA) have proven discriminatory in chickpea (Weising **et al.**, 1989) and barley (Beyerman **et al.**, 1992).

Robert **et al.** (1992) worked on the RFLP based phylogeny of **Musa** species in Papua New Guinea. Random genomic probes were used to detect restriction fragment length polymorphisms (RFLPs) in 26 accessions of **Musa** representing eight species from Papua New Guinea. The results agreed with previous morphology based phylogenetic analysis. A genetic map of citrus based on the segregation of isozymes and RFLPs in an intergeneric cross was constructed by Jarvell **et al.** (1992). The map was based on the segregation of 8 isozymes, one protein and 37 RFLP loci in 60 progenies of a cross of two intergeneric hybrids. They found that eight loci in three linkage groups and one unlinked locus deviated significantly from the Mendelian segregation.

Comparison of allozyme, RFLP and RAPD markers for revealing genetic variation within and between trembling aspen, (**Populus tremuloides**) and big tooth aspen, (**P. grandidendata**) popular tree species of North America was done by Liu and Furnier (1993). They examined genetic variation in allozyme loci, nuclear DNA restriction fragment length polymorphisms (RFLPs)

and random amplified polymorphic DNAs (RAPDs) in 130 trembling aspen and 105 big tooth aspen trees. This provided a very powerful tool for fingerprinting aspen individuals.

Genome relationship between mungbean (**Vigna radiata**) and cowpea (**V. unguiculata**) based on the linkage arrangement of random genomic RFLP markers was investigated by Menancio-Hautea **et al.** (1993). A partial molecular linkage map of the **Musa acuminata** diploid genome presented by Faure **et al.** (1993). This map is based on 58 RFLP, four isozyme and 28 RAPD markers segregating in a population of 92 F_2 individuals.

The level of polymorphism using genomic and cDNA probes with a number of restriction enzymes and the inheritance of RFLP loci were investigated in **Eucalyptus nitens** by Byrne **et al.** (1994). The polymorphism detected with 366 genomic and cDNA probes and three to six restriction enzymes was analysed in three generation outbred pedigrees. No differences in the level of polymorphism detected with genomic versus cDNA probes were observed. They did not find a difference in the efficiency of detection of polymorphism with six different restriction enzymes with three of the enzymes (Bg III, Dra I and Eco RI) showing substantially more polymorphism than the others. They also found no significant correlation between size of the DNA fragments generated by the enzymes and the detection of polymorphism. They concluded that high polymorphisms, large number of alleles and ease of interpretation of RFLPs in **E. nitens** means that they will be useful in a range of application such as genetic linkage maps and paternity analysis.

Ahuja **et al.** (1994) worked on constructing a high density genetic map based on RFLPs for loboly pine (**Pinus taeda**). Consequently they had

made a large number of DNA probes from pine available for use in other species. They have also used these probes to detect RFLPs in 12 conifers and angiosperms.

Genetic diversity in cocoa revealed by cDNA probes was studied by Laurent **et al.** (1994). They investigated the variability of the cocoa (**Theobroma cacao**) nuclear genome. A total of 203 cocoa clones was surveyed for RFLPs using four restriction endonucleases and 31 seed cDNA probes.

A study of genetic variation and evolution of **Phyllostachys** (Bambusoideae) using nuclear RFLP was conducted by Friar and Kochert (1994). Phylogenetic and taxonomic difficulties are common within the woody bamboos due to their unique life cycle which severely limits the availability of floral characters. To address some of these problems, 20 species of woody bamboos were analysed using RFLP and it was found that RFLPs can be used for species identification and the delineation of species limits.

Jack and Mayes (1993) exploited the DNA marker system in oil palm (**Elaeis guinensis**). They started with genetic finger printing for confirming the identity of tissue culture derived clones with the original selected palms. They developed two probes PoPg 54 and PoPg 95 which generated at least 50 distinct banding patterns in 124 genotypes. A hypervariable clone (smp6) has been identified and in this case DNA sequence analysis reveals a complex mosaic of interspersed repetitive domains. Recently they have initiated a programme on oil palm RFLP linkage map.

Rohde **et al.** (1992) from the Max-Plank Institute, Germany made the first effort to characterize the coconut genome. They detected highly repetitive sequences in the genome of coconut palm by analysis of EcoRI

restricted genomic DNA. A sequence comparison of EcoR1 digested fragments showed that the repetitive sequence belonged to a single element family and they were highly related to copia and copia-like retrotransposons. They have concluded that plant transposable elements are present in the repetitive elements of coconut, indicating that trasposons may have contributed to genetic diversity among coconut genotypes.

2.4.4. Random Amplified Polymorphic DNA markers (RAPD)

The detection and exploitation of naturally occurring DNA sequence polymorphism represents one of the most significant recent developments in molecular biology. The technical complexity of performing RFLP analysis coupled with the widespread use of short lived radioisotopes in detection method, has prompted a debate on whether the routine and large scale application of RFLPs in crop improvement programmes is feasible. Since its development, the polymerase chain reaction (PCR) of Saiki **et al.** (1988) has revolutionized many standard molecular biological techniques with modifications of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular markers termed Random Amplified Polymorphic DNAs (RAPDs).

RAPD markers are electrophoretic band polymorphism generated by PCR amplification of genomic DNAs from different sources using short (usually 10mer) oligonucleotide primers of arbitrary sequence (Welsh and McClelland 1990; Williams **et al.**, 1990). The RAPD fragments show great potential as genetic markers for genome mapping (Williams **et al.**, 1990), gene tagging (Mulcahy **et al.**, 1992 and Quiros **et al.**, 1991), population and phylogenetic studies (Van Heusaden and Bachman, 1992) and variety identification (Caetano-Anolles **et al.**, 1991; Hu and Quiros, 1991).

RAPD markers are more preferred to RFLP, because they are easy to analyse, require very little DNA and do not need radioactivity handling facilities. Reproducible amplification of RAPD markers have been demonstrated in many crop species including corn (Huen and Helentjaris 1993), wheat (Devos and Gale, 1992; Vierling and Nguyen, 1992), barley (Tinker **et al.**, 1993), rice (Fukuoka **et al.**, 1992), Sorghum (Pammi **et al.**, 1994) and others (Hu and Quiros, 1991). Apart from the annuals, RAPD markers have also been utilized in the characterisation of long term annuals and perennials also.

RAPD markers had been used to characterise **Cocoa** clones representing the three main clones (Criollo, Forastero and Trinitario) (Wilde **et al.**, 1992). The use of single primer of arbitrary nucleotide sequence resulted in the selective amplification of DNA fragments which were unique to the individual cocoa clones studied. The use of a single primer allowed each of the clones evaluated to be unequivocally characterised.

A molecular marker based linkage map of diploid bananas was constructed by Faure **et al.** (1993). They used 58 RFLP, four isozyme and 28 RAPD markers segregating in an F_2 population of 92 individuals. A total of 90 loci was detected, 77 of which were placed on 15 linkage groups, while 13 segregated independently.

Castiglione **et al.** (1993) detected RAPD finger prints for identification and for taxonomic studies of elite poplar (**Populus** sps.) clones. RAPD analysis was performed on 32 clones belonging to different species of the genus **Populus**. Four primers out of the 18 tested, were selected on the basis of the number and frequency of the polymorphism produced. With these a total of 120

different DNA bands were reproducibly obtained, 92 per cent of which were polymorphic. Results showed that RAPD analysis allows one to discriminate all the tested clones and can therefore be recommended as a convenient tool to defend plant breeder's rights.

Cai **et al.** (1994) initiated genetic mapping with RAPD markers in citrus. Reproducible polymorphism of amplifed DNA fragments was obtained with approximately half of the 140 random primers tested, revealing 266 segregating loci. These were tested for linkage using 60 backcrossed progenies from an intergeneric cross of citrus.

Stiles **et al.** (1993) applied the technique of RAPD to the analysis of the relationships among 10 cultivars of papaya (**Carica papaya** L.). Eleven tenbase synthetic oligonucleotides were chosen that gave multiple PCR amplification products using papaya DNA as the template. These 11 primers amplified a total of 102 distinct fragments. Cultivars were scored for presence or absence of RAPD fragments and grouped by cluster analysis using simple matching coefficients of similarity.

Eleven apple cultivars were differentiated using randomly amplified polymorphic DNA (RAPD) markers by Koller **et al.** (1993). A set of bands consistent in their presence or absence was chosen to create a differentiating band pattern. A key was proposed by which one can differentiate apple cultivars using commercially available primers.

The use of random amplified DNA fragments as genetic markers in **Coffea** was investigated by Lashermes **et al.** (1993). Intraspecific variation was easily detected in **Coffea comephora** and **Coffea liberica** whereas the primers assayed failed to reveal polymorphism between **Coffea arabica**

accessions. Extensive interspecific variation was observed. Genetic relationship between **Coffea** species was deduced from the degrees of similarity in amplified product profiles. RAPD markers appeared to be of high value for characterisation analysis and utilisation of coffee genetic resources. Orozco-Castillo **et al.** (1994) further studied the use of RAPD markers in coffee. Material originating from Ethiopia and the arabica sub-groups **C. arabica** var. **typica** and **C. arabica** var. **bourban** were clearly distinguished. Species specific amplification products were identified but more importantly amplification products specific to **C. camephora** were identified in **C. arabica** genotypes indicating gene flow and selective introgressive hybridization in coffee. This study again emphasized the use of PCR technology for the generation of genetic markers for long lived perennial tree and bush crops.

Keil and Griffin (1994) carried out four separate studies using RAPD markers to analyse samples of **Eucalyptus** supplied by several different organizations. The objective was to examine the reproducibility of the RAPD technique and its ability to discriminate between individual genotypes for verification of clonal identities. They found RAPD profiles that are unique to a genotype and can be generated reliably and simply and that even closely related genotypes can be distinguished. In addition, cases of missampled or mislabelled plant materials were detected when RAPD profiles were not consistent with the identification numbers.

Identification of cultivars and validation of genetic relationships in **Mangifera indica** L. using RAPD markers was done by Schnell **et al.** (1995). Twenty five accessions of mango were examined for RAPD genetic markers with 80, 10mer random primers. Of the 80 primers screened, 33 did not

amplify, 19 were monomorphic and 28 gave reproducible, polymorphic DNA amplification patterns. Eleven primers were selected from the 28 for the study. The number of bands generated was primer and genotype dependent.

Identification of 21 fig (**Ficus carica** L.) accessions representing different varieties was performed using RAPD finger prints by Khadari **et al.**(1995). They found RAPD markers showed sufficient polymorphism for genotype discrimination, clonal stability, environmental stability and experimental reproducibility. They also reported that the genetic variability observed in the fig genotypes was not structured into distinct subgroups probably because an important gene flow occurred in the natural populations from which cultivars originated.

Comparison of two RAPD maps using selfed and open pollinated seeds of the same individual, was done by Plomion **et al.** (1995) in maritime pine (**Pinus pinaster**) . Two genomic maps were constructed for one individual tree of maritime pine using a common set of 263 RAPD markers. The results showed that frame work maps constructed using RAPD markers were repeatable and that differences in locus order for maps of different genotypes or species could result from chance.

Shah **et al.** (1994) studied the utility of RAPD markers for the determination of genetic variation in oil palm (**Elaeis guineensis**). The genetic variation among different accessions of oil palm germplasm collected from Africa was estimated using random primers and the polymerase chain reaction. The study revealed high levels of genetic variation in these accessions. Electrophoresis of the amplification products indicated that nine out of 20 primers were able to generate polymorphic products ranging in length from 0.2 Kb to 2.3 Kb.

In coconut, the use of RAPD markers for assessing genetic diversity has not so far been dealt in detail. But Rohde **et al.** (1993) reported the use of polymerase chain reaction (PCR) for detecting mycoplasma-like organisms (MLO) associated with lethal disease of coconut palm. In their study, PCR was used in conjunction with oligodeoxy nucleotide primers for conserved regions of the 16s rRNA gene to amplify corresponding DNA fragments from DNA of healthy coconut and coconut palms infected with MLO and showing the typical symptoms of lethal disease. The amplified DNA fragments were characterised by sequence analysis and on this basis, oligodeoxynucleotide primers were synthesised that allowed the specific amplification of MLO DNA from highly diluted extracts of coconut tissue. The authors are of opinion that this technique could become the standard technique for MLO identification under field conditions and hence a prerequisite for disease control by epidemiological studies and for breeding programmes by the indexing of germplasm.

Coconut palm is yet to be subjected to detailed studies of molecular biology and hence published reports regarding this field is very much limited in coconut palm.

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

Protein studies and isozyme studies were conducted in the laboratory of School of Genetics, TNAU and RFLP study was conducted in the Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore. RAPD was done in the laboratory of the Institute of Forest Genetics and Tree Breeding, Coimbatore. Plant material was collected from the coconut collection and maintenance nursery under the Department of Horticulture, TNAU.

3.1. Morphological studies

Morphological data (20 characters) in 30 genotypes were recorded. The 30 varieties were planted in 1974 in three replications in the coconut seed nursery under TNAU. The trees were 21 years old at the time of observation. Each replication had one palm of the respective genotype. The exotic varieties and hybrid seed units were originally procured from Central Plantation Crop Research Institute, (CPCRI), Kasargod. All the standard crop management practices were undertaken uniformly for all the palms. Yield data is the average of yearly data over 4 years.

3.1.1 Genotypes studied

Tall varieties

1. Jamaica Tall (JT)
2. San Ramon (SR)
3. Borneo (Bor)
4. Kenya Tall (KT)
5. Zanzibar Tall (ZT)
6. Cochin China (CCT)
7. British Solomon Islands (BSI)

8. Fiji Tall (FT)
9. Andaman Giant (AG)
10. Federated Malay States (FMS)
11. Andaman Ordinary (AO)
12. Philippines Ordinary (PO)
13. Laccadive Ordinary (LO)
14. Laccadive Micro (LM)
15. Semitall Yellow (STY)
16. West Coast Tall (WCT)
17. East Coast Tall (ECT)
18. Seychelles (Sy)
19. Gonthembli (GO)

Dwarf varieties

20. Chowghat Orange Dwarf (COD)
21. Kulasekharam Yellow Dwarf (KYD)
22. Kulasekharam Green Dwarf (KGD)
23. Malayan Yellow Dwarf (MYD)

Hybrids

24. West Coast Tall x Gangabondham (WCT x GB)
25. Semi-Tall Yellow x Jamaica (STY x JT)
26. Chowghat Orange Dwarf x West Coast Tall (COD x WCT)
27. West Coast Tall x Cochin China (WCT x CCT)
28. West Coast Tall x Kulasekharam Green Dwarf (WCT x KGD)
29. East Coast Tall x Malayan Green Dwarf (ECT x MGD)
30. Natural Cross Dwarf (NCD) (Dwarf x Tall)

3.1.2. Characters studied

1. Plant height - from the ground level to the crown (Pl. ht.)(m)
2. Stem circumference;150 cm from the ground level (C-150) (cm)

3. Stem circumference 20 cm from the ground level (C-20) (cm)
4. Number of leaf scars between one and two metres from the ground level (LS)
5. Length of leaf petiole (PL) (cm)
6. Length of rachis (RL) (cm)
7. Theoretical leaf area (LA)(m^2) (N'Cho **et al.** (1994))
   Number of leaflets on one side of leaf (NL)
   Leaflet length (LL) and width (LW), the product of the three giving theoretical leaf area (LA) expressed here in m^2
8. Inflorescence Peduncle Length (IPL)(cm)
9. Inflorescence axial length (IAL)(cm)
10. Inflorescence spikelet length (ISL)(cm)
11. Number of spikelets (INS)
12. Distance from the point of spikelet insertion on the axis to the insertion part of the first female flower on the spikelet (IFFD) (cm)
13. Number of female flowers (NF)
14. Anther length (AL)(cm) (Average of five anthers from each palm measured using travelling microscope)
15. Number of bunches (MNB)
16. Number of nuts/bunch (NNB)
17. Mean fruit weight (FW)(kg)
18. Fruit polar perimeter (Circumference across the poles) (FPP) (cm)
19. Equatorial perimeter - (Circumference through the equator)(FPE) (cm)
20. Nuts/palm/year - Average number of nuts for four years from 1992 to 1995.

3.1.3. Statistical analysis

Elementary statistics (mean, standard deviation and coefficient of variation) were calculated for each variable on all the individuals. Single

variable analysis of variance was carried out to detect the amount of total variability represented by the differences between ecotypes. The significance of these differences became even clearer when a multivariate analysis was carried out. For discriminating the desirable genotypes from the undesirable ones on the basis of their phenotypic performance, selection indices were worked out (Smith, 1936).

The thirty varieties of coconut under study were classified into clusters using the non-heirarchial euclidean cluster analysis done with "SPAR1" computer programme (Spark, 1973).

3.2. Seed storage protein studies

Fully matured ripe coconuts were collected from eight varieties of coconut.

1. West Coast Tall (WCT)
2. East Coast Tall (ECT)
3. Jamaica Tall (JT)
4. Kulasekharam Green Dwarf (KGD)
5. Chowghat Orange Dwarf (COD)
6. Malayan Yellow Dwarf (MYD)
7. Chowghat Orange Dwarf x West Coast Tall (DxT)
8. East Coast Tall x Malayan Green Dwarf (TxD)

Nuts were dehusked and grated endosperm was collected separately. The grated coconut was oven dried at 50°C for three hours without charring. The dried samples were wrapped in filter papers kept in petroleum ether overnight for oil removal. The fat-free desiccated coconut was powdered well

and stored in polythene bags at 4°C. Samples for all protein studies were taken from this.

3.2.1. Extraction of total soluble protein from endosperm

For the extraction of total soluble protein from endosperm, the following buffers were used :

Phosphate buffer (pH 7.0)

Sodium dihydrogen phosphate 10 mM

Disodium hydrogen phosphate 10 mM

Two hundred and fifty mg of the oven dried fat free endosperm from the eight varieties was homogenised separately with 1.0 ml of phosphate buffer in a pestle and mortar and transferred to a centrifuge tube and centrifuged at 72,000 rpm for 6 min at 4°C. The supernatant was used for analysis.

3.2.2. Estimation of protein content

Quantification of protein was done by the method described by Bradford (1976) using the Beckman Spectrophotometer Du 4.

A. Preparation of reagents

a. Bradford reagent

i. Stock Bradford dye solution

One hundred mg of Coomassie Brilliant Blue-G250 was dissolved in 50 ml ethanol (95% v/v) to which 100 ml concentrated orthophosphoric acid was added and finally the volume was made upto 200 ml with distilled water.

ii. Working dye solution

The stock Bradford dye solution was diluted five times with distilled water and filtered before use.

b. Protein standard

i. Stock standard

Ten mg bovine serum albumin dissolved in 10 ml distilled water.

ii. Working standard

One ml stock standard was diluted 100 times to get working standard solution containing 100 mg protein per ml.

B. Protocol

Protein concentrations ranging from 10 to 100 µg were prepared by pipetting out 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard solution into a series of test tubes containing required quantity of distilled water to make up the volume to 1 ml. In each tube, 5.0 ml working dye solution was added to make the final volume to 6.0 ml. The contents were mixed well and allowed for 5 min. but no longer than 30 min. A reagent blank was maintained essentially as above except adding working standard solution. Absorbance was measured at 595 nm against the reagent blank. A standard graph was drawn by plotting concentration of protein along the'X' axis and absorbance along the'Y' axis. From the standard graph, the amount of protein in the samples was calculated.

3.2.3. Electrophoresis

Electrophoretic separation of protein was achieved with polyacrylamide gel electrophoresis as described by Laemmli (1970).

a. Materials

i. Stock acrylamide solution

Acrylamide 30% (w/v)

Bis-acrylamide 0.8% (w/v) (stored at 4°C in the dark)

ii. Running gel buffer (4x) pH 8.8

Tris-HCl	1.5 M
(Adjust the pH with HCl)	

iii. Stacking gel buffer (pH 6.8).

Tris-HCl	0.5 M

iv. Polymerising agent

Ammonium persulphate	5.0% (w/v)

TEMED

v. Sodium dodecyl sulphate (SDS) 10% (w/v)

vi. Electrode buffer (pH 8.2 - 8.4)

Tris-HCl	0.05 M
Glycine	0.192 M
SDS	0.1%
(No pH adjustment required)	

vii. Sample buffer (5x)

Stacking gel buffer	50% (v/v)
Sucrose	50% (w/v)
2-mercaptoethanol	2.5% (v/v)
SDS	5% (w/v)
Bromophenol blue	10% (v/v)
(0.5 % w/v in distilled water)	

(Stored frozen in small aliquots and diluted to (1x) concentration and used)

viii. Standard marker protein

Lactalbumin	14,200 (MW) daltons
Trypsin inhibitor protein	20,100
Tripsinogen	24,000
Carbonic anhydrase	29,000
Glyceraldehyde-3-phosphate dehydrogenase	36,000

Albumin (egg)	45,000
Albumin (Bovine)	66,000

The above proteins were dissolved in single strength sample buffer at a concentration each of 1.0 mg/ml and used at 25 - 50 µl per well.

ix. Destaining solution

Methanol	40 %
Acetic acid	10 %
Distilled water	50 %

x. Staining solution

Coomassie brilliant blue (G 250) 1.0 % in staining solution.

(First the dye was dissolved in methanol and proceeded, used fresh preparation every time).

Separating Gel composition

	Acrylamide Gel		
	15%	12.5%	7 %
Stock acrylamide solution	20.0 ml	16.6 ml	9.2 ml
Separating gel buffer	8.0 ml	8.0 ml	8.0 ml
Water	11.4 ml	18.1 ml	22.4 ml
Degas using a water pump and then add			
Ammonium persulphate solution	0.2 ml	0.2 ml	0.2 ml
10 % SDS	0.4 ml	0.4 ml	0.4 ml
TEMED	20.0 µl	20.0 µl	20.0 µl

Stacking gel composition	4 .0 %
Stock acrylamide solution	1.35 ml
Tris HCl (pH 6.8)	1.0 ml
Water	7.5 ml

Degas the above and add Ammonium persulphate solution 5.0% 50.0 ml

10% SDS	0.1 ml
TEMED	10.0 µl

Procedure

i. The glass plates, spacers and comb were cleaned thoroughly and wiped. The spacers were placed on the edges, in between the plates and clamped with clips lightly.

ii. Molten agar 1.0 % was poured along the sides and was allowed to solidify to seal.

iii. Thirty ml of the separating gel mixture 12.5 % was prepared and poured carefully between the glass plates. A layer of distilled water was added above the gel layer and was allowed to polymerise for 30 minutes.

iv. Stacking gel 4% was prepared and poured on the separating gel after removing the layer of water.

v. The comb was placed in the gel and allowed to set for 30 minutes.

vi. After polymerising, the gel was installed in the electrophoresis apparatus.

vii. The electrode buffer was poured slowly and air bubbles removed.

viii. Equal quantity of protein was loaded in each well, by adjusting the protein content with sample buffer, to have equal protein per unit volume.

ix. Before loading, the sample solutions were heated in boiling water for 5 minutes, to ensure complete reaction between proteins and SDS for effective denaturing.

The electrophoresis was conducted at constant current (30 mA) at room temperature till the blue dye reached the bottom of the gel. The gel was removed immediately after electrophoresis and incubated in the staining solution for atleast three hours. Then the gel was soaked in destaining solution to visualise protein bands.

3.2.4. Protein classification

Depending upon the solubility characteristics, the different protein fractions of the whole endosperm were separated by the method of Sauvaire *et al.* (1984). Four different solvents, distilled water, NaCl (0.86 M), 70 per cent ethanol and 0.05 N NaOH were used successively with 20 ml solvent per gram sample.

The samples were stirred in the respective solvent for 30 min at room temperature with a magnetic stirrer. The extracts were centrifuged at 10,000 g for 20 min and the supernatants filtered through Whatman No.1 filter paper. For each solvent, three extractions were carried out and the supernatants pooled. Nitrogen content of the supernatants was analysed by micro-Kjeldahl method.

3.2.5. Electrophoresis of the fractions of protein

Electrophoresis of the fractionated protein was done as described in section 3.2.3.

		gel %
Albumin - water extracted protein	-	12.5%
Globulin NaCl extracted protein	-	12.5%
Glutelin NaOH extracted protein	-	12.5%
Prolamin Ethanol extracted protein	-	15.0%

For water extractable protein, 250 mg of oven dried fat free endosperm was homogenised in 1.0 ml of distilled water and the protein quantified by Bradford reagent. Equal quantity of protein was loaded in each well and run at 30 mA. For NaCl extractable protein, the NaCl solution containing protein was dialysed against water for 24 hours. Protein was precipitated in the dialysis tube. The precipitate separated by centrifuging and was redissolved in phosphate buffer. Electrophoresis of this fraction also was done as in the previous fractions. Alcohol extracted protein was isolated by homogenising with 70% alcohol (250 mg in one ml of alcohol). The supernatant after centrifugation was boiled with sample buffer for five minutes in boiling water bath. The sample was loaded in 15% gel and electrophoresis was carried out at 50 mA at 4°C. For extraction of glutelin for electrophoresis, the following procedure was used.

i) Two hundred mg flour was suspended (ground) in 50 ml of 0.04 M NaCl It was shaken for 50 min with sufficient vigour to keep it suspended.

ii) The tube was then centrifuged and the supernatant discarded

iii) The pellet was again resuspended in 50 ml of 0.04 M NaCl vortexing and the extraction was repeated.

iv) The residue was again extracted twice with 70% ethanol to remove gliadin (Prolamins).

v) The pellet was then suspended in 20 ml of 0.7% acetic acid and shaken for 30 min and 56 ml of 95% ethanol was added to bring the ethanol concentration 70% and the tube again shaken for 30 min.

vi) The pH was adjusted to 6.8 with 1.0 ml of 2.0 N NaOH and the tube was cooled to 3-4°C (overnight in a refrigerator to reprecipitate glutelin which was previously solubilised.

vii) Then centrifuge and lyophilize the pellet.

viii) Fifty mg of extracted powder was again suspended in 0.4 ml sample buffer (10 mM Tris-HCl and buffer containing -2.5% SDS, 1.0 mM EDTA and 10% Sucrose and 5% mercaptoethanol for reduction).

Electrophoresis of glutelin was done in 12.5% gel at room temperature (30°C)

3.3. Isoenzyme analysis

The variation between the eight varieties of coconut used was also detected by isoenzyme analysis like (1) esterase (2) peroxidase and (3) polyphenol oxidase.

3.3.1. Reagents

i) Extraction buffer

a) Esterase (EST)

Sodium phosphate (pH 9.5)	10.0 mM
EDTA (Disodium salt)	1.0 mM

b) Peroxidase (PRX)

Phosphate buffer	0.1M Na_2HPO_4
(pH 7.0)	0.1M NaH_2PO_4

2-mercaptoethanol

Insoluble poly vinyl pyrolidone pinch

c) Polyphenol oxidase

Potassium phosphate buffer	0.01 M
pH 7	
2-mercaptoethanol	
Insoluble poly vinyl pyrolidone pinch	

ii) Staining solutions for isoenzymes

a. Esterase

Sodium dihydrogen phosphate	1.40 g
Disodium hydrogen phosphate	0.55 g
Fast blue RR salt	0.10 g
Alpha-Naphthyl acetate	0.015 g
Distilled water to	100.00 ml

b. Peroxidase

Benzidine	1.04 g
Hydrogen peroxide (30%)	1.50 ml
Acetic acid	9.00 ml
Distilled water to	100.00 ml

c. Polyphenol oxidase

p-phenylenediamine	0.1 g
Potassium phosphate buffer	pH 7 100.00 ml
Catechol	0.11 g

3.3.2. Procedure

i) Enzyme extraction

For isozyme analysis, crude enzyme extract was prepared from young leaves. About 0.5 g leaf sample was homogenised in 2.0 ml of extraction buffer (specific for each enzyme) in a prechilled pestle and mortar kept in the ice box and was immediately transferred to a prechilled eppendorf tube. The

suspension was centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was used for isozyme analysis.

ii) Gel electrophoresis

Gel electrophoresis was carried out as described for endosperm protein except that wherever required SDS was replaced with distilled water and the samples were not heat denatured before loading. All the isozymes were separated in the 7.0% gels. The electrophoresis was conducted at a constant current of 30 mA at 4°C till the blue dye reached the bottom of the gel.

iii) Isoenzyme staining

The staining procedure was different for each isozyme. Esterase isozymes were detected by incubating the gel for 10-20 minutes at 37°C, preferably in dark, in esterase staining solution prepared as suggested by Smith **et al.** (1970). After the bands had clearly appeared, the enzyme reaction was stopped by soaking the gel in a mixture of methanol, water, acetic acid and ethanol in the ratio 10:20:2:1. The gel was photographed and the position of the bands was drawn schematically.

Peroxidase isozyme was detected by incubating the gel for one to two minutes in a mixture prepared as described before by Reddy and Gasber (1971). When the blue coloured bands were visible in the gel, the enzyme reaction was stopped by soaking the gel in 7.0% acetic acid. The gel was photographed before the blue colour of the band changed to dark. The position of the bands was schematically drawn.

For polyphenol oxidase, the gel was incubated at 30°C for half an hour in the above solution till the lanes of activity appeared. The gel was then photographed.

3.3.3. Nomenclature of isozymes

. The norms of nomenclature described by Vanden Berg et al (1982) for peroxidase were followed for all the enzymes. The standard acronyms for esterase, polyphenol oxidase and peroxidase are EST, PPO and PRX respectively. Corresponding to the relative mobility of the isozymes, they were given a number relative to the dye front, preceded by the standard acronym for the enzyme.

The relative mobility (Rm) of each band was calculated as

$$Rm = \frac{\text{Distance of band from origin}}{\text{Total distance run}}$$

The similarity index (Si) between two samples was calculated according to Nei and Li (1979) formula.

3.4. DNA studies

DNA studies were conducted in six varieties of coconut.

1. West Coast Tall (WCT)
2. East Coast Tall (ECT)
3. Kulasekharam Green Dwarf (KGD)
4. Chowghat Orange Dwarf (COD)
5. Chowghat Orange Dwarf x West Coast Tall (D x T)
6. East Coast Tall x Malayan Green Dwarf (TxD)

Explant used for the study was very tender, white, unopened coconut leaves collected freshly from the crown of each palm.

3.4.1. DNA isolation

DNA extraction from the tender leaves of coconut was done by the following protocol. This was developed by Uta Pilch and Indo Schubert (1993) for plants with high content of polyphenols. It is a modified version of method by Dellaporta **et al.**(1983).

Materials

1. Extraction buffer (pH 8.0)

Tris-HCl	100 mM
EDTA	50 mM
NaCl	500 mM

   150 µl mercaptoethanol added immediately before use.

2. PVP - Poly Vinyl Pyrolidone - 20%
3. Solid SDS.
4. Potassium acetate/Ammonium acetate - 5.0 M
5. Sodium acetate - 3.0 M (pH 5.4)
6. TE buffer (pH 8.0)

Tris-HCl	10.0 mM
EDTA	1.0 mM

All the solutions used for DNA isolation were sterilised by autoclaving at 15 lb for 20 minutes. Centrifuge tubes, eppendorfs, mortar and pestle, scoop, scissors gloves all were autoclaved each time before use.

Procedure

a) 1.0 g leaf frozen in liquid nitrogen was powdered with porcelain mortar and pestle.

b) The powder was transferred into 50 ml centrifuge tubes with 15 ml extraction buffer .

c) Mixture was thawed and kept on ice.

d) 4.5 ml of ice cold 20% stock solution of PVP (stored at -20°C) was added.

e) The lysate was kept on ice after thawing.

f) Solid SDS (0.4g) was added.

g) Extract was mixed slightly (crushed solid SDS) with the tip of a Pasteur pipette for easy dissolving.

h) Incubated on a water bath at 65°C for 10 min.

i) 5.0 M potassium acetate/ammonium acetate-1.5 ml was added followed by 30 min incubation on ice and centrifugation at 13000xg, 10min at 4°C.

j) The supernatant was transferred into a new tube, mixed with 1.2 vol isopropanol by inverting the tubes three times.

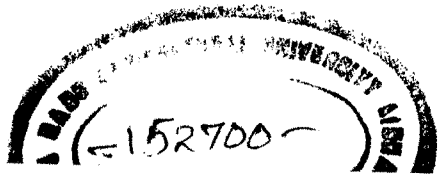
k) Incubated on ice for 10 min.

l) After another centrifugation (13000xg, 10 min at 4°C), the supernatant was discarded completely.

m) The pellet was dried under vacuum, dissolved in 100 µl 1x TE (pH 8.0) and extracted once or twice with 1 vol. phenol-chloroform-isoamyl alcohol (25 : 24 :1) mixture.

n) After centrifugation the aqueous phase was transferred and nucleic acid precipitated by adding 100% ethanol 2 vol + 1 drop of sodium acetate solution and keeping at -20°C overnight. During this time tubes were inverted at least five times.

o) Next day centrifuged and collected the pellet, washed the pellet with 70% ethanol by centrifuging at 13000xg for 10min at 4° C.



p) Dried the pellet under vacuum, dissolved in 50 µl 1x TE (H-8.0).

3.4.2. Estimation of DNA

Spectral characteristics of DNA can be used to assess the quality of DNA preparations. An absorbance of 1.0 at 260 nm is equal to a concentration of 50 µg/ml.

A ratio of 1.0 between the absorbance at 280 nm and 260 nm is an indication of good DNA preparation without impurities. The absorbance of DNA preparations was measured at 280 nm and 260 nm in a Beckman Spectrophotometer Du 64 to assess the quality and quantity of the DNA.

3.4.3. DNA electrophoresis in agarose gel

DNA can be checked for size intactness, homogeneity and purity by this technique.

Materials

i. TBE (Tris-Borate buffer) 1x (pH 8.3)

Tris-HCl	89.0 mM
$Na_2EDTA.H_2O$	2.0 mM
Boric acid	89.0 mM

ii. Agarose 0.8% (w/v) in TBE (1x)

iii. Sample buffer

Sucrose	30.00%
Bromophenol blue	0.25%
Xylene cyanol (FF)	0.25%

(all w/v) in TBE 1x)

iv. Staining solution

Ethidium bromide	1 mg/ml.

v. DNA molecular weight marker

Lambda DNA/Eco RI - Hind III double digest (Bangalore Genei)

vi. Submarine gel apparatus with running tank

vii. Power supply unit

Electrophoretic separation of genomic DNA was done to check its quality (Maniatis **et al.**, 1989).

I. The running tray was cleaned and both the ends were closed with cellophene tape.

II. Required amount of 1.0% agarose was melted and cooled to 50°C.

III. Both TBE (1x) and ethidium bromide were added to final concentration of 1.0% and 1.0 mg/ml respectively.

IV. The gel solution was poured into the running tray and appropriate size comb was set in place.

V. The gel was allowed to solidify for 30 min.

VI. When the gel was ready, the cellophene tape and the comb were removed carefully.

VII. The gel along with the running tray was placed on the platform of submarine gel apparatus, the well side facing towards cathode.

VIII. TBE (0.5x) was poured in both the buffer chambers, so that the gel was submerged and about 1.0 mm buffer covered the gel.

IX. DNA samples were mixed with 0.1 volume of sample buffer (10x) and loaded in the wells with the help of a micropipette. The molecular marker after warming was loaded in a separate well.

X. The apparatus was connected to the power supply unit and power applied at 5 v/cm.

XI. DNA bands were visualized by UV illumination and photographed.

3.4.4. Restriction digestion of genomic DNA

Materials

Genomic DNA

Restriction endonuclease (EcoRI, EcoRV, Hae III, Sau III, Hind - III, Bam HI and Hinf-1)

Restriction buffer (10x) (supplied with the enzyme)

Nuclease free water

Water bath 37°C.

Protocol

i. DNA samples of the two in each of tall varieties, dwarf varieties and two hybrids were digested with each of the enzymes for hybridization.

ii. The DNAs and the reagents were taken out of the freezer and thawed on ice.

iii. In sterile tubes the reagents were mixed as follows.

DNA (1mg/ml)	10 µl
Restriction buffer (10x)	2 µl
Restriction endonuclease	20 units
Nuclease free water	20 µl

iv. The reaction components were collected with momentary spinning in a microfuge at 4°C.

v. The components were mixed by gently tapping the bottom of the microfuge tube and again gathered at the bottom of the tube by gentle spinning.

vi. The tubes were sealed with parafilm and incubated at 37°C in a waterbath overnight.

vii. The reaction was stopped by addition of EDTA (5.0M) and fractionated on 1.0% agarose gel.

3.4.5. Southern blotting

Materials

i. Denaturation solution

NaCl	1.5 M
NaOH	0.5 M

ii. Neutralization solution (pH 7.0)

Tris	0.5 M
NaCl	3.0 M

iii. 20 x SSC (pH 7.0)

NaCl	3.0 M
Sodium citrate	0.3 M (filter before use)

iv. Gene screen plus membrane

Protocol

The digested DNA of the varieties and hybrids with each of the seven enzymes were fractionated in 1.0% agarose gel and transferred to gene screen plus membrane by semidry blotting. Simplified Southern blot protocol (Schuler and Zielinski, 1989) was followed.

i. The gel was carefully removed from running tray and the denaturation solution added to immerse the gel and gently shook for 15 min.

ii. The denaturation solution was decanted and 1.0 M ammonium acetate added and kept at a gentle speed on a rotary shaker.

iii. Genescreen membrane was cut exactly to the size of the gel and a slant cut given at left corner for orientation and immersed in 1.0 M ammonium acetate.

iv. The gel was taken after 30 min from 1.0 M ammonium acetate and gently placed on glass plate. The concave side of the membrane was placed on the gel and rolled with a glass rod to remove the air bubbles.

v. Five sheets of Whatman 3 mm filter paper were placed over the membrane and several layers of country filter paper placed above. A weight of 250-500 g was placed over this and left for 14-16 h.

vi. The completion of transfer was checked by viewing the gel on UV transilluminator.

vii. The blots were baked between two 3 mm filter papers at 80°C under vacuum for 3 h, to fix the DNA and this filter was used for Southern hybridization.

3.5. Probe preparation

3.5.1. Transformation in *E. coli* cells

Materials

i. LB medium (with 1.5% agar)

ii. **E. coli** strain DH5 α cells)

iii. Calcium chloride 0.1 M (stock)

iv. Magnesium chloride 1.0 M (stock)

v. Ampicillin 25 µg/ml (stock)

vi. Sterile petriplates

vii. Oil palm clones CL 54 and CL 95 in pUC 13 vector obtained from Dr. Sean Mayer, PBI, Cambridge, UK.

Protocol

The recombinant plasmids received from Dr. Sean Mayer were introduced into competent cells of **E. coli** (DH5α) by transformation (Maniatis **et al.**, 1982).

Details of the oil palm genomic clones

Probe	Vector	Excision enzyme	Selectable marker	Insert size
CL 54	pUC13	EcoR1	Ampicillin	1.5 kbp
CL95	pUC13	EcoR1	Ampicillin	1.9 kbp

i. A single colony of DH5 α was picked by sterile tooth pick and was grown overnight in LB broth (5.0 ml) incubated at 37°C at 200 rpm without ampicillin.

ii. This 5.0 ml culture (in 10 g phase) was transferred into 100 ml LB broth under sterile conditions, and grown at 37°C, 200 rpm for 3 h. till the OD value reached 0.5-0.7 at 550 nm.

iii. The cells were harvested by centrifuging at 5,000 rpm for 5 min at 4°C in a refrigerated centrifuge and the supernatant was discarded.

iv. The cell pellet was resuspended in 10 ml of 0.1 M calcium chloride (ice cold) and again centrifuged at 5,000 rpm for 5 min at 4°C.

v. The supernatant was drained off, and the pellet again suspended in 2.0 ml of 0.1 M calcium chloride and incubated on ice for 20 min after which the cells were pelletted by centrifugation (5,000 rpm, 5 min, at 4°C).

vi. These cells were dissolved in 0.8 ml of 0.1 M calcium chloride (final volume 1.0 ml) to get competent cells. These cells were used for transformation.

vii. 2.0 ml of competent cells were taken in sterile eppendorf tubes and the DNA received was dissolved in 10 ml sterile water. 2.0 ml of the resulting materials were used for transformation.

viii. The DNA and the competent cells were mixed gently and incubated in ice for one hour. The cells were given heat shock at 42°C for 2 min. Immediately 900 ml of LB broth was added and incubated at 37°C at 100 rpm for one h.

ix. 10 ml of the above cells were plated on LB medium containing 50 mg/ml ampicillin and incubated at 37°C overnight.

x. The transformants were selected on ampicillin medium. The white colonies which are recombinant plasmids were selected and inoculated into 10 ml. LB medium with ampicillin 50 mg/ml was used for plasmid isolation and for hybridization. Negative control was included without adding the ligation mixture.

3.5.2. Plasmid DNA isolation from the clones

Materials

1. LB broth (pH 7.0)

Yeast extract	5.0 g
Tryptone	10.0 g
Sodium chloride	10.0 g
Distilled water to	1.0 l
Ampicillin	50.0 mg/ml.

ii. Solution 1

Glucose	50.0 mM
EDTA	10.0 mM
Tris-HCl	25.0 mM (pH 8.0)

(to which lysozyme is added freshly at the rate of 2.0 µg/ml)

iii. Solution 2

Sodium hydroxide	0.2 M (or)
SDS	1.0%

Prepared freshly in sterile water

iv. Solution 3

Potassium acetate	3.0 M (pH 4.8)

v. Absolute ethanol (kept in -70°C)

vi. 70% ethanol

vii. TE (pH 8.0)

Tris-HCl	10.0 mM
EDTA	1.0 mM

Protocol

Plasmid DNA was isolated from the clone by alkali lysis procedure of Maniatis.C(1982).

i. Inoculated a single tooth picked bacterial colony into a 3.0 ml LB broth with ampicillin 50 µg/ml and incubated at 37°C with shaking at 200 rpm overnight.

ii. Decanted 1.5 ml of overnight culture into an eppendorf tube and spun at 13000 rpm for one minute in a benchtop centrifuge.

iii. Removed the supernatant completely from the bacterial pellet.

iv. The pellet was carefully suspended in 200 ml of solution 1 and then 400 ml of freshly prepared solution 2 was added, mixed thoroughly by inversion and kept on ice for five minutes.

v. Then 300 ml of solution 3 was added, the tube was inverted several times and kept on ice for 25 min.

vi. Then it was centrifuged at 13,000 rpm for 5 min and the supernatant was removed and transferred carefully to a clean 1.5 ml eppendorf and one volume of absolute ethanol was added, mixed thoroughly and placed in -70°C for 2 h.

vii. After 2 h the eppendorf were centrifuged at 13,000 rpm for 20 min and pellet formed was rinsed with 70% ethanol, dried thoroughly and dissolved in 40 ml TE buffer.

3.5.3. Agarose gel electrophoresis of plasmid DNA

The isolated plasmid DNA was fractionated in an agarose gel electrophoresis as detailed in section 3.4.3.

3.5.4. Restriction digestion of plasmid DNA

Materials

Plasmid DNA (isolated as given in the section 3.3.2.).

Restriction endonuclease Eco RI (Bangalore Genei)

Restriction buffer (supplied with the enzyme).

Nuclease free water

Water bath 37°C

Protocol

The plasmid DNA was digested with Eco RI to separate the vector DNA from the insert as detailed in section 3.7.

The digest was kept overnight and the reaction was stopped by adding EDTA (5.0 M). The digested sample was electrophoresed in agarose (0.8%). The insert was electroeluted as follows.

3.5.5 Electro-elution of insert DNA from the agarose gel

Materials

I. Tris saturated phenol (pH 8.0)

II. Absolute ethanol (Kept in -70° C)

III. TE buffer

IV. Chloroform : isoamyl alcohol (24:1)

V. Ethanol

VI. Sterile syringe

Protocol

DNA fragment corresponding to 1.5 Kb and 1.9 Kb on agarose gel was cut and eluted from the gel by slightly modifying the procedure of Maniatis **et al**. (1982).

i. The agarose gel along with DNA band corresponding to 1.2 Kb (approximately) was excised with a sterile scalpel blade by viewing over an UV transilluminator.

ii. The excised band was made into small pieces, and transferred to 5 ml sterile syringe, and 500 ml of TE buffer was added.

iii. The syringe was pressed and the crushed gel pieces were pierced out of the nozzle into a sterile tube.

iv. To this, 500 ml of phenol (Tris saturated) was added and mixed thoroughly by vortexing, and placed in -70° C overnight.

v. The tubes were centrifuged in a microfuge at 10,000 rpm for 5min.

vi. The supernatant was transferred into a sterile tube and equal volume of chloroform: isoamyl alcohol added, mixed by inversion and centrifuged at 10,000 rpm for 5 min.

vii. The supernatant was added with 2 volumes of absolute ethanol and placed in -70°C for 3-4 hrs.

viii. The tubes were centrifuged at 15,000 rpm for 20 min. and the pellet formed was rinsed with 70% ethanol, dried in vacuum and dissolved in 20 ml TE buffer.

iv. The insert DNA was checked on 1.5% agarose gel.

3.5.6. Radiolabelling the probe

Nick translation

Materials

i. Nick translation kit (BRIT, India)

ii. 32_{p} labelled dCTP (BRIT, India)

iii. Eluted genomic DNA insert

iv. Boiling water bath

v. STE buffer

Tris-HCl	10.0 mM
EDTA	1.0 mM
NaCl	0.1 M

Protocol

The probe DNAs were restriction digested with Eco R1 as described earlier, fractionated on 1.5% agarose gel and used against the restricted genomic DNA of the six varieties of coconut.

1. The probe DNAs were labelled using Nick translation kit supplied by BRIT, India following the manufacturers protocol. Accordingly, recipe was prepared in tubes placed on ice bath by adding in the following order.

i.	DNA solution	30 µl
ii.	dNTPs - 6.4 µl each of (dATP, dGTP, and dTTP)	20 µl
iii.	Enzyme mix (dNase and polymerase)	10 µl
iv.	Labelled dCTP (P) 3000ci/m mol	5 µl
v.	Distilled water to (mixed the contents several times)	100 µl

2. Incubated at 15°C for 1 1/2 hrs.
3. The reaction was stopped by adding 5.0 ml of 0.5M EDTA.
4. The probe was purified using Sephadex G 50 spun columns.
5. Plugged a 1.0 ml syringe with small piece of siliconized glass wool.
6. Equilibrated Sephadex G 50 with STE buffer.
7. Filled the syringe with equilibrated Sephadex and placed in a 10 ml conical tube in which decapped 1.5 ml conical polypropylene reaction tube was inserted, centrifuged at 2,000 rpm for 3 min.
8. Added 0.1 ml of STE to the top of the column and centrifuged at 2,000 rpm for 3 min so as to obtain 0.1 ml of fraction.
9. Applied DNA sample to the column in a total volume of 0.2 ml (STE was used to make up the volume) and centrifuged as before and collect the purified DNA in fresh 1.5 ml tube.
10. About 1.0 ml of probe DNA eluted from the column was taken and 1.0 ml of scintillation fluid added and the cpm counted in liquid scintillation counter. The probe DNA was denatured by boiling for 5 min before adding to the prehybridization buffer.

3.6. Southern hybridization

Materials

1. Denhardt's solution (50 x)

Ficol	1.0 %
Polyvinyl pyrolidone	1.0 %
Acylated BSA	1.0 %
EDTA	1.25 mM

2. Prehybridization solution

SSC	6.0 %
SDS	0.5 %
Denhart's solution	5.0 %
Denatured Salmon sperm DNA	10.0 ng/ml
EDTA	10.0 mM

3. Hybridization solution

Labelled denatured DNA probe in prehybridization solution.

4. Washing buffer

SSC	2.0 %
SDS	0.2 %

Protocol

I. The fixed blots were floated on 6x SSC and immersed for 2 min.

II. Ten ml of prewarmed (42°C) prehybridization solution was poured in the bottle and transferred to the hybridization chamber.

III. The blots were allowed to incubate for 4 hrs in prehybridization solution at 42°C.

IV. The prehybridization solution was removed and the probe (denatured) was introduced in 10 ml hybridization solution into the tube taking care not to pour the solution directly on the blot.

V. The blots were hybridized for 16 h.

VI. The blots were removed from the chamber carefully and incubated in washing buffer at room temperature for 15 min.

VII. The blots were given stringency wash as required.

VIII. Blots were covered with saranwrap and x-ray film (Kodak XAR2) was placed over it with an intensifying screen, inside a X-ray cassette and kept at -70°C for three days.

IX. The autoradiogram was developed using premium X-ray developer (Allied Photochemicals, India) and fixed according to manufacturer's instruction, washed and drip dried.

3.7. Random Amplified Polymorphic DNA analysis (RAPD)

Procedure suggested by Shah *et al.* (1994) was followed with relevant modification.

3.7.1. Purification of DNA for amplification

The genomic DNA extracted from the six varieties of coconut was purified by passing through sephadex G-50 column.

Materials

1.	Sephadex G-50	10.0 g
2.	STE Tris-HCl pH 8.0	10.0 mM
	EDTA	1.0 Mm
	NaCl	100.0 mM

3. Spun column

Preparation of spun column

* 100 ml of STE added to 10 g sephadex G50 and kept for one to two hours and autoclaved at 15 psi for 15 - 20 min.

* The bottom of the syringe plugged with sterile glass wool.
* Sephadex G 50 equilibrated in STE was packed into the syringe till the column volume was 0.9 ml.
* Syringe placed inside a microfuge tube whose lid was cut.
* The microfuge tube with the syringe was placed inside 15 ml centrifuge tube.
* It was centrifuged for 5 minutes at 4000 rpm. This was done till the column was packed.
* 100ml of STE was added to the column and centrifuged as above.
* The amount of fluid collected in the microfuge tube was measured.
* This process continued till the fluid collected was correctly 100 ml.

Method

50 µl of the DNA sample was passed through the spun column and collected in the eppendorf at the bottom by centrifuging at 1600xg for four minutes. Purified DNA was quantified by taking the spectrophotometer reading at 260 nm.

Cocktail for the amplification

Taq DNA polymerase and DNTPs were procured from Bangalore Genei, Bangalore. The random primers used were from the Operon Technologies (USA). OPC series primers 1, 2, 4, 6, 7,8, 10, 11,14 and 20 were used. After a number of trials, the cocktail for amplification was standardised as follows:

Sample DNA	90.0 ng
10x Taq Buffer	2.5 µl
Primer	2.0 µl
DNTP (0.2 mM) each	2.0 µl

Taq DNA polymerase	1.0 µl
Sterile water	11.5 µl
TOTAL	20.0 µl

Procedure

i. In 0.5 ml autoclaved microfuge tube kept on ice, all the reaction components were mixed as indicated above and centrifuged for five seconds. To prevent evaporation, 40 µl of mineral oil was added into each tube.

ii. The tubes were placed in thermocycler and the amplification was programmed as follows:

	Temperature	Time (min)
Step-1	94°C	5
Step-2	94°C	1
Step-3	37°C	1
Step-4	72°C	2
Step-5	72°C	7
Step-6	Hold at 15°C	1.5
Step-7	Storage 4°C	overnight

Step 2-4 repeated 45 cycles.

iii. When the amplification was over, the tubes were taken from the thermocycler. After centrifuging, 10 µl of the reaction volume was taken into a fresh eppendorf tube to which 10x tracking dye was added. The amplified DNA was run on agarose gel (1.4%) as described in the section 3.4.3.). The gel was stained with ethidium bromide solution and the amplification products observed under UV transilluminator and photographed.

DATA analysis for RAPD

The RAPDs separated electrophoretically on 1.4% agarose gel were schematically drawn and were sequentially numbered as 1,2, 3 and so on. Pairwise comparison of genotypes based on the presence or absence of unique and shared polymorphic products was done. The fraction of bands (F) in common between two genotypes was estimated using the formula of Nei and Li (1979).

$$F = \frac{2\,Mxy}{Mx + My}$$

Where,

Mxy - number of bands shared by the genotypes.

Mx and My - number of bands in each genotype.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

In the present investigation in coconut (**Cocos nucifera** L.), selected varieties and hybrids were subjected to biochemical and molecular studies like protein electrophoresis, isozymes, RAPD and RFLP. As a basic step, the morphological characters which distinguish the varieties of coconut were also examined and the data analysed statistically. The results of the above study are presented in this chapter.

4.1. Morphological markers

Twenty morphological traits were studied for thirty genotypes. Analysis of variance, mean, standard error, critical difference and coefficient of variation were worked out for each variable. Correlation between the variables was also worked out. The genotypes were grouped into six clusters following a non-heirarchial euclidean cluster analysis. Analysis of variance showed significant F value for all the variables suggesting significant variation among genotypes for these parameters. F values ranged from 28.26 to 1893.84 for the different traits. Mean, SE and coefficient of variation for the twenty variables are given in Table 2. Coefficient of variation for the twenty variables compared in the histogram (Fig.1).

1. Plant height : Pl.ht (m)

Plant height showed a high coefficient of variation (4.53%). Range for this character was from 13.00 m (WCT) to 4.21 m (COD). Among the tall varieties, (S.No. 1 to 17) (Table 1) the exotic tall varieties were shorter than the local varieties, the range among the talls was from 13.00 m (WCT) to 5.61

Table 2. Mean values for morphological traits in coconut genotypes

	Varieties	Pl.ht (m)	C-150 (cm)	C-20 (cm)	LS	PL (cm)	RL (cm)	LA (m^2)	IPL (cm)	IAL (cm)	ISL (cm)	INS	IPFD (cm)	NF	AL (cm)	MNB	NNB	FW (kg)	FPE (cm)	FPP (cm)	NPY
1.	JT	8.18	95.17	167.00	14.67	139.33	406.50	8.37	62.67	62.33	42.33	60.33	11.17	39.33	0.80	13.33	11.67	1.76	61.67	51.33	76.67
2.	SP	7.17	97.33	202.00	17.67	136.50	411.50	5.99	40.67	43.17	45.83	39.33	7.33	17.33	0.57	9.00	8.33	4.90	77.33	75.17	50.67
3.	Bor	7.25	92.00	142.33	15.00	129.00	328.00	5.97	48.33	37.83	42.33	33.00	5.83	9.33	0.59	11.33	7.33	2.03	55.33	49.83	56.33
4.	KT	8.42	94.67	162.00	11.33	125.67	393.33	4.97	40.35	36.52	22.00	34.67	6.33	6.33	0.73	9.67	5.67	1.08	53.17	41.67	44.67
5.	ZT	5.71	80.33	87.83	15.33	130.17	363.50	5.05	42.17	36.60	33.67	27.33	6.67	6.33	0.52	9.67	5.33	0.64	42.83	37.00	59.83
6.	CCT	9.40	103.67	201.00	11.33	126.00	390.17	6.88	52.67	45.17	33.50	39.67	11.50	14.67	0.69	15.67	5.67	1.55	61.17	54.67	65.33
7.	BSI	7.75	92.17	156.33	12.00	127.17	390.83	5.86	35.67	37.83	36.50	33.33	8.17	14.67	0.76	12.33	8.33	1.18	49.67	47.83	38.33
8.	FI	5.85	93.50	133.83	14.33	140.00	364.00	5.71	47.00	36.00	36.50	29.00	7.67	11.33	0.68	15.33	10.67	0.65	43.83	40.50	80.00
9.	AG	6.72	100.67	193.50	16.33	159.17	596.00	7.67	39.83	32.67	41.33	31.00	8.83	10.33	0.60	14.67	8.33	2.38	65.17	59.50	53.28
10.	FMS	7.11	99.33	150.02	17.67	119.17	477.00	6.62	46.50	37.33	41.50	36.00	6.50	18.67	0.64	15.33	11.67	1.48	60.17	52.83	83.94
11.	AO	9.13	98.17	161.00	10.67	137.50	392.33	6.17	62.17	47.67	35.83	41.67	9.00	17.33	0.71	17.33	10.33	1.17	55.67	49.33	94.93
12.	PC	7.07	97.67	143.33	12.67	119.67	354.67	6.04	43.67	37.83	40.83	35.33	8.33	16.00	0.62	13.33	15.33	0.79	48.17	44.33	92.96
13.	LO	8.31	73.00	101.17	18.33	122.00	367.00	5.39	45.83	37.00	45.83	31.67	12.33	22.33	0.70	12.33	19.33	1.34	53.00	43.67	87.95
14.	STY	8.50	72.00	100.33	15.33	98.17	311.17	3.91	27.17	22.67	33.50	31.33	5.67	10.67	0.67	8.67	8.67	0.85	51.33	47.00	135.60
15.	LM	9.87	89.50	151.83	11.67	123.00	554.00	4.78	51.00	38.33	45.50	32.67	14.33	50.00	0.66	10.00	41.67	0.40	37.00	34.52	292.78
16.	WCT	13.00	87.83	172.33	8.33	116.00	361.00	5.76	51.17	39.00	46.50	35.67	11.67	21.00	0.82	14.33	18.67	1.98	59.00	50.17	112.33
17.	ECT	8.79	89.17	135.33	12.33	110.67	354.67	6.60	51.50	37.50	34.33	37.33	11.17	20.67	0.69	12.67	13.67	1.32	62.33	51.33	83.11
18.	Sy	5.61	91.50	177.17	20.67	117.17	412.17	7.33	40.67	40.67	50.38	35.3	7.67	14.67	0.70	9.67	7.33	1.11	63.33	41.33	40.78
19.	GO	12.61	102.33	191.33	10.67	162.83	403.17	7.75	55.50	53.50	40.50	52.00	10.67	21.67	0.85	13.33	17.67	2.01	67.17	52.33	101.40
20.	COD	4.21	64.17	86.83	18.67	102.07	309.83	3.84	33.00	29.50	41.17	25.33	6.67	11.33	0.46	10.33	10.33	0.57	44.67	39.50	67.78
21.	KYD	4.83	66.00	70.50	19.00	103.50	304.33	2.99	26.00	28.00	27.33	31.33	5.33	8.00	0.57	12.67	11.33	0.86	51.83	42.50	73.61
22.	KGD	4.16	64.00	72.33	23.33	118.50	311.67	5.19	35.50	24.17	33.83	33.67	5.83	20.00	0.55	11.67	16.67	0.74	46.00	40.67	72.17
23.	MYD	4.52	61.83	67.00	19.67	102.17	276.33	3.38	33.33	25.67	30.17	30.67	5.33	8.67	0.47	12.67	7.67	0.63	48.50	41.17	86.61
24.	WCTxGB	11.46	92.83	195.50	10.67	122.33	389.33	7.65	54.73	40.50	45.50	38.33	12.67	18.33	0.75	14.67	15.33	2.31	66.50	57.67	108.05
25.	STYxJT	7.85	87.50	117.33	12.67	133.33	315.50	7.10	37.83	44.33	55.00	35.00	11.00	17.67	0.69	14.33	16.33	0.92	56.83	41.83	132.94

contd.....

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
26.	CODxWCT	4.20	70.67	97.83	22.33	134.17	322.83	5.36	51.00	30.17	41.00	29.00	7.00	11.33	0.62	10.67	9.67	0.82	54.00	42.83	108.44
27.	WCTxCCT	7.37	92.33	146.33	13.67	138.83	385.17	6.41	73.67	41.17	46.00	37.33	11.67	16.33	0.63	14.33	23.67	1.50	61.50	51.17	105.78
28.	WCTxKGD	8.11	78.17	131.83	23.00	122.83	343.33	6.81	45.50	42.83	38.83	43.00	9.17	38.00	0.80	14.67	24.67	1.05	60.17	57.17	145.28
29.	ECTxMGD	7.18	81.33	131.17	20.00	136.33	413.33	5.19	55.17	35.33	38.67	36.33	8.07	19.33	0.65	11.33	15.67	1.51	57.67	47.50	106.00
30.	NCD	4.51	91.50	115.17	20.00	162.00	395.67	7.99	58.83	47.17	51.67	40.67	8.23	12.67	0.61	9.67	8.33	1.4	55.50	47.17	132.61
TM		7.39	86.34	134.71	15.64	127.17	379.94	5.94	46.30	38.28	39.96	35.91	8.74	17.47	0.66	12.50	13.17	1.36	55.68	47.88	93.00
SE		0.19	0.74	1.18	0.42	0.74	1.55	0.117	0.89	1.06	0.67	0.60	0.32	0.94	0.01	0.43	0.51	0.06	1.014	1.08	3.70
CD		0.54	2.08	3.34	1.20	2.12	4.44	2.12	2.54	3.00	1.88	1.72	0.92	2.64	0.03	1.22	1.46	0.170	2.86	3.06	10.48
CV		4.53	1.48	1.48	4.70	1.02	0.762	3.42	3.38	4.80	2.90	2.93	6.54	9.25	3.32	5.98	6.82	7.63	3.15	3.93	6.89

TM - Treatment mean
SE Standard error of mean
CD - Critical difference
CV Coefficient of variation
F - All characters showed significant difference and F values ranged from 28.26 to 1893.84

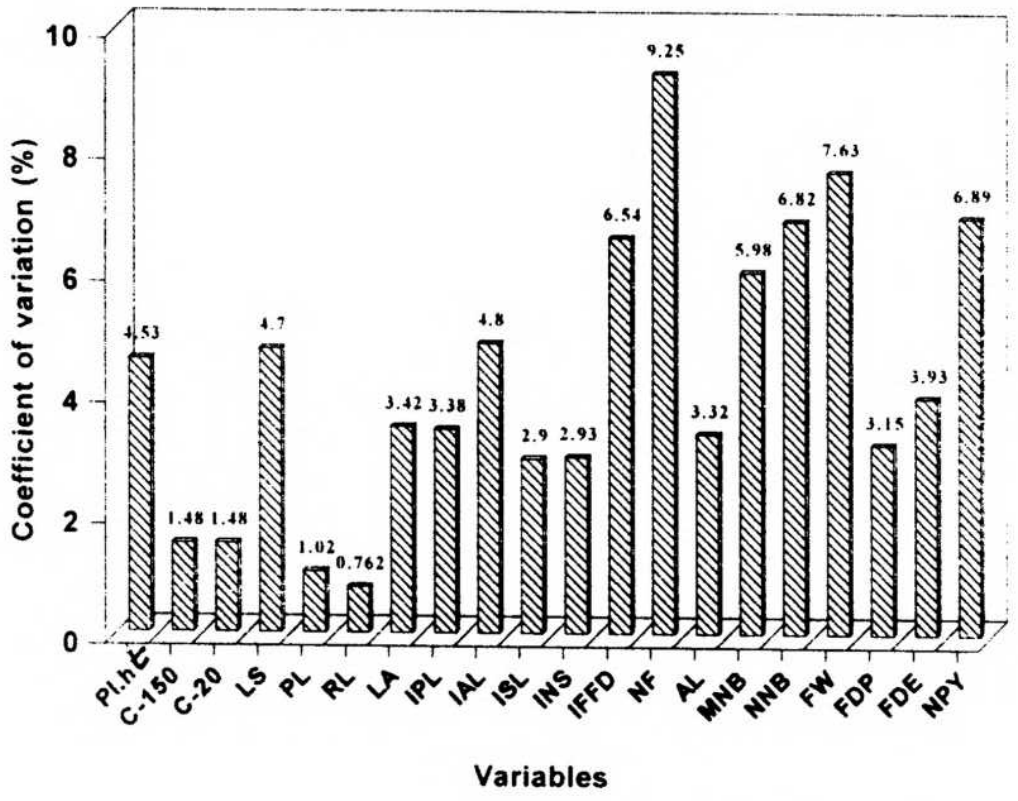
JT Jamaica Tall
ZT Zanzibar Tall
AG Andaman Giant
LO Laccadive Ordinary
ECT East Coast Tall
KYD Kulasekharam Yellow Dwarf
SR San Ramon
CCT Cochin China
FMS Federated Malay States
LM Laccadive Micro
SY Seychelles
KGD Kulasekharam Green Dwarf
Bor Borneo
BSI British Solomon Islands
AO Andaman Ordinary
STY Semi-Tall Yellow (STY)16
GO Gonthembli
MYD Malayan Yellow Dwarf
KT Kenya Tall
FT Fiji Tall
PO Philippines Ordinary
WCT West Coast Tall
COT Chowghat Orange Dwarf

WCT x GB West Coast Tall x Gangabondham
COD x WCT Chowghat Orange Dwarf x West Coast Tall
WCT x KGD West Coast Tall x Kulasekharam Green Dwarf
NCD Natural Cross Dwarf
STY x JT Semi-Tall Yellow x Jamaica
WCT x CCT West Coast Tall x Cochin China
ECT x MGD East Coast Tall x Malayan Green Dwarf

Pl. ht. Plant height from the ground level to the crown
C-20 Stem circumference 20 cm from the ground level
PL Length of leaf petiole
LA Theoretical leaf area
IAL Inflorescence axial length
INS Number of spikelets
C-150 Stem circumference 150 cm from the ground level
LS Numberof leaf scars between oneandtwo metres from the ground level
RL Length of rachis
IPL Inflorescence Peduncle Length
ISL Inflorescence spikelet length
IFFD Distance from the point of spikelet insertion on the axis to the first female flower on the spikelet

NF Number of female flowers
FW Mean fruit weight
AL Anther length
FPP Fruitpolarperimeter
MNB Number of bunches
FPE Equatorial perimeter
NNB Number of nuts/bunch
NPY Nuts/palm/year

Fig.1. Coefficient of variation for twenty morphological markers



C-150 Stem circumference 150 cm from the ground level
C-20 Stem circumference 20 cm from the ground level
LA Theoretical leaf area
INS Number of spikelets
NF Number of female flowers
FPP Fruit polar perimeter
IPL Inflorescence Peduncle Length
IFFD Distance from the point of spikelet
AL Anther length
FPE Equatorial perimeter
Pl. ht. Plant height from the ground level to the crown
LS Number of leaf scars between one and two metres from the ground level
PL Length of leaf petiole
RL Length of rachis
IAL Inflorescence axial length
ISL Inflorescence spikelet length
insertion on the axis to the first female flower on the spikelet
MNB Number of bunches
NNB Number of nuts/bunch
NPY Nuts/palm/year
FW Mean fruit weight

m (Seychelles). Among the four dwarf varieties studied the range was narrow from 4.16 m (KGD) to 4.83 (KYD). Hybrids with tall variety as the female parent (varieties 25, 27, 28 and 29) had values around the mean value except for WCT x GB (11.46 m). Hybrids with dwarf variety as the female parent (varieties 26 and 30) showed mean value which was closer to the lower range of the variable.

2. Collar girth at 150 cm height: C-150 (cm)

All the genotypes differed significantly with respect to this character. Range for this variable was from 103.00 cm (CCT) to 61.83 cm (MYD). Coefficient of variation was 1.48%. Similar to the previous character of height, the tall varieties showed a wider range from 103.67 to 73 cm (LO). In the dwarf varieties, the range was very narrow (61.83 to 66.00 cm). The hybrids irrespective of the female parent showed the mean values clustered around the general mean (86.34 cm).

3. Collar girth at 20 cm height: C-20 (cm)

All the varieties were significantly different from each other for this character. Coefficient of variation was 1.48%. Range for this character was from 202.00 cm (SR) to (67.00 cm) (MYD). Here also the tall varieties showed a wider range (202.00 cm to 87.83 cm) and the range for dwarf varieties was narrower (67.00 - 86.8 cm). Hybrids did not show a general trend and the character ranged from 195.5 cm to 97.8 cm.

4. Leaf scars: LS

Number of leaf scars between 1 and 2 m height from the ground level also showed significant difference between the varieties. Range for this character was from 23.33 (KGD) to 8.33 (WCT). The tall varieties showed very

wide range (20.67 to 8.33). Dwarf varieties showed a narrow range (18.67 to 19.67), Hybrids did not show any general trend for the character.

5. Peduncle length: PL

Varieties differed significantly for this character. Mean of this character was 127.17 cm and the range was from 98.17 (STY) to 162.83 cm (GO). The tall varieties showed a range from 98.17 cm to 162.83 cm. Peduncle length in dwarf varieties ranged from 102.17 cm to 118.5 cm. Hybrids did not show any specific pattern of variation and all were around the general mean . Coefficient of variation was only 1.02%.

6. Rachis length: RL (cm)

Rachis length differed significantly between the varieties with a mean of 379.94 cm and ranging from 596.00 cm (AG) to 276.33 cm (MYD). Tall varieties showed a range from 596.00 cm to 328.00 cm which was much higher than dwarf varieties which showed a range from 276.33 to 311.67 cm. This character recorded the lowest coefficient of variation (0.76%) among all the variables studied.

7. Theoretical Leaf area: LA (m^2)

Theoretical leaf area differed significantly between the varieties with a coefficient of variation 3.42%. The mean for this character was 5.94 m^2 and it ranged from 2.99 (KYD) to 8.37 m^2 (JT). Tall varieties ranged from 8.37 to 3.91 m^2 and dwarf varieties from 2.99 to 5.19 m^2. Hybrids exhibited values within the range of tall varieties.

8. Inflorescence peduncle length: IPL (cm)

All the genotypes differed significantly with respect to this character, with a coefficient of variation of 3.38% and mean value of 46.30 cm. The

range for this character is between 73.67 cm (WCT x CCT) to 26.00 cm (KYD). Tall varieties showed a range from 27.17 to 62.67 cm. and dwarf varieties showed a range from 26.00 to 33.00 cm. Hybrids showed the widest range from 37.83 to 73.67 cm.

9. Inflorescence axis length: IAL (cm)

All the genotypes differed significantly with respect to inflorescence axis length. The coefficient of variation for this character was 4.8%. The inflorescence axis length ranged from 62.33 cm (Jamaica tall) to 22.67 cm (STY), with a mean of 38.28 cm. Tall varieties ranged from 62.33 to 22.67 cm and the dwarf varieties ranged from 24.17 to 29.50 cm.

10. Inflorescence spikelet length : ISL (cm)

Inflorescence spikelet length differed significantly between the varieties. The range for this character was from 55.00 cm (STY x JT) to 22.00 cm (KT). The mean for this character was 39.96 cm. Tall varieties showed a wide variation ranging from 22.00 cm to 50.38 cm. Dwarf varieties ranged from 27.33 cm to 41.17 cm. Hybrids did not show a uniform specific variation. The coefficient of variation was 2.90%.

11. Number of spikelets/inflorescence: NSL

Jamaican tall (JT) variety had the maximum number of spikelets/ inflorescence (60.33) and Chowghat Orange Dwarf (COD) variety had the least number of spikelets (25.30) per inflorescence. Tall varieties varied widely for this character (27.33 to 60.33). Dwarf varieties had a range from 25.33 to 33.67. All the hybrids except Chowghat Orange Dwarf x West Coast Tall (COD x WCT) had values above or around the mean value. Coefficient of variation for this character was 2.93%.

12. Distance between the point of spikelet insertion on the axis to the first female flower: IFFD (cm)

All the genotypes differed significantly with respect to this character with a high coefficient of variation of 6.54%. This distance ranged from 14.33 (laccadive micro) to 5.33 cm (MYD) with a mean 8.74 cm. Tall varieties showed a range from 14.33 to 5.67 cm and dwarf varieties had a range from 5.33 to 6.67 cm. Hybrids differed significantly but within the range of tall varieties.

13. Number of female flowers / inflorescence: NF

Laccadive micro variety had the maximum number of female flowers/ inflorescence (50.00). Range for this character was from 6.33 (Bor, KT to 50.00 (LM) with a highest coefficient of variation of 9.25%. Tall varieties showed a range from 6.33 to 50.00. Dwarf varieties ranged from 8.00 to 20.00. Hybrids fell within the range of tall varieties. Laccadive micro showed highly distinct value almost three times that of the mean (17.47).

14. Anther length: AL (cm)

The anther length varied significantly between the varieties with a range from 0.85 cm (Gonthembly) to 0.46 cm (Chowghat Orange Dwarf). This character showed a coefficient of variation of 3.32% with a mean of 0.66 cm. Tall varieties showed a range from 0.85 cm to 0.52 cm. Dwarf varieties showed a lower range for this character also from 0.46 cm to 0.57 cm. Hybrids showed values around the mean value except for the two hybrids, West Coast Tall x Kulasekharam Green Dwarf and West Coast Tall x Gangabondham (0.80 and 0.75 cm) respectively.

15. Mean number of bunches / year: MNB

. Andaman Ordinary variety had the highest mean number of bunches (17.33). The general mean for this variable was 12.50 with a coefficient of variation of 5.98%. All the varieties differed significantly with respect to this character. Tall varieties showed a range from 8.67 to 17.33.

In the dwarf varieties, number of bunches ranged from 10.33 to 12.67. Among the seven hybrids, WCT x GB, STY x JT, WCT x CCT and WCT x KGD had mean number of bunches that were significantly more than the overall mean. Hybrids showed a range from 9.67 to 14.67 with respect to this character.

16. Number of nuts per bunch: NNB

Laccadive micro variety showed the highest number of nuts per bunch (41.67) and the lowest by Zanzibar Tall (5.33). This character showed a comparatively high coefficient of variation (6.82%). Tall varieties, dwarf varieties and their hybrids did not show any distinct pattern of variation. The overall mean number of nuts was 13.17. Laccadive micro showed the highest deviation from the general mean. Number of nuts per bunch for the hybrids WCT x KGD and WCT x CCT were 24.67 and 23.67 respectively. These hybrids had the highest number of nuts per bunch next to Laccadive Micro.

17. Fruit weight: FW (kg)

Fruit weight showed a coefficient of variation (7.63%). The mean fruit weight was 1.36 kg. Highest fruit weight was seen for San Ramon (4.9 kg) and lowest was in Laccadive Micro (0.40 kg). Tall varieties showed a range from 0.40 to 4.90 kg and dwarf varieties from 0.57 to 0.86 kg. Hybrids

showed values around the mean but West Coast Tall x Gangabondham (WCT x GB) showed a high fruit weight (2.31 kg).

18. Fruit equatorial perimeter: FPE (cm)

This character is determined by the shape of the fruit. San Ramon had the highest value (77.33 cm) and it had the biggest fruit among all the varieties. Treatment mean for this character was 55.68 cm and it ranged from 37.00 cm (laccadive Micro) to 77.33 cm (San Ramon). Tall varieties had a wider range (37.00 to 77.33 cm) and dwarf varieties showed within this range but were more close to the lower limit. Hybrid varieties clustered around the mean value. Coefficient of variation for this character was 3.15%.

19. Fruit polar perimeter: FPP (cm)

This character is also determined by the shape of the fruit. So the variety with the biggest fruit, San Ramon had the highest value and it ranged from 34.52 (Laccadive Micro) to 78.17 cm (San Ramon). For all the varieties excepting for San Ramon, the FPE value was higher to FPP value suggesting slightly oblong shape; But for San Ramon, it was somewhat equal (77.33 and 78.17 cm) suggesting a near spherical shape. Range for this character in tall varieties, dwarf and hybrids showed a similar trend as to that of fruit equatorial perimeter.

20. Nuts/palm/year: NY

Average yield of nuts for four consecutive years was calculated. Yield showed a coefficient of variation of 6.89%. Average yield was 93 nuts/palm/year. Highest number of nuts/year was harvested from Laccadive micro (292.78) and lowest yielder was from Seychelles (40.78). Exotic varieties

produced nuts below the overall mean. All the hybrids yielded more than the overall mean. In the dwarf varieties, number of nuts/palms/year ranged from 67.78 to 86.61.

4.1.2. Correlation studies

Correlation between the 20 variables were estimated and the correlation matrix is given in Table 3.

Of all the characters studied, only the IFFD (Distance between the point of spikelet insertion on the axis and insertion point of the first female flower), NF (number of female flowers) and NNB (Number of nuts/bunch) had significant positive correlation with yield (number of nuts/palm/year). Plant height did not have significant positive correlation with any of the characters but had significant negative correlation with number of leaf scars. Stem circumference at 20 cm and 150 cm had significant positive correlation with peduncle length, rachis length, theoretical leaf area, inflorescence peduncle length, inflorescence axis length, number of spikelets, inflorescence axis to insertion point of first female flower, anther length and fruit characters. They also had significant negative correlation with number of leaf scars.

Number of leaf scars had significant negative correlation with anther length and distance from inflorescence axis to the insertion point of first female flower. Peduncle length and rachis length had significant positive correlation with each other and with theoretical leaf area. Peduncle length had significant positive correlation with inflorescence peduncle length and inflorescence axis length. Theoretical leaf area had significant positive correlation with inflorescence peduncle length, inflorescence axis length, spikelet length,

Table 3. Correlation matrix for twenty morphological traits

Varieties	Pl.ht (m)	C-150 (cm)	C-20 (cm)	LS	PL (cm)	RL (cm)	LA (m^2)	IPL (cm)	IAL (cm)	ISL (cm)	INS	IFFD (cm)	NF	AL (cm)	MNB	NNB	FW (kg)	FPE (cm)	FPP (cm)
C-150	0.35																		
C-20	0.35	0.88**																	
LS	-0.44*	-0.60**	-0.55*																
PL	0.07	0.61**	0.45*	-0.11															
RL	0.01	0.63**	0.61**	-0.22	0.49*														
LA(m^2)	0.14	0.72**	0.67**	-0.21	0.67**	0.44*													
IPL	0.20	0.50*	0.44*	-0.30	0.56**	0.30	0.59**												
IAL	0.24	0.70**	0.61**	-0.39	0.59**	0.29	0.77**	0.67**											
ISL	0.12	0.26	0.27	0.00	0.35	0.24	0.55*	0.36	0.42										
INS	0.17	0.50*	0.50*	-0.22	0.43	0.17	0.67**	0.56**	0.84**	0.19									
IFFD	0.17	0.43	0.50*	-0.52*	0.26	0.40	0.51*	0.59**	0.56**	0.49*	0.40								
NF	0.07	0.14	0.23	-0.07	0.07	0.36	0.29	0.36	0.45*	0.33	0.52	0.65**							
AL	0.29	0.51*	0.58**	-0.48*	0.27	0.21	0.55	0.39	0.60	0.16	0.63	0.56	0.46						
MNB	0.11	0.36	0.30	-0.35	0.17	0.10	0.37	0.37	0.31	0.02	0.28	0.35	0.13	0.36					
NNB	0.06	-0.04	0.02	-0.11	-0.01	0.27	0.01	0.29	0 10	0.32	0.12	0.63**	0.79**	0.26	0.11				
FW	0.28	0.49*	0.64**	-0.13	0.37	0.30	0.39*	0.20	0.36	0.29	0.36	0.16	0.00	0.15	0.00	-0.16			
FDE	0.17	0.52**	0.66**	-0.07	0.35	0.22	0.61**	0.32	0.47*	0.30	0.55*	0.23	0.01	0.37	0.21	-0.18	0.81**		
FDP	0.20	0.48*	0.63**	-0.08	0.30	0.26	0.45*	0.22	0.35	0.18	0.43	0.16	0.07	0.23	0.21	-0.14	0.90**	0.85*	
NPY	0.01	-0.08	-0.08	-0.10	-0.00	0.22	-0.05	0.22	0.05	0.30	0.04	0.49*	0.66**	0.16	-0.04	0.82**	-0.28	-0.30	-0.23

* Significant at 5% ** Significant at 1%

C-150 Stem circumference 150 cm from the ground level

C-20 Stem circumference 20 cm from the ground level

LA Theoretical leaf area

INS Number of spikelets

NF Number of female flowers

FPP Fruit polar perimeter

IPL Inflorescence Peduncle Length

IFFD Distance from the point of spikelet insertion on the axis to the first female flower on the spikelet

AL Anther length

FPE Equatorial perimeter

Pl. ht. Plant height from the ground level to the crown

LS Number of leaf scars between one and two metres from the ground level

PL Length of leaf petiole

RL Length of rachis

IAL Inflorescence axial length

ISL Inflorescence spikelet length

MNB Number of bunches

NNB Number of nuts/bunch

NPY Nuts/palm/year

FW Mean fruit weight

number of spikelets, anther length distance from the inflorescence axis to the first female flower and with fruit perimeters.

Number of spikelets had significant positive correlation with fruit perimeter. Number of female flowers and distance from inflorescence axis to the first female flower had significant positive correlation with number of nuts/bunch, fruit weight and fruit equatorial perimeter. Inflorescence peduncle length and axial length had significant positive correlation with each other and also with number of spikelets and the distance from inflorescence axis to the point of insertion of female flower, which inturn had positive correlation with anther length number of female flowers and number of nuts per bunch. Number of female flowers at significant positive correlation with anther length and number of nuts per bunch. Fruit weight had significant positive correlation with fruit perimeters.

4.1.3. Selection indices in coconut

Selection indices in the 30 genotype calculated by the Smith simultaneous selection model are given in table 4. Genotypes are arranged in descending order of the selection rank in the table. San Ramon (SR) showed the highest value followed by Gonthembli (GO). Among the dwarf varieties, Kulasekharam Yellow dwarf occupied the highest rank. Among the hybrids, WCT x GB was found to be superior to all the hybrids.

4.1.4 Cluster analysis

A non heirarchial euclidean cluster analysis was conducted for the data using 'SPAR1' computer programme. Analysis brought out six clusters.

Cluster I : This cluster had 7 members (Zanzibar, Chowghat Orange Dwarf, Kulasekharam Yellow Dwarf, Kulasekharam Green Dwarf, Malayan

Table 4. Selection rank for different varieties in coconut

Rank	Genotype	Index value
1.	San Ramon (SR)	8029.38
2.	Gonthembli (GO)	6285.50
3.	West Coast Tall x Gangabondham (WCT x GB)	5993.63
4.	West Coast Tall (WCT)	5634.69
5.	Andaman Giant (AG)	5624.13
6.	West Coast Tall x Cochin	5453.89
7.	Jamaican Tall (JT)	5218.74
8.	Cochin China (CCT)	4958.96
9.	Borneo (Bor)	4897.67
10.	East Coast Tall x Malayan Green Dwarf (ECT x MGD)	4736.62
11.	Andaman Ordinary (AO)	4631.13
12.	East Coast Tall (ECT)	4542.84
13.	Natural Cross Dwarf (NCD)	4443.39
14.	Chowghat Orange Dwarf x West Coast Tall (WCT)	4383.72
15.	Federated Malayan States (FMS)	4342.51
16.	British Solomon Islands (BSI)	4200.38
17.	Laccadive Ordinary (LO)	4165.17
18.	Semi-Tall Yellow x Jamaican Tall (STY x JT)	4163.68
19.	Tall x Dwarf	4155.36
20.	Laccadive Micro (LM)	4094.49
21.	Philippines Ordinary (PO)	4060.92
22.	Kenya Tall (KT)	3916.60
23.	Semi-Tall Yellow (STY)	3614.24
24.	Fiji Tall (FT)	3546.01
25.	Kulasekharam Yellow Dwarf (KYD)	3518.71
26.	Seychelles (Sy)	3418.51
27.	Kulasekharam Green Dwarf	3203.82
28.	Zanzibar Tall (ZT)	3004.01
29.	Malayan Yellow Dwarf (MYD)	2963.97
30.	Chowghat Orange Dwarf (COD)	2881.13

Yellow Dwarf, Semi Tall Yellow and Chowghat Orange Dwarf x West Coast Tall.

Cluster II : This cluster had 2 members (San Ramon and Andaman Giant).

Cluster III : There were 9 members. They are : Federation Malay States, Philippines, Laccadive Ordinary, East Coast Tall, Seychelles, Semi Tall Yellow x Jamaica, West Coast Tall x Kulasekharam Green Dwarf, East Coast Tall x Malayan Green Dwarf and Natural Cross Dwarf (NCD).

Cluster IV : This cluster had only one member. (Laccadive Micro).

Cluster V : This cluster had four exotic varieties as members. They are : Bourneo, Kenya Tall, British Solomon Islands and Fiji Tall.

Cluster VI : This cluster had 7 members. (Jamaica Tall, Cochin China, Andaman Ordinary, West Coast Tall, Gonthembli, West Coast Tall x Gangabondham and West Coast Tall x Cochin China.

Means of variables and standard deviation in each group are given in Table 5. Average distances of cluster members from cluster centroids and the distances between cluster centroids are given in the Table 6. Cluster diagram is shown in Figure 2.

Average distance of cluster members from the cluster centres did not vary much. Cluster II and Cluster IV were the widest ones with the inter cluster distance 11.09. Cluster IV was far from almost all the clusters. Cluster III and Cluster V were nearer to each other than other clusters.

From the cluster mean values, it is seen that Group IV with Laccadive Micro as the highest number of nuts/palm/year is followed by the Cluster III. For all the fruit characters (FW, FPE and FPP), Group II had the highest

Table 5. Cluster mean and Standard Deviation (SD) for six clusters

Varieties		Pl.ht. (m)	C-150 (cm)	C-20 (cm)	LS	PL (cm)	RL (cm)	LA (m^2)	IPL (cm)	IAL (cm)	ISL (cm)	INS	IFFD (cm)	NF	AL (cm)	MNB	NNB	FW (kg)	FPE (cm)	FPP (cm)	NPY
I	Mean	6.16	68.43	83.24	19.10	115.68	314.24	4.25	35.45	28.11	34.38	29.81	6.07	10.90	0.55	10.90	9.95	0.73	48.45	41.52	86.29
	Sd	1.57	6.41	13.44	3.09	14.83	26.03	0.95	8.71	4.64	5.15	2.80	0.69	4.43	0.08	1.51	3.55	0.11	4.13	3.11	26.85
II	Mean	6.95	99.00	197.75	17.00	147.83	503.75	6.83	40.25	37.92	43.58	35.17	8.33	13.83	0.59	11.83	8.33	3.64	71.25	68.63	51.97
	Sd	0.31	2.36	6.01	0.94	14.03	130.46	1.19	0.59	7.42	3.18	5.89	0.71	4.95	0.02	4.01	0.00	1.77	8.60	13.20	1.85
III	Mean	6.84	86.57	133.61	17.48	127.02	381.48	6.56	47.28	40.00	44.11	36.74	9.16	20.00	0.68	12.59	14.70	1.21	57.46	47.46	100.62
	Sd	1.68	7.93	22.17	3.98	15.30	48.40	0.90	6.76	3.99	6.96	3.32	1.92	7.38	0.06	2.07	5.36	0.254	4.79	5.38	32.55
IV	Mean	9.87	89.50	151.83	11.67	123.00	554.00	4.78	51.00	38.33	45.50	32.67	14.33	50.00	0.66	10.00	41.67	0.40	37.00	34.52	292.78
	Sd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
V	Mean	7.31	93.08	148.62	13.17	130.46	369.04	5.50	42.84	37.05	34.33	32.50	7.00	10.42	0.69	12.17	8.00	1.23	50.50	44.96	54.83
	Sd	1.05	1.25	12.87	1.77	6.51	30.41	0.39	5.92	0.93	8.67	2.44	1.10	3.50	0.07	2.38	2.09	0.57	5.02	4.57	18.36
VI	Mean	10.17	96.05	176.36	11.43	134.69	389.67	7.00	58.94	47.05	41.60	43.57	11.19	21.24	0.75	14.71	14.71	1.75	61.81	52.38	94.95
	Sd	2.20	5.69	20.16	2.11	15.36	14.80	0.95	7.86	8.38	5.19	9.13	1.14	8.35	0.08	1.41	5.99	0.38	4.02	2.88	17.53

Pl.ht. Plant height from the ground level to the crown
C-150 Stem circumference 150 cm from the ground level
C-20 Stem circumference 20 cm from the ground level
LA Theoretical leaf area
IPL Inflorescence Peduncle Length
IAL Inflorescence axial length
ISL Inflorescence spikelet length
INS Number of spikelets
IFFD Distance from the point of spikelet insertion on the axis to the first female flower on the spikelet
NF Number of female flowers
AL Anther length
MNB Number of bunches
NNB Number of nuts/bunch
FPP Fruit polar perimeter
FPE Equatorial perimeter
NPY Nuts/palm/year
FW Mean fruit weight
LS Number of leaf scars between one and two metres from the ground level
PL Length of leaf petiole
RL Length of rachis

Table 6. Average distances within and between clusters

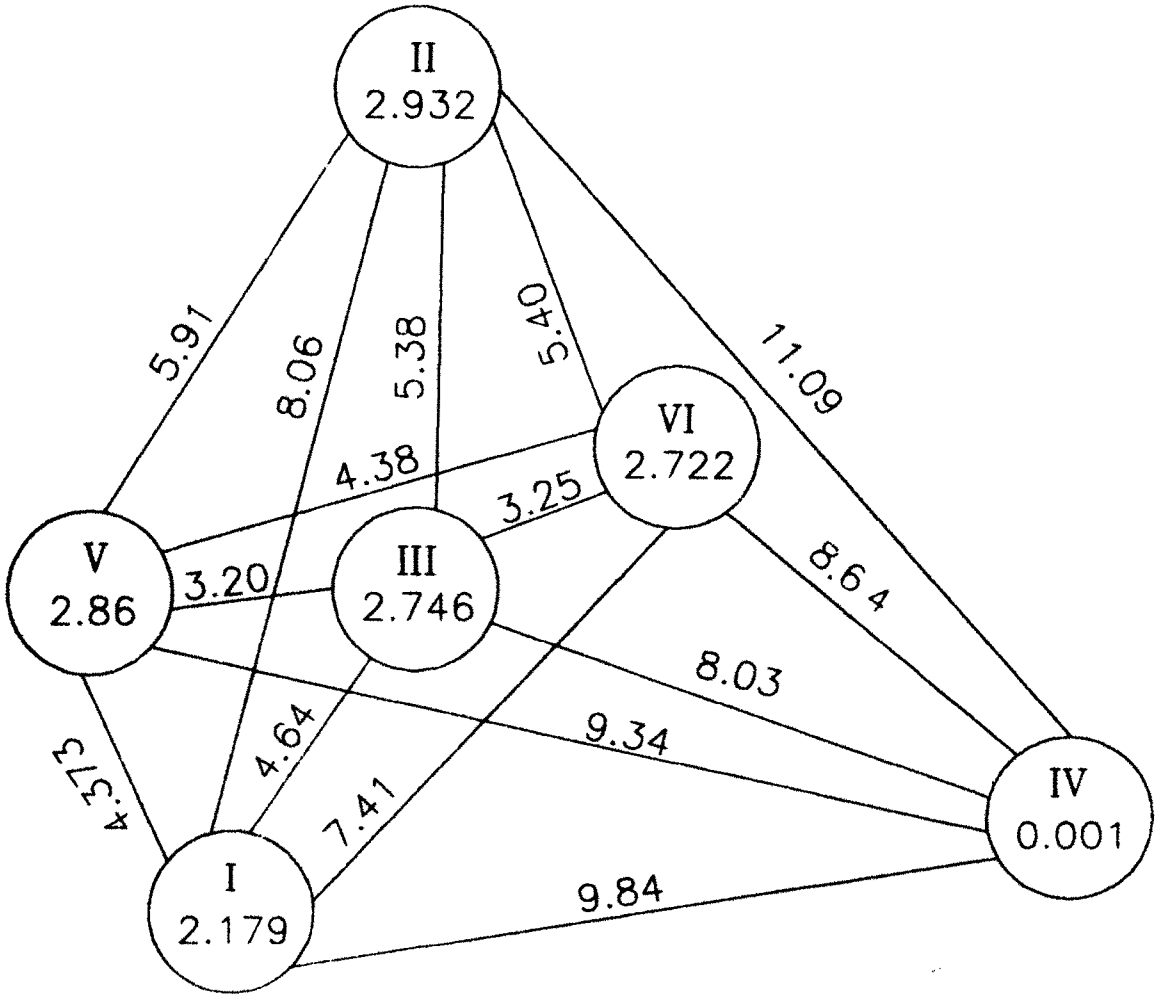
i. Average distance within clusters

Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI
2.179	2.932	3.746	0.001	2.860	2.722

ii. Distances between cluster centroids

	I	II	III	IV	V
II	8.05				
III	4.65	5.38			
IV	9.84	11.09	8.03		
V	4.37	5.91	3.19	9.34	
VI	7.40	5.40	3.25	8.64	4.38

Fig.2 . CLUSTER DIAGRAM



mean value. Mean number of nuts/bunch was highest for Cluster IV followed by Cluster VI. Cluster VI also had the highest mean number of bunches. Number of female flower production also was highest in Cluster IV followed by Cluster VI. Theoretical leaf area also was highest for Cluster VI. Tall palms with cluster mean 7.31 m were grouped in Cluster V. Short palm with a mean height of 6.16 m were grouped in Cluster I. All the dwarf varieties are found in this cluster. Tall palms with a mean height of 10.17 were grouped in Cluster VI. Exotic varieties like Bo rneo , BSI, Kenya and Fiji were included in the cluster V.

4.2. Protein profile analysis

The electrophoretic separation of total soluble protein from the endosperm of eight different coconut varieties is shown in Plate 2. The proteins were resolved on 12.5% gel and the electrophoregram is given in Figure 3.

The total soluble protein in coconut endosperm resolved into 16 bands ranging from 10 to 70 KDa molecular weight. The four major bands (band no 5, 8, 11 and 15) resolved between 14 and 66 KDa molecular weight. These four major bands were found uniformly in all the varieties with high intensity. Minor bands showed difference between the varieties in intensity as well as number. Band No.2 was found with low intensity and was absent in the West Coast Tall variety and Kulasekharam Green Variety. Band No.3 was also seen with low intensity and was absent in the 1st lane (WCT variety). Band No.6 was seen only in the two dwarf varieties (COD and MYD) and the two hybrids (T x D and D x T). Band No.10 was seen in the Jamaican Tall variety, in the two dwarf varieties (COD Orange and MYD) and the two hybrids (lanes 3, 5, 6, 7 and 8). Band No.12 and 14 were also found only in these varieties

Plate 2. SDS-PAGE pattern of total endosperm protein in coconut genotypes

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

M Protein weight marker.

Plate 2

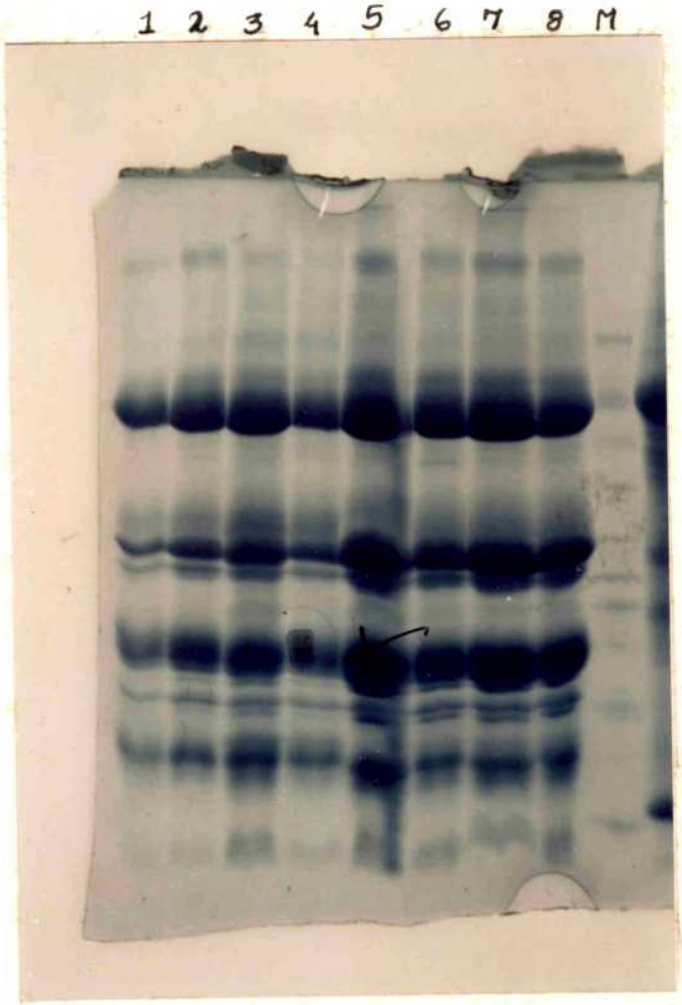


Fig.3. SDS-PAGE pattern of total endosperm storage proteins in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x
West Coast Tall (D X T)

Lane 8. East Coast Tall x
Malayan Green Dwarf (T X D)

M Protein weight marker.

Fig.3. SDS-PAGE PATTERN OF TOTAL ENDOSPERM STORAGE PROTEINS IN COCONUT VARIETIES

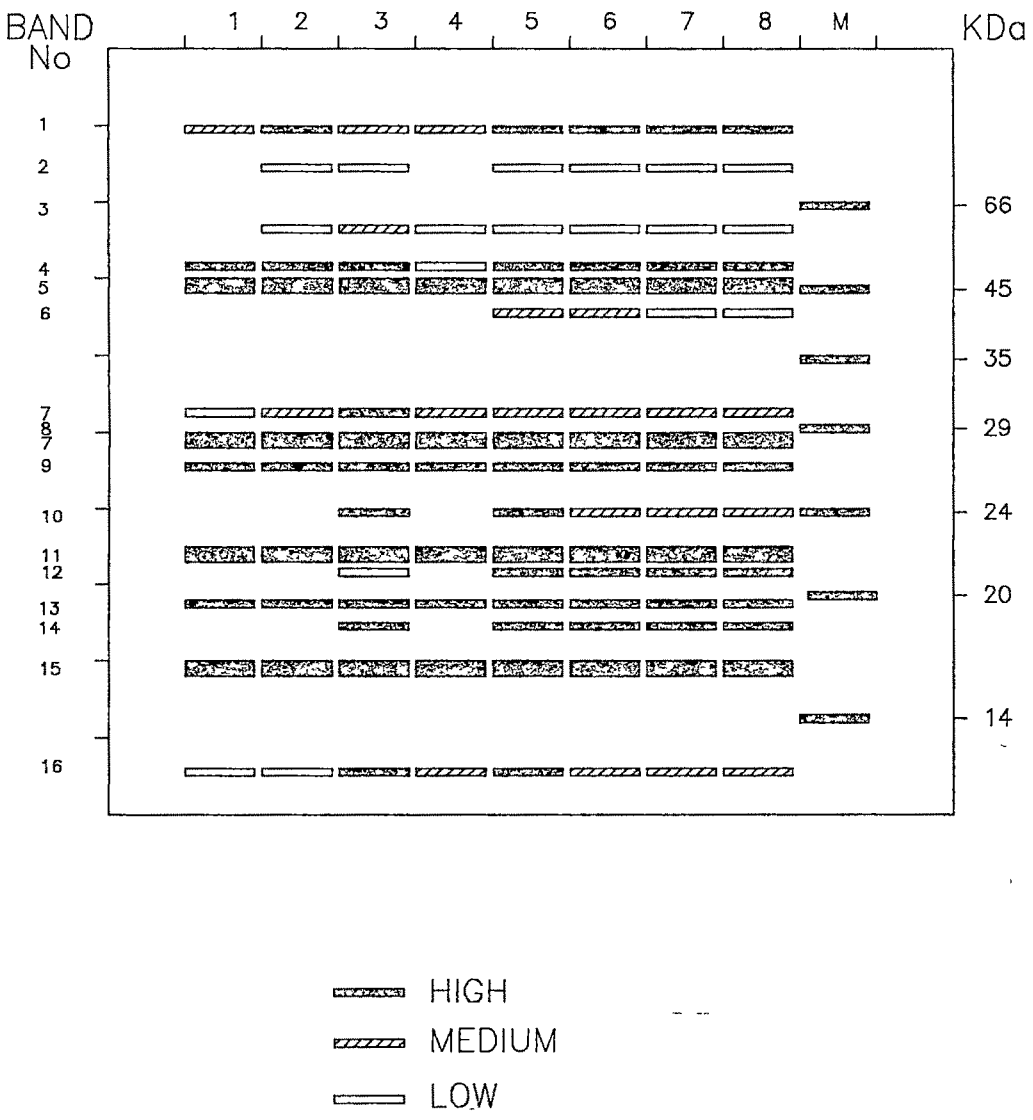


Table 7. Similarity indices for total endosperm protein SDS profile in coconut genotypes

	WCT	ECT	JT	KGD	COD	MYD	DxT
ECT	0 90						
JT	0.80	0 88					
KGD	0.95	0 95	0 84				
COD	0 76	0 85	0 96	0.81			
MYD	0 76	0 85	0 96	0 81	1 00		
D x T	0 76	0 85	0 96	0.81	1 00	1 00	
T x D	0 76	0 85	0.96	0 81	1.00	1 00	1 00

WCT - West Coast Tall
ECT - East Coast Tall
JT - Jamaica Tall
KGD - Kulasekharam Green Dwarf
COD - Chowghat Orange Dwarf
MYD - Malayan Yellow Dwarf
D x T - Chowghat Orange Dwarf x West Coast Tall
T x D - East Coast Tall x Malayan Green Dwarf

(lanes 3, 5, 6, 7 and 8). Similarity indices between the varieties are given in Table 7. There was no specific difference between the similarity indices. All the combinations showed more than 75% similarity.

4.2.1. Water soluble protein (albumins)

The electrophoretic separation of water soluble protein in the coconut endosperm of the eight varieties is shown in Plate 3. The proteins were resolved in 12.5% gel and the electrophoregram is given in Figure 4.

Water soluble proteins of coconut endosperm resolved into four major bands and 7 minor bands. All the bands were within the range of 66 KDa to 14 KDa molecular weight. Band-1 was not seen with high intensity in any of the varieties. Varieties ECT, KGD, KYD and DxT had medium activity (lanes 2, 4, 6 & 7). It showed low activity in the Jamaican Tall variety (lane 3). Band 2 was seen with medium activity in all the lanes. Bands 3 to 6 were uniformly present in all the varieties. Band 7 was uniform for all the varieties excepting in COD in which it was absent. In T x D hybrid, it had only low activity. Band No.8 was very specific with medium activity and was seen only in the KYD and D x T (lane 6 and 7). Bands 9, 10 and 11 were uniform for all the varieties.

4.2.2. Salt soluble protein (globulins)

Electrophoretic separation of salt soluble protein in the coconut endosperm of the eight varieties is shown in plate 4 and the electrophoregram is given in Figure 5.

Salt soluble protein of coconut endosperm resolved into 7 bands within the molecular weight range of 20 to 45 KDa. Band No.1 was seen uniformly in

Plate 3. SDS-PAGE pattern of albumin endosperm storage protein in coconut genotypes

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

M Protein weight marker.

Plate 3

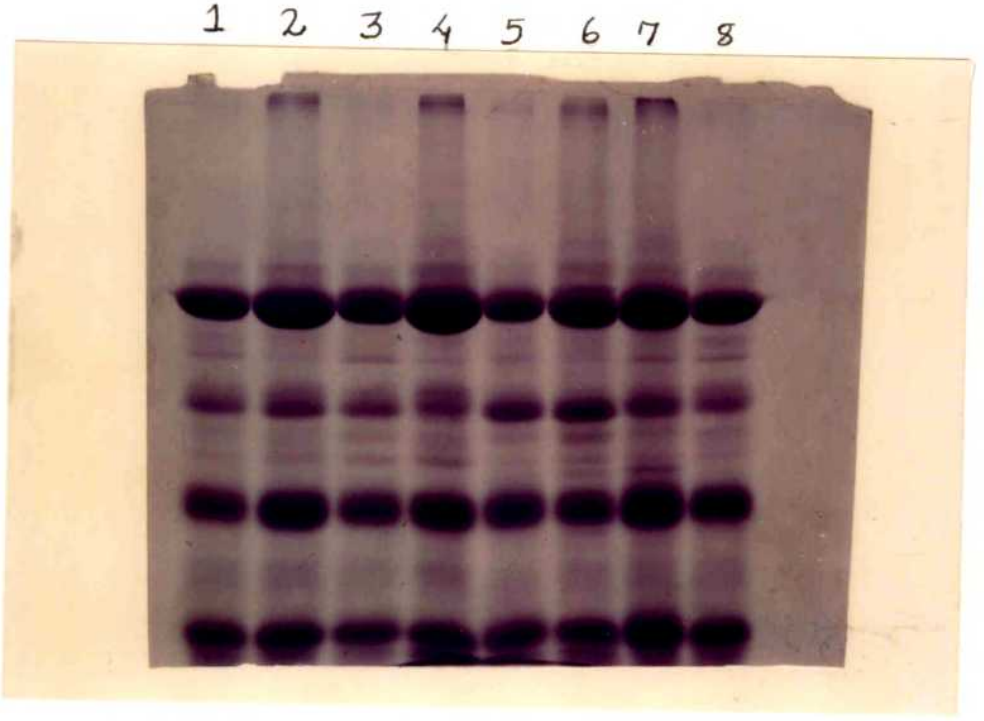


Fig.4. SDS-PAGE pattern of albumin endosperm storage protein in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2 East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

M Protein weight marker.

Fig.4. SDS-PAGE PATTERN OF ALBUMIN ENDOSPERM STORAGE PROTEIN IN COCONUT VARIETIES

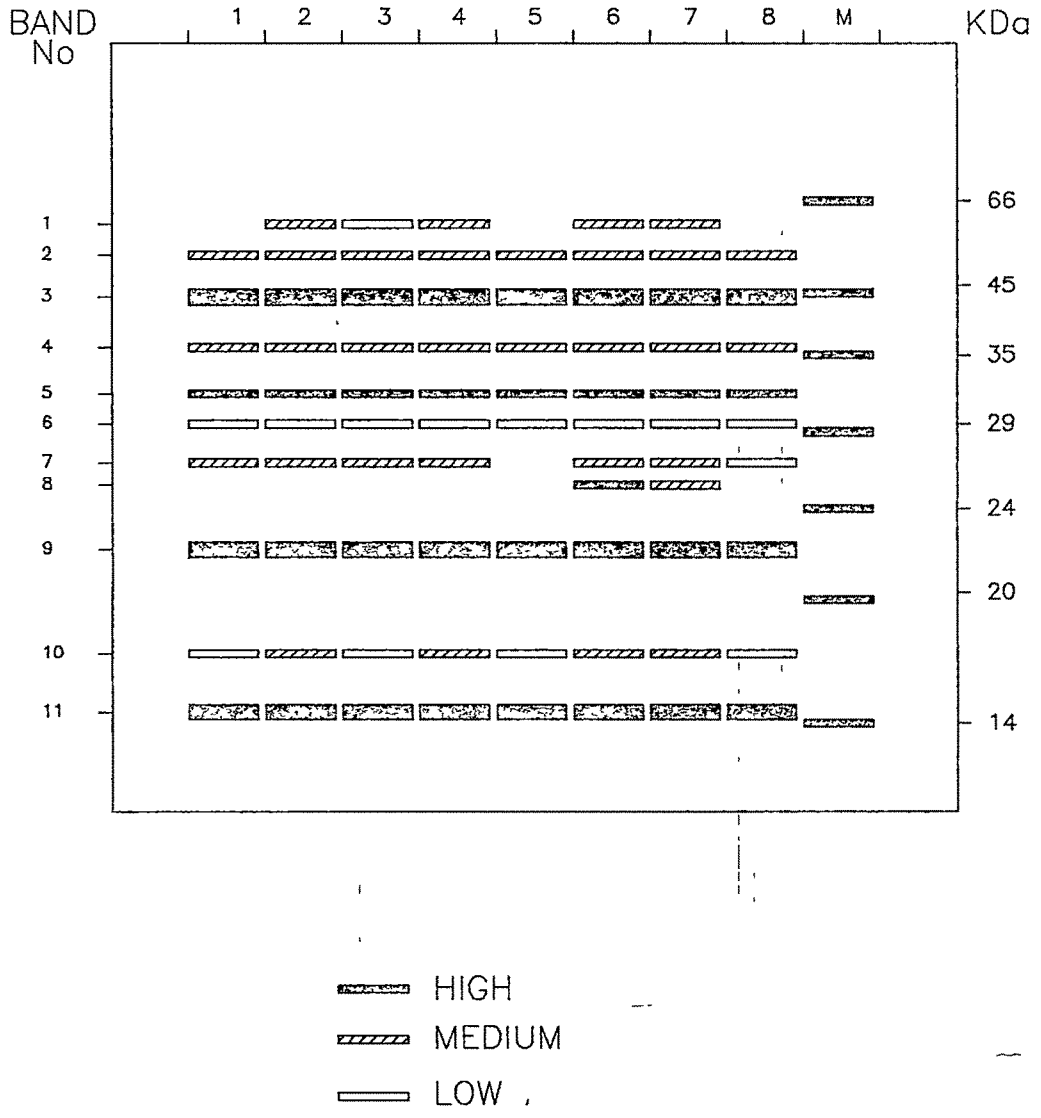


Plate 4. SDS-PAGE pattern of globulin endosperm storage protein in coconut genotypes

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7 Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

M Protein weight marker.

Plate 4

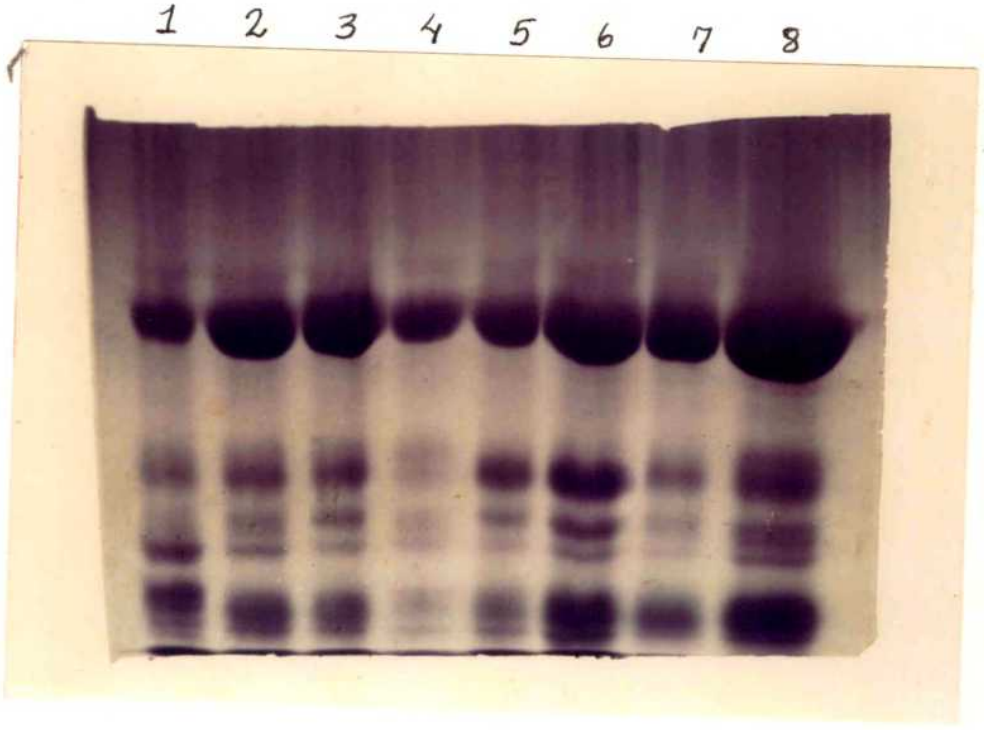


Fig.5. SDS-PAGE pattern of globulin endosperm storage protein in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

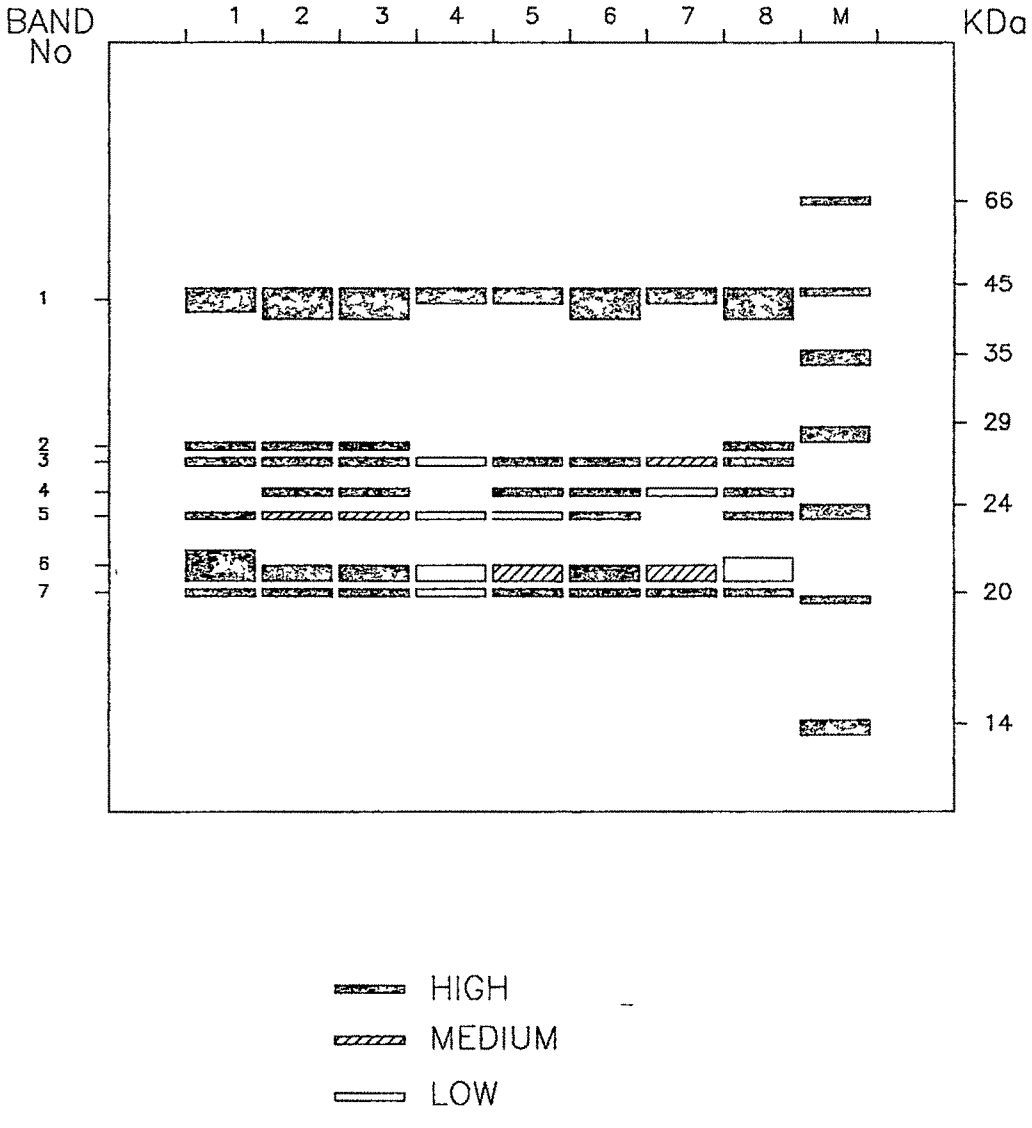
Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf X West Coast Tall (D X T)

Lane 8. East Coast Tall X Malayan Green Dwarf (T X D)

M Protein weight marker.

Fig.5. SDS-PAGE PATTERN OF GLOBULIN ENDOSPERM STORAGE PROTEIN IN COCONUT VARIETIES



all the varieties with high intensity. But the width of the band varied between varieties. Band No.2 was around molecular weight 29 KDa and was present only in the tall varieties WCT, ECT and Jamaican Tall (lanes 1, 2 and 3), and the T x D hybrid. Band 3 was seen uniformly in all the varieties but the intensity was low in KGD (lane 4) and medium in D x T hybrid (lane 7). Band 4 was absent in the WCT variety (lane 1) and KGD variety (lane 4) and had low intensity in D x T hybrid (lane 7). Band 6 and 7 were uniform in all the varieties but differed in intensity Polymorphism was mostly seen between 29 KDa and 24 KDa molecular weight region.

4.2.3. Alkali soluble protein (glutelins)

Electrophoretic separation of alkali soluble protein is shown in Plate 5. The electrophoregram is given in Figure 6

Alkali soluble protein in coconut endosperm resolved into an array of bands between 25 and 45 KDa molecular weights. Of these, only 6 were distinctly visible. Band number 1, 2, 3 and 6 were uniformly seen in all the varieties. Only band 4 and 5 gave some polymorphism. Band 4 was present in all except COD variety (lane 5). The hybrids and MYD variety showed only medium activity for this band. Band No.5 was very specific and seen only in the tall varieties (lane 1, 2 & 3). In this fraction also polymorphism was found between the 29 KDa and 24 KDa molecular weights.

4.2.4. Alcohol soluble protein (prolamins)

Electrophoretic pattern of alcohol soluble protein is shown in plate 6. Alcohol soluble protein was resolved in a 15% gel at 4°C for 3 hours, at 50 m A. Electrophoregram was shown in Figure 7. Since the protein content of this fraction was very low only bands of light intensity were obtained. It

Plate 5. SDS-PAGE pattern of glutelin endosperm storage protein in coconut genotypes

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5 Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7 Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8 East Coast Tall x Malayan Green Dwarf (T X D)

M Protein weight marker.

Plate 5

1 2 3 4 5 6 7 8

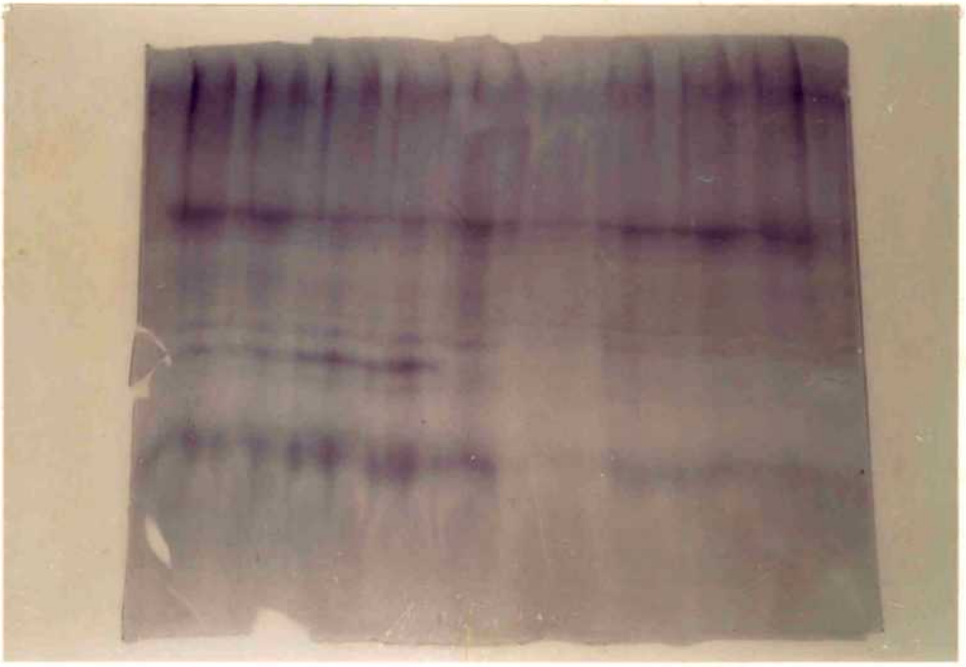


Plate 11. Genomic DNA of coconut digested with enzymes Hae III and Eco RV

Hae III

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

EcoRV

Lane 7	-	West Coast Tall (WCT)
Lane 8		East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Fig.6. SDS-PAGE pattern of glutelin endosperm storage protein in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf X West Coast Tall (D X T)

Lane 8. East Coast Tall X Malayan Green Dwarf (T X D)

M Protein weight marker.

Fig.6. SDS-PAGE PATTERN OF GLUTELIN ENDOSPERM STORAGE PROTEIN IN COCONUT VARIETIES

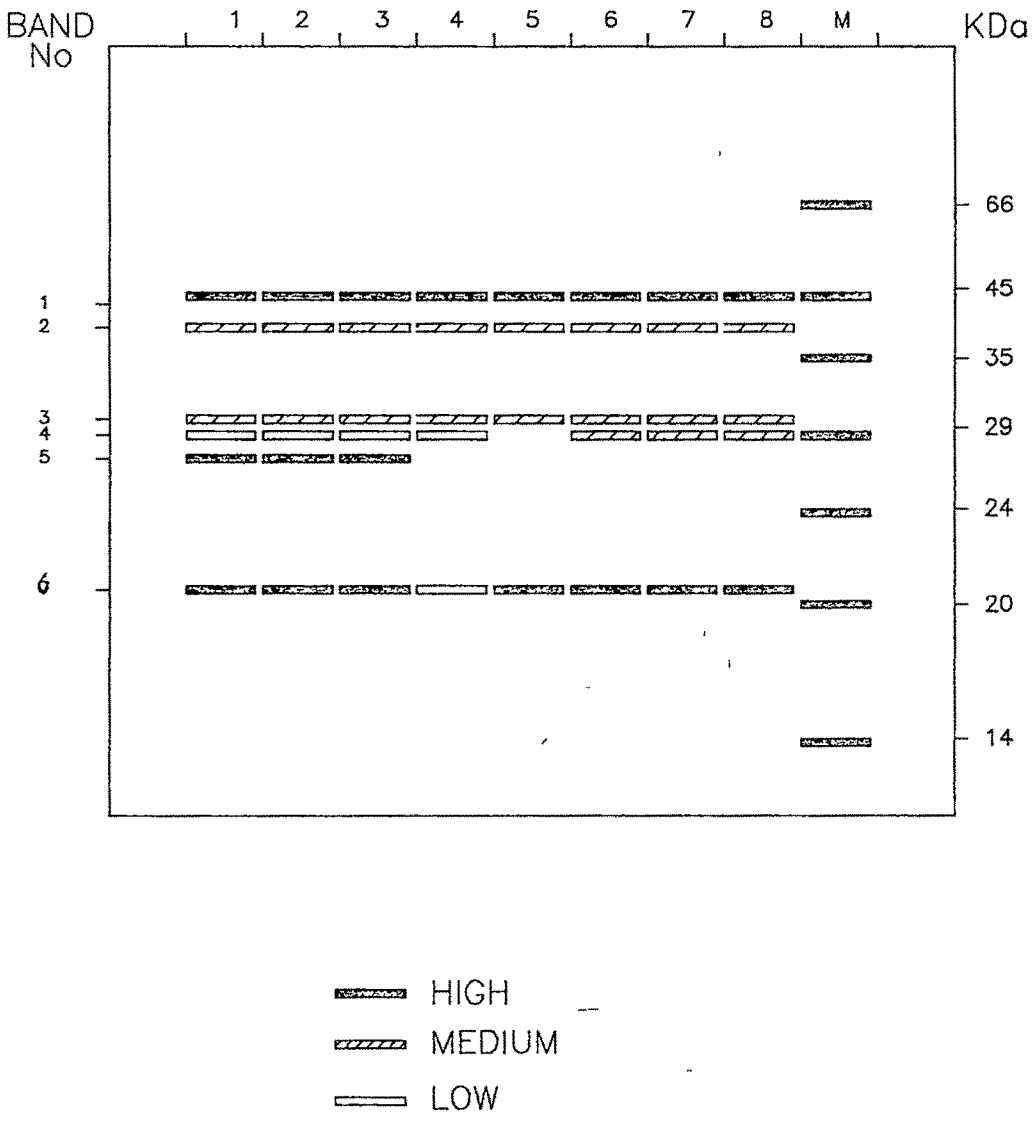


Plate 6. SDS-PAGE pattern of prolamin endosperm storage protein in coconut genotypes

Lane 1.	West Coast Tall (WCT)
Lane 2.	East Coast Tall (ECT)
Lane 3.	Jamaica Tall (JT)
Lane 4.	Kulasekharam Green Dwarf (KGD)
Lane 5.	Chowghat Orange Dwarf (COD)
Lane 6.	Malayan Yellow Dwarf (MYD)
Lane 7.	Chowghat Orange Dwarf x West Coast Tall (D X T)
Lane 8.	East Coast Tall x Malayan Green Dwarf (T X D)
M	Protein weight marker.

Plate 6

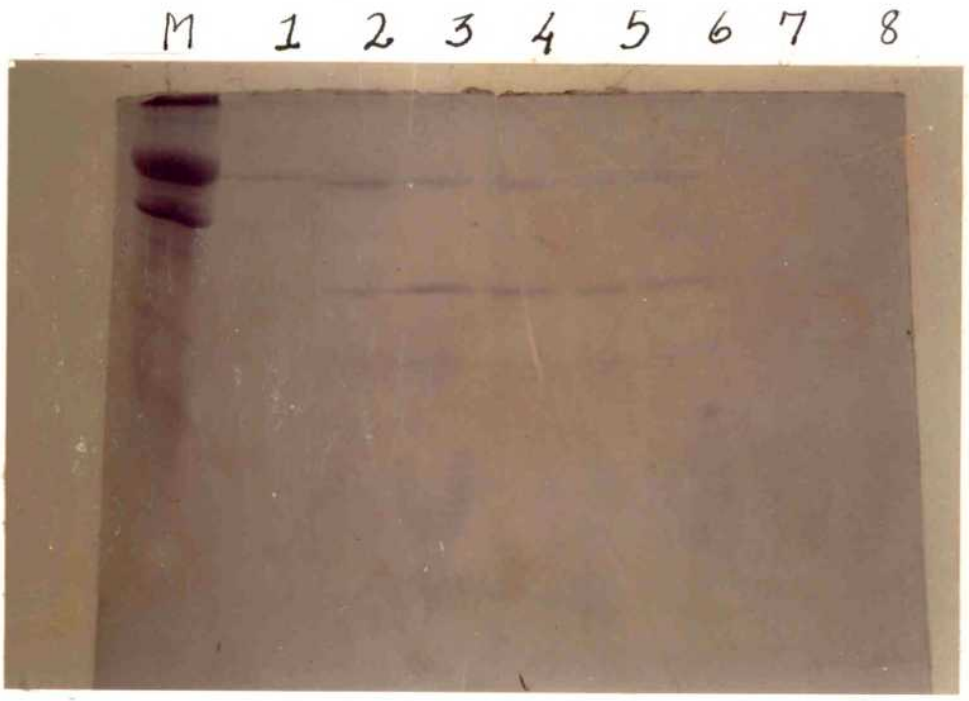
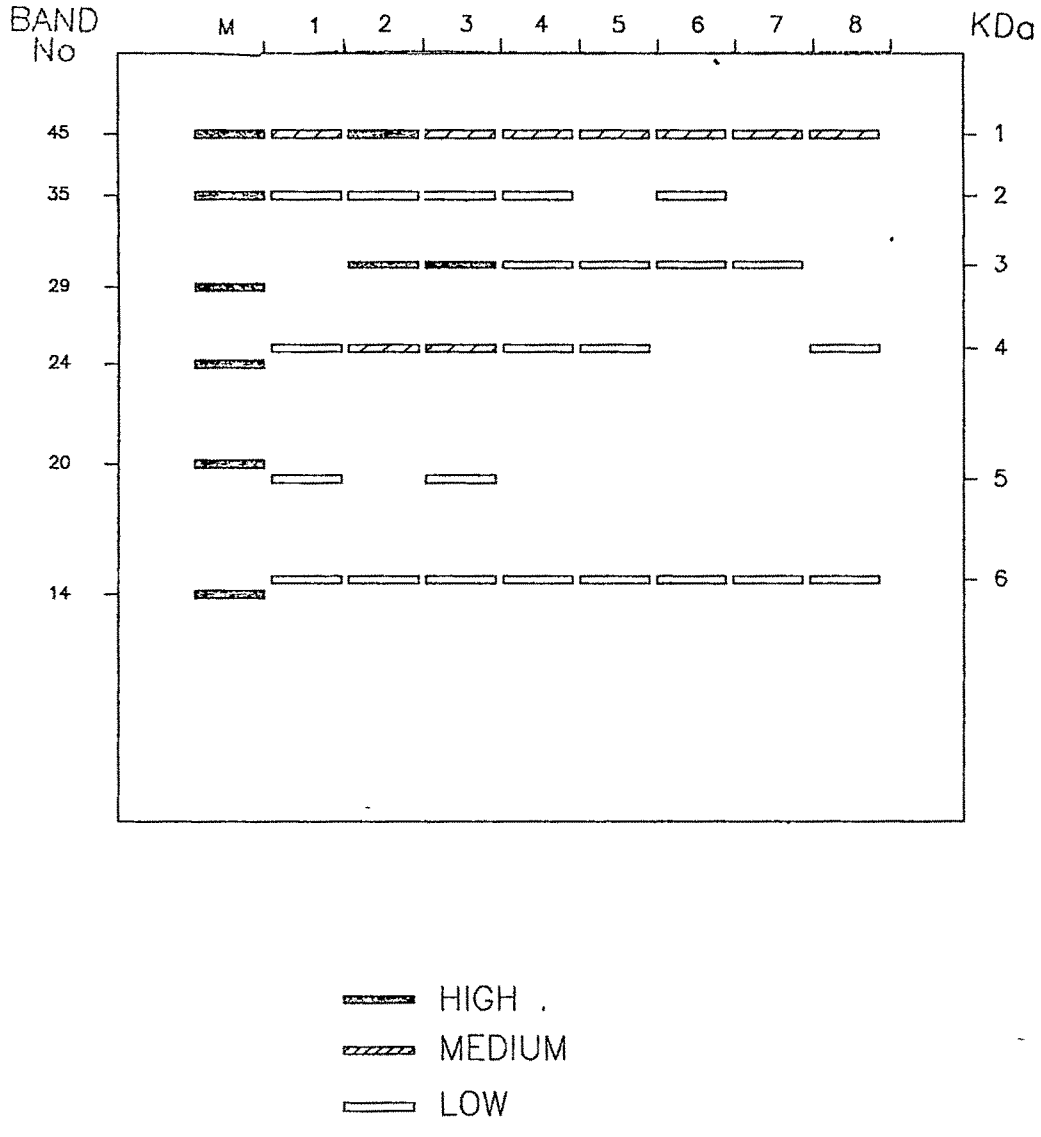


Fig.7. SDS-PAGE pattern of prolamin endosperm storage protein in coconut varieties

Lane 1	West Coast Tall (WCT)
Lane 2.	East Coast Tall (ECT)
Lane 3.	Jamaica Tall (JT)
Lane 4	Kulasekharam Green Dwarf (KGD)
Lane 5	Chowghat Orange Dwarf (COD)
Lane 6	Malayan Yellow Dwarf (MYD)
Lane 7	Chowghat Orange Dwarf x West Coast Tall (D X T)
Lane 8	East Coast Tall x Malayan Green Dwarf (T X D)
M	Protein weight marker.

Fig.7. SDS-PAGE PATTERN OF PROLAMIN ENDOSPERM STORAGE PROTEIN IN COCONUT VARIETIES



resolved into 6 bands between 45 and 14 KDa protein. Band 1 and 6 were uniformly present in all the varieties. Band 2 was absent in the COD variety and hybrid Band 3 was not seen in the WCT variety and T x D hybrid. Band No.4 was absent in the KYD variety and D x T hybrid. Band 5 was present only in the two tall varieties WCT and Jamaican Tall variety.

4.2.5. Percentage of protein fraction of different varieties

Amount of different fractions of proteins (based on solubility) in the coconut endosperm of eight varieties of coconut was estimated by microkjeldahl method. Mean, SE and coefficient of variation for each fraction is given in Table 8.

Total protein as well as the 3 soluble fractions (globulin, albumin, and glutelin) varied significantly between the varieties. All fractions gave coefficient of variation above 2.0%. Alubumin fraction showed the highest cv of 4.35%. Highest percentage of total protein was shown by T x D hybrid and was significantly superior than all the varieties. ECT among the talls and KGD among the dwarf varieties, showed the highest percentage of 27.35 and 25.65 respectively. T x D hybrid also had the highest percentage of albumin and globulins. But glutelin fraction was the highest for KGD variety. Prolamin fraction was the lowest and it did not differ significantly between the varieties. In all the varieties, globulin protein (soluble in 5.0% NaCl) fraction was the major fractional protein. Comparison of percentage fraction of different proteins in different genotypes of coconut is shown in Figure 8.

4.3. Isozyme analysis

Electrophoretic separation of crude enzyme extracts of the eight varieties of coconut for peroxidase , esterase and polyphenol oxidase are

Table 8. Fractionation of endosperm proteins of different coconut genotypes**

Varieties		Total protein %	Protein type solvent: Albumin (water soluble) %	Globulin (5% NaCl soluble) %	Prolamin (70% EtOH soluble) %	Glutelin (0.05 N NaOH soluble) %
1	WCT	21 84	5 7	13 04	1.04	2.06
2.	ECT	27 35	8 72	15 49	1 00	2 14
3	JT	23 98	8.68	12 08	1.10	2 12
4	KGD	25.65	4 18	16 73	1.06	3 68
5.	COD	23 70	5.16	13.87	1 55	3 12
6	MYD	20 21	4.87	12.07	1.11	2 16
7	DxT	27 80	6.24	16 92	1.13	3 51
8.	TxD	29 97	8 75	17 15	1.55	2.52
	Mean	24 90	6 50	14 67	1 13	2.60
	SE	0 35	0.16	0 19	0 16	0.02
	CD	1 06	0.49	0.59	0.49	0 05
	CV	2 44	4.35	2 35	NS	2 10

* Values are average of three determinations expressed on moisture and fat free basis

** N X 6 25

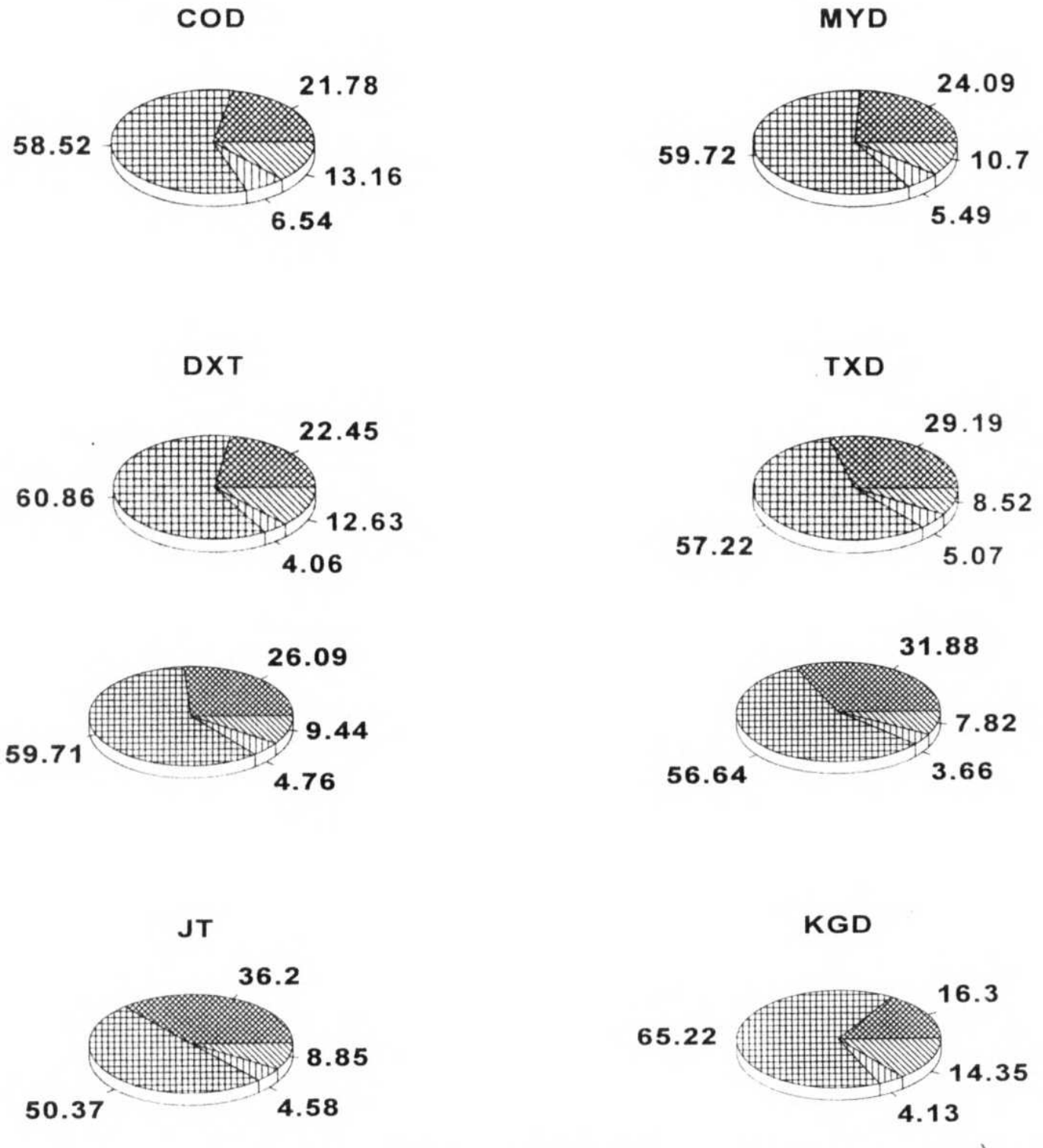
NS - Non-significantWCT - West Coast Tall

ECT - East Coast Tall JT - Jamaica Tall
KGD - Kulasekharam Green Dwarf COD - Chowghat Orange Dwarf
MYD - Malayan Yellow Dwarf DxT - Chowghat Orange Dwarf x West Coast Tall
TxD - East Coast Tall x Malayan Green Dwarf

Fig.8. Comparison of percentage of protein fractions in coconut genotypes

WCT	- West Coast Tall
ECT	- East Coast Tall
JT	- Jamaica Tall
KGD	- Kulasekharan Green Dwarf
COD	- Chowghat Orange Dwarf
MYD	- Malayan Yellow Dwarf
D X T	- Chowghat Orange Dwarf X West Coast Tall
T X D	- East Coast Tall X Malayan Green Dwarf

Fig.8. Comparison of percentage of protein fractions in coconut genotypes



shown in plates 7, 8 and 9 respectively. The isozymes were schematically drawn based on relative mobility. The zymogram of peroxidase esterase and polyphenol oxidase are given in figures 9, 10 and 11 respectively.

An analysis of the zymograms of each enzyme revealed that number and intensity of isozymes varied between the eight varieties of coconut studied. The details are presented here, under separate headings.

4.3.1. Peroxidase

Two zones of peroxidase activity were observed (Plate 7 and Fig. 9). In zone one, two isozymes PRX 7 and PRX 22 were seen. Isozyme PRX 7 was seen only in the two tall varieties (lanes 2 and 3) ECT and JT and the hybrids D x T and T x D (lanes 7 and 8). PRX22 isozyme was found uniformly with same intensity in all the varieties.

In zone two, again two isozymes PRX 42 and PRX 50 were found. Isozyme PRX 42 was found only in the two tall varieties, WCT and the Jamaican Tall and the two hybrids (Lanes 1, 3, 7 and 8) D x T and T x D. PRX 50 isozyme was found in two tall varieties (lanes 1 and 2) West Coast Tall and East Coast Tall but the activity was high in East Coast Tall and low in West Coast Tall. This isozyme was seen in all the three dwarf varieties as well (lanes 4,5 and 6) but the activity was low in KGD. High intensity of this isozyme was seen in the Tall x Dwarf hybrid but was absent in the D x T hybrid (lane 8 and 7). Similarity indices between the varieties are given in table 9. WCT variety showed more similarity with the dwarf varieties and the hybrid T x D. ECT variety also showed a similar trend. But the JT variety showed more similarity with the hybrids. Dwarf varieties were

Plate 7

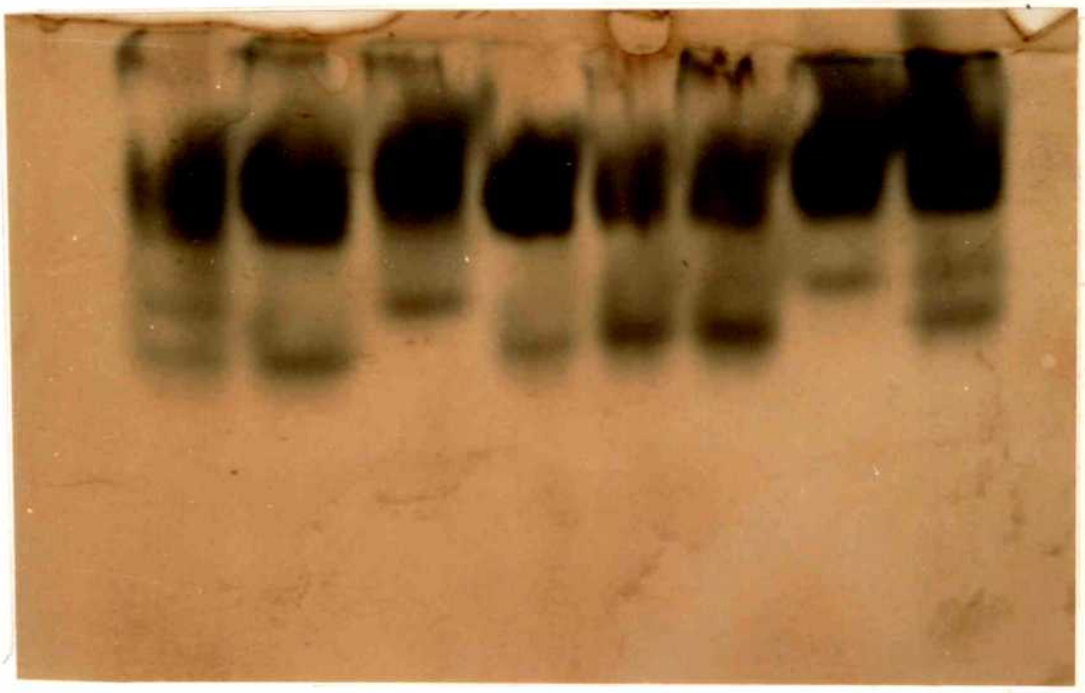


Fig.9. Zymogram of leaf peroxidase in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

Fig.9. ZYMOGRAM OF LEAF PEROXIDASE IN COCONUT VARIETIES

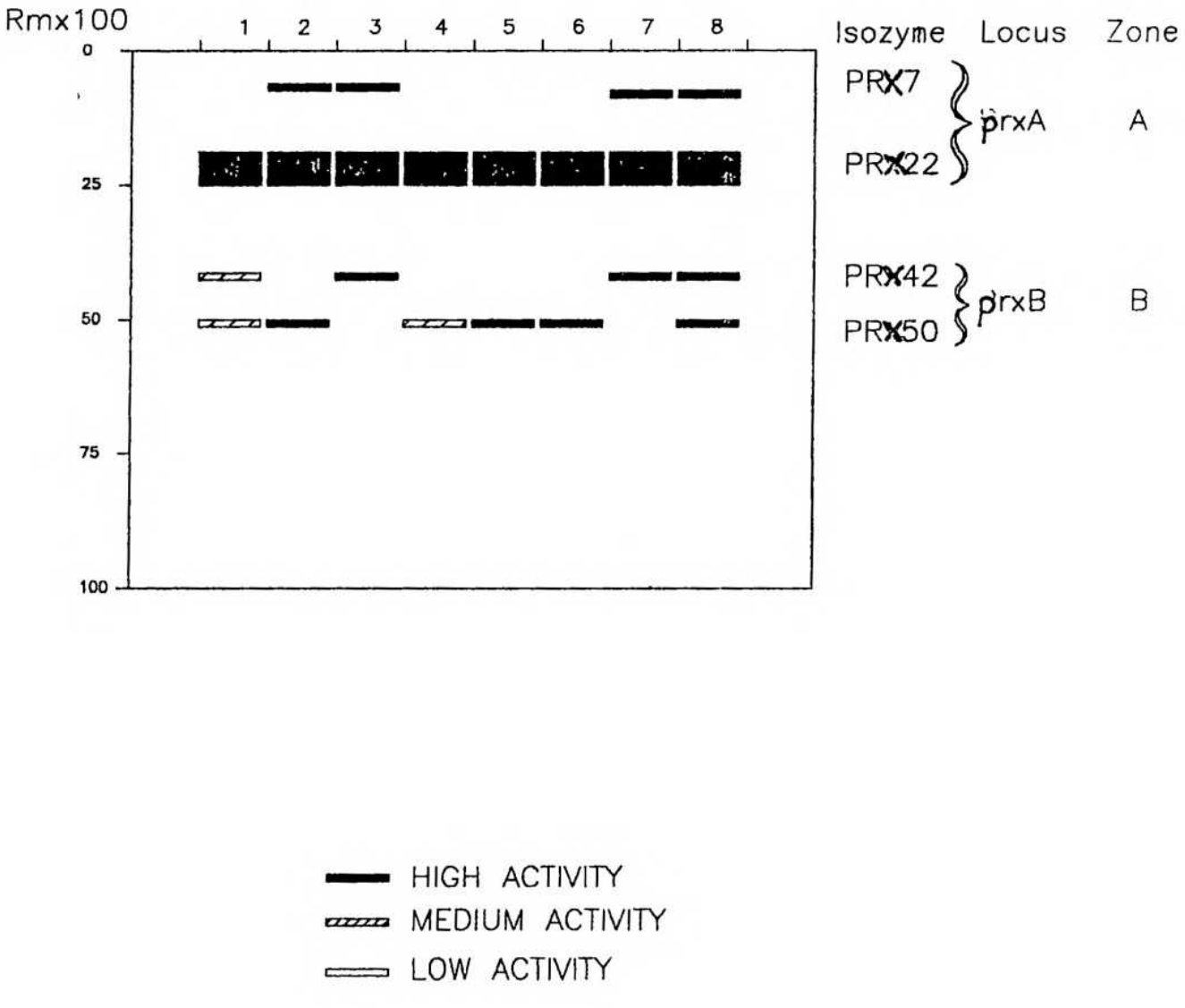


Table 9. Similarity indices for Peroxidase isozyme in coconut genotypes

	WCT	ECT	JT	KGD	COD	MYD	D x T
ECT	0 66						
JT	0 66	0 66					
KGD	0 80	0 80	0.40				
COD	0.80	0 80	0.40	1 00			
MYD	0.80	0 80	0 40	1 00	1 00		
D x T	0 66	0 66	1.00	0 40	0 40	0 40	
T x D	0.85	0 85	0.85	0 80	0 80	0.80	0 85

WCT - West Coast Tall
ECT - East Coast Tall
JT - Jamaica Tall
KGD - Kulasekharam Green Dwarf
COD - Chowghat Orange Dwarf
MYD - Malayan Yellow Dwarf
D x T - Chowghat Orange Dwarf x West Coast Tall
T x D - East Coast Tall x Malayan Green Dwarf

completely similar and were more similar to the T x D hybrid than D x T hybrid.

4.3.2. Esterase

Two zones of esterase activity were observed. (Plate 8 and Fig. 10). In zone 1, two isozymes EST 25 and EST 30 were seen. Isozyme EST 25 had only medium activity and was present in all the three tall varieties (lane 1, 2 and 3) and the COD variety (lane 5) and the two hybrids D x T and T x D (lanes 7 and 8). EST 30 isozyme was seen uniformly in all the eight varieties but at varying intensity. West Coast Tall and the Kulasekharam Dwarf, Green Dwarf varieties showed medium activity for this isozyme (lanes 1 and 4).

In the second zone, five isozymes were seen namely EST 50, EST 55, EST 62, EST 67 and EST 75. EST 50 was found to be an isozyme of low activity. It was observed in the two tall varieties, ECT and JT (lanes 2 and 3) and in the three dwarf varieties (lanes 4, 5 and 6) and the T x D hybrid (lane 8). Isozyme EST 55 was seen in all the 7 varieties excepting West Coast Tall (lane 1). But the KGD variety showed only a medium activity (lane 4). EST 62 isozyme was seen uniformly in all the eight varieties with high intensity. Isozyme EST 67 was seen to have high activity in the two tall varieties East Coast Tall and Jamaican Tall (lanes 2 and 3) and the COD variety (lane 5). Isozyme EST 75 was observed only in two varieties, the COD variety (lane 5) and the D x T hybrid (lane 7). Similarity indices among the varieties are given in Table 10. WCT variety showed more similarity with the two hybrids and with the other two tall varieties. East Coast Tall variety had highest similarity with the COD and the T x D hybrid. JT variety also showed a similar trend. Dwarf varieties also showed high similarity with T x D hybrid

Plate 8. Leaf esterase pattern in coconut genotypes

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3 Jamaica Tall (JT)

Lane 4 Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

Plate 8

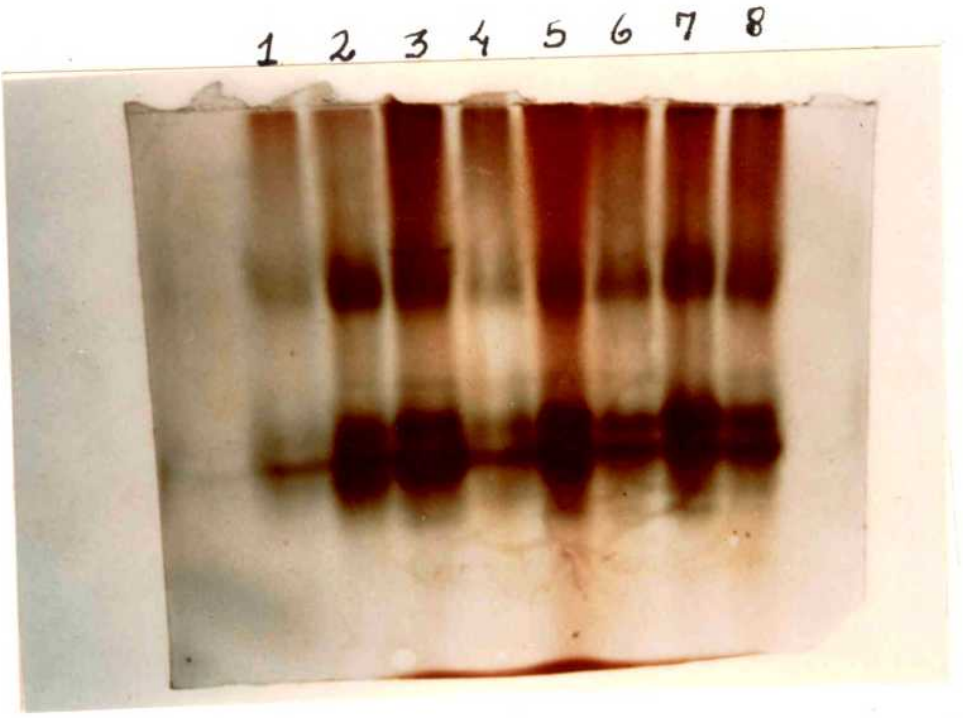


Fig.10. Zymogram of leaf esterase in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3. Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

Fig. 10. ZYMOGRAM OF LEAF ESTERASE IN COCONUT VARIETIES

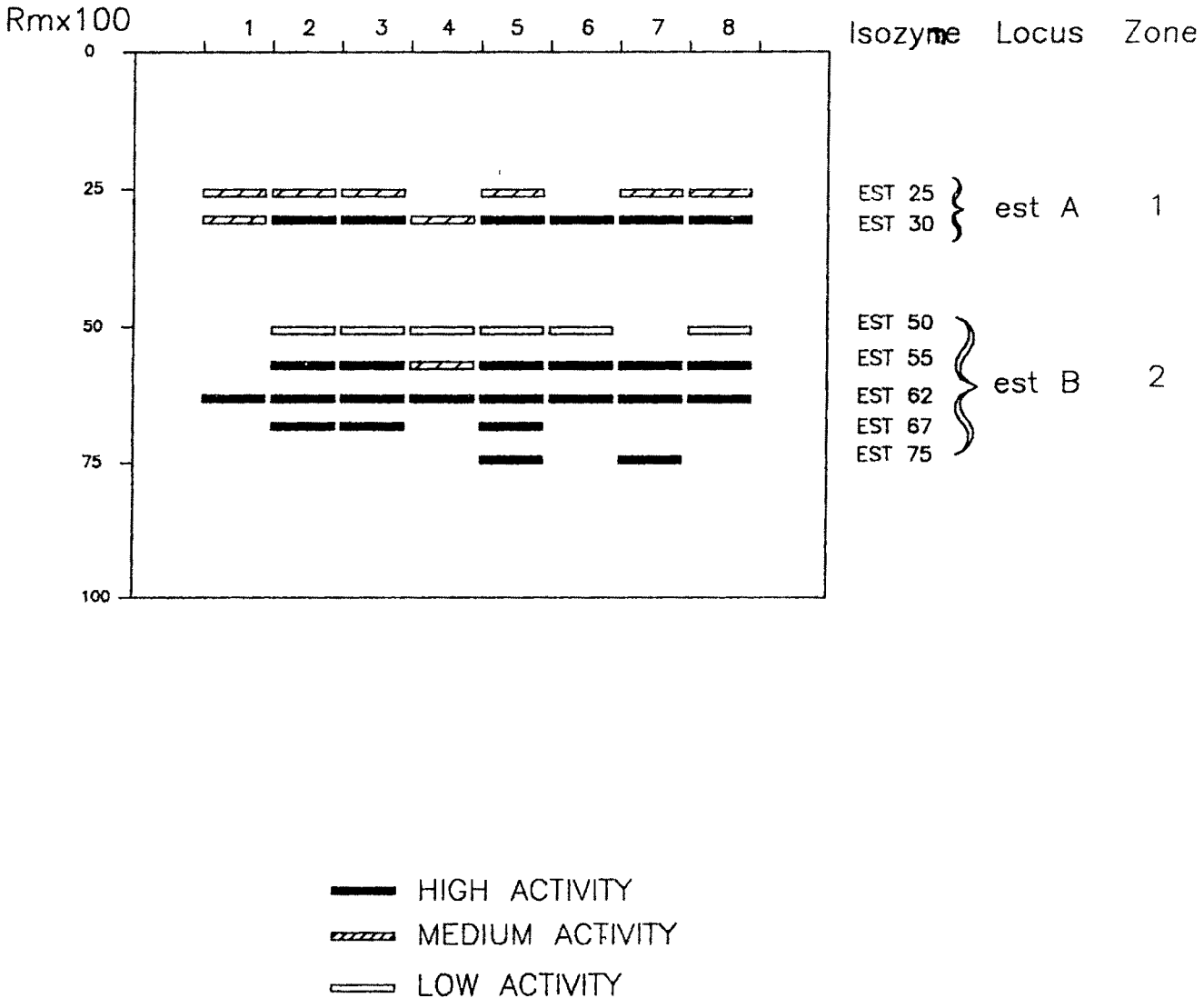


Table 10. Similarity indices for Esterase isozyme in coconut genotypes

	WCT	ECT	JT	KGD	COD	MYD	D x T
ECT	0.66						
JT	0.66	1 00					
KGD	0.57	0 80	0.80				
COD	0.60	0.92	0.92	0 72			
MYD	0 57	0 80	0.80	1 00	0 72		
D x T	0 75	0 72	0 72	0.66	0.66	0 66	
T x D	0 75	0 90	0 90	0 88	0.83	0 88	0.80

WCT - West Coast Tall
ECT - East Coast Tall
JT - Jamaica Tall
KGD - Kulasekharam Green Dwarf
COD - Chowghat Orange Dwarf
MYD - Malayan Yellow Dwarf
D x T - Chowghat Orange Dwarf x West Coast Tall

4.3.3. Polyphenol oxidase

Polyphenol oxidase had only one zone of activity as shown in the Plate 9 and Fig. 11). Three isozymes PPO 28, PPO 35 and PPO 43 were seen in this zone. Isozyme PPO 28 was seen in all the eight varieties with medium activity in the two tall varieties, East Coast Tall and Jamaican Tall (lanes 2 and 3). Isozyme PPO 34 was found only in the West Coast Tall variety (lane 1) and in hybrids D x T and T x D (lanes 7 and 8). This isozyme expressed only medium activity. PPO 43 isozyme was seen only in the three dwarf varieties (lanes 4, 5 and 6) and in the hybrid T x D (lane 8) where the isozyme had only medium activity. Similarity indices among varieties are given in Table 11.

Dwarf varieties had high similarity between themselves and with the hybrid T x D. Tall varieties did not show a similar trend.

4.4. DNA studies

4.4.1. Isolation of genomic DNA

Genomic DNA was isolated and the purity and intactness were tested in 0.8% agarose gel and are presented in Plate 10. First two lanes represent the genomic DNA from the tall varieties. Lanes 3 and 4 from DNA from the dwarf varieties and Lanes 5 and 6 from the hybrids. In general, all the lanes had the intact and high molecular weight DNA. The intact band shows that good quality DNA was obtained for all the varieties. Ratio of Spectrophotometric reading at 260 nm and 280 nm of the extracted DNA was below 1 indicating that the DNA extracted was pure with less of salts and proteins and that further purification is not necessary.

Plate 9. Leaf polyphenol oxidase pattern in coconut genotypes

Lane 1 West Coast Tall (WCT)

Lane 2 East Coast Tall (ECT)

Lane 3 Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

Plate 9

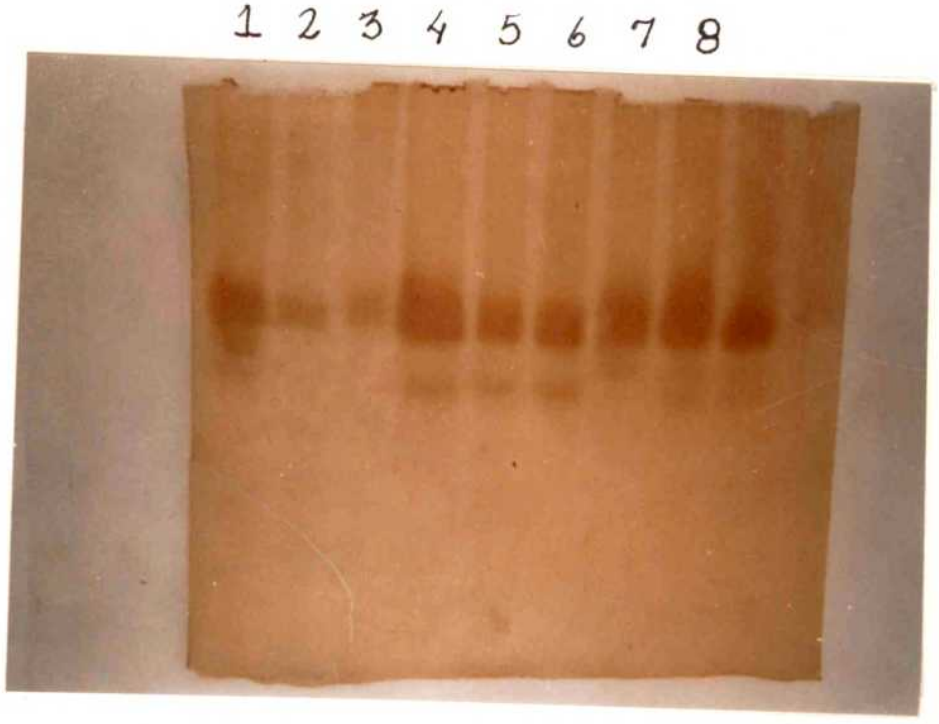


Fig. 11. Zymogram of leaf poly phenol oxidase in coconut varieties

Lane 1. West Coast Tall (WCT)

Lane 2. East Coast Tall (ECT)

Lane 3 Jamaica Tall (JT)

Lane 4. Kulasekharam Green Dwarf (KGD)

Lane 5. Chowghat Orange Dwarf (COD)

Lane 6. Malayan Yellow Dwarf (MYD)

Lane 7. Chowghat Orange Dwarf x West Coast Tall (D X T)

Lane 8. East Coast Tall x Malayan Green Dwarf (T X D)

Fig.11. ZYMOGRAM OF LEAF POLY PHENOL OXIDASE IN COCONUT VARIETIES

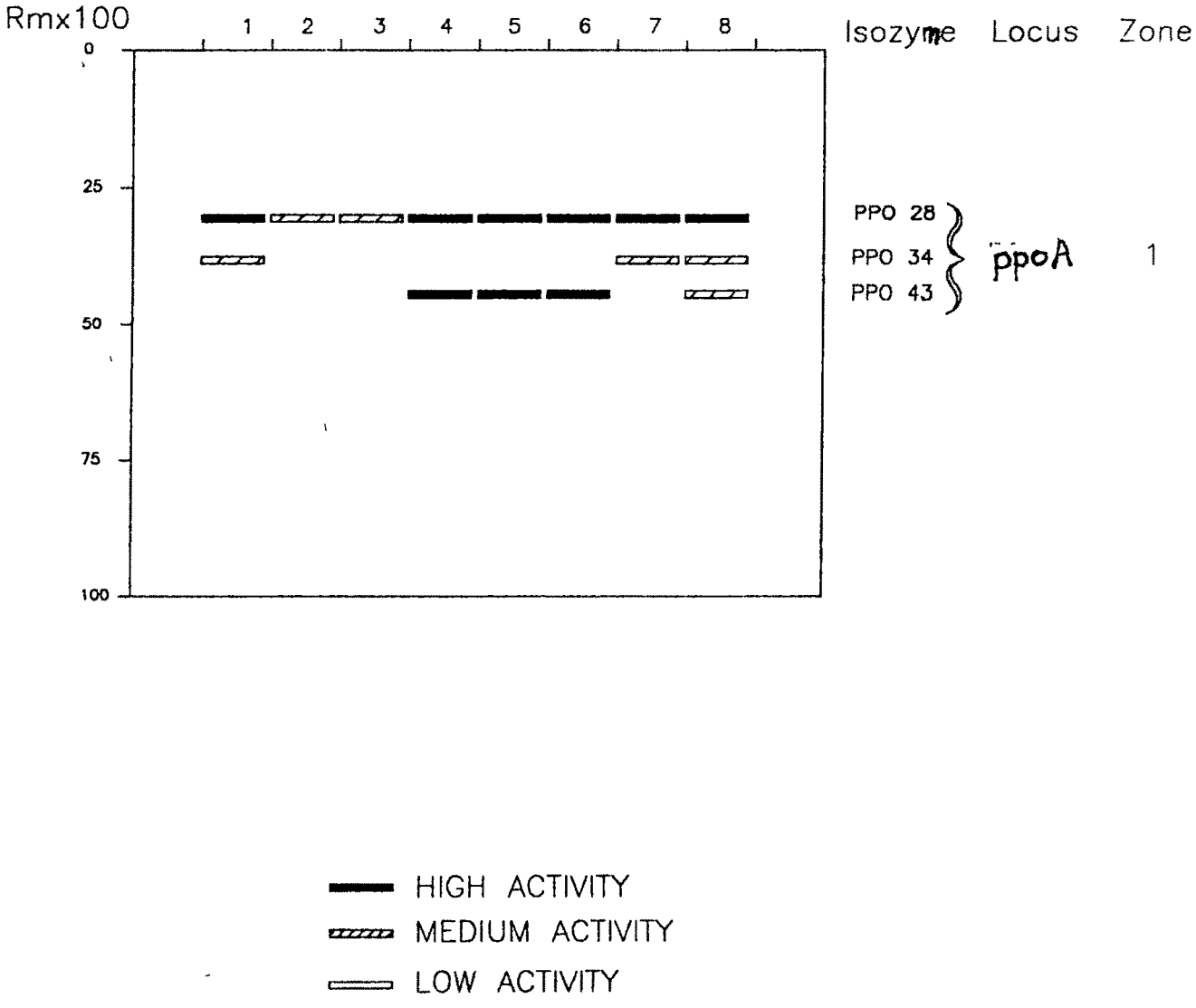


Table 11. Similarity indices for Polyphenol oxidase-isozyme in coconut genotypes

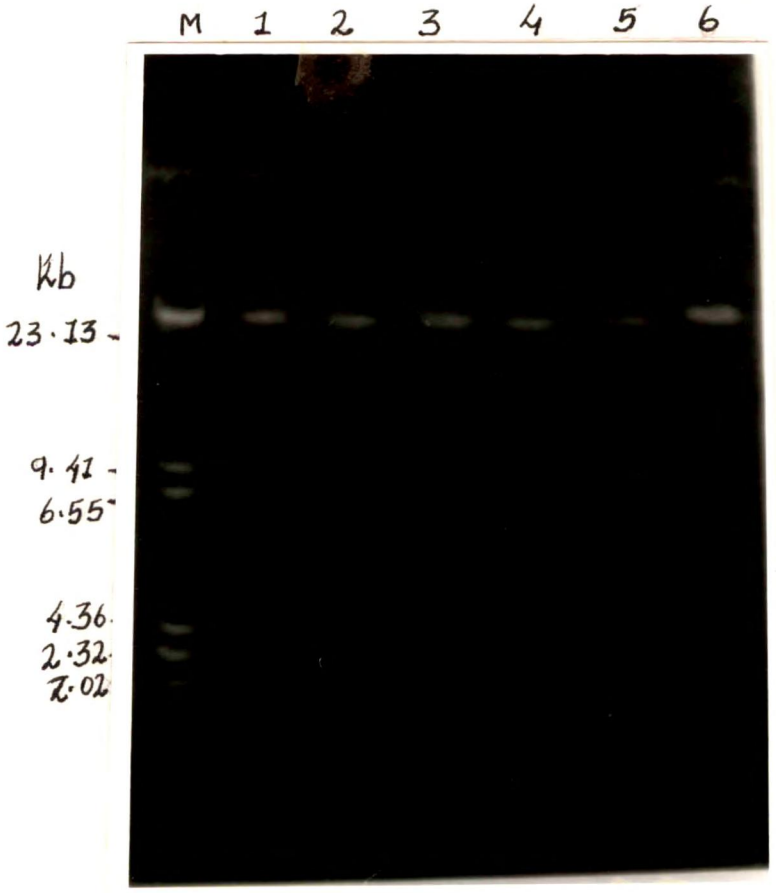
	WCT	ECT	JT	KGD	COD	MYD	D x T
ECT	0.66						
JT	0 66	1 00					
KGD	0 50	0 66	0.66				
COD	0.50	0 66	0 66	1.00			
MYD	0.56	0 66	0.66	1 00	1.00		
D x T	1.00	0 66	0 66	0 50	0 50	0 50	
T x D	0 86	0 50	0 50	0 80	0 80	0 80	0 80

WCT - West Coast Tall
ECT - East Coast Tall
JT - Jamaica Tall
KGD - Kulasekharam Green Dwarf
COD - Chowghat Orange Dwarf
MYD - Malayan Yellow Dwarf
D x T - Chowghat Orange Dwarf x West Coast Tall
T x D - East Coast Tall x Malayan Green Dwarf

Plate 10. Genomic DNA of coconut varieties

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 10



4.4.2. Restriction digestion of genome DNA

. The genomic DNA isolated from the six varieties of coconut were digested with EcoRI, EcoRV, Hind III, Bam HI, Hae III, Hinf I and Sau III restriction enzymes used gave a complete digestion of the genomic DNA uniformly in all the varieties of coconut. The digestion pattern of the restriction enzymes EcoRV and Hae III is shown in Plate 11.

The restricted DNA was transferred into Nytran-N-membranes by southern blotting. The gel was completely blank when viewed under UV transilluminator indicating complete transfer of DNA.

4.4.3. Probe preparation

Oil palm genomic probes CL 54 and CL 95 were obtained from Dr.Sean Mayer, PBI, Cambridge as a lyophilised powder. This was reconstituted in the prescribed quantity of sterile water. The probes were transformed into DH 5α bacterial cells. Transformation was confirmed by plating on ampicillin plates. Both the probes gave enough single colonies as shown in the plates 12&13.

Single colonies from the plates were grown separately and the enture plasmid was isolated. Good plasmid with four bands were obtained as shown in the plate 14.

4.4.4. Southern hybridisation

Cloned DNA probes were hybridised to blots containing individual restriction enzyme digest of total genomic DNA from the six varieties of coconut. The hybridised membranes were put for autoradiogram at -70°C for one week. It was taken out after exposure period, developed and fixed.

Plate 11

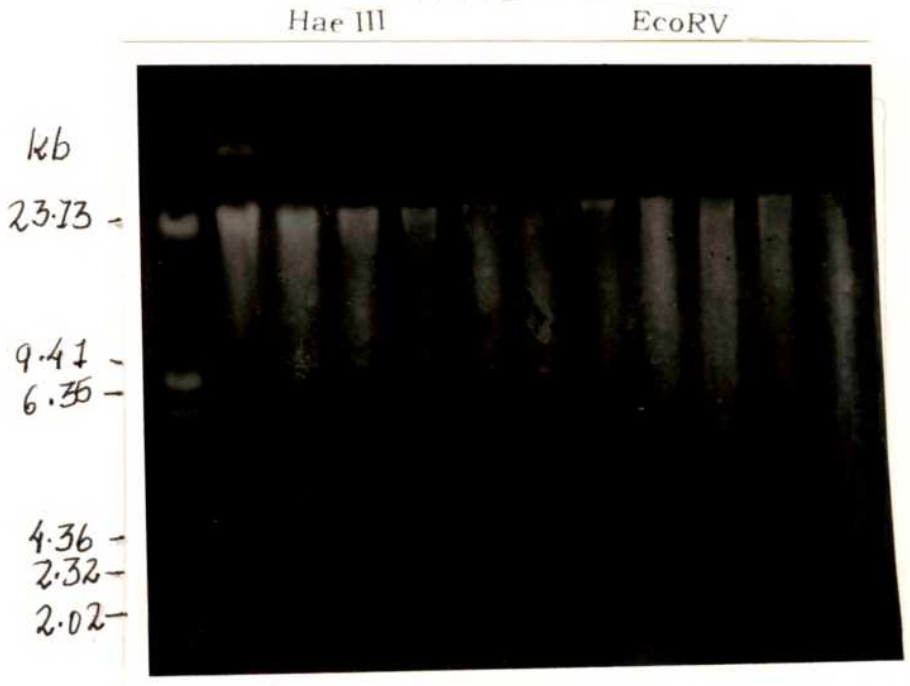


Plate 12. Transformed colonies of *E. coli* grown in ampicillin plates for CL 54

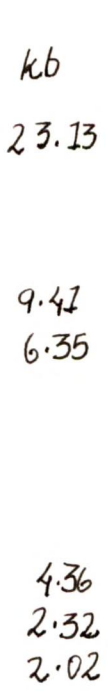
Plate 13. Transformed colonies of *E. coli* grown in ampicillin plate for CL 95

Plate 12

Plate 13

Plate 14. Profile of the plasmids with the insert for CL 54 & CL 95.

Plate 14



4.4.5. Autoradiogram of genomic DNA digested and probed

Autoradiogram of the blots probed with the probe CL 95 did not show any signal indicating the failure of the probe CL 95 to hybridise the coconut genome. Autoradiogram of the blots probed with CL 54 gave faint signals as shown in the plate 15. The signal was prominent in the blot of the genomic DNA of the dwarf orange variety digested with SauIII and EcoR1. Genomic DNA digested with other restriction enzymes did not give any signal in the autoradiogram with neither of the probes.

4.4.6. RAPD analysis

Twelve random primers from OPC series were tried for producing amplification products in coconut varieties. Ten primers gave good result but two primers OPC-13 and OPC-15 did not produce any amplification. Primer number and its sequence and the number of amplification products produced are given in Table 12. Details of the results obtained for each primer are listed below.

OPC-1 and OPC-20

Pattern of amplification of DNA of the 6 varieties for the two primers are shown in plate 16. OPC-1 primer produced 6 amplification products in each variety. But it failed to produce polymorphism in the varieties except for the fifth band whose intensity was high in the tall varieties WCT and ECT (lane 1 and 2) but seen with low intensity in the other varieties.

OPC-20 primer could produce 9 amplification products numbered serially from 7-15. This primer could produce polymorphism between the varieties. Band 10 and 12 were missing in the WCT variety (7th lane)

Plate 15. Autoradiogram of the genomic DNA digested with enzymes Eco RI and Sau III, hybridized with oil palm probe CL 54

EcoRI

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

Sau III

Lane 7	-	West Coast Tall (WCT)
Lane 8	-	East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 15

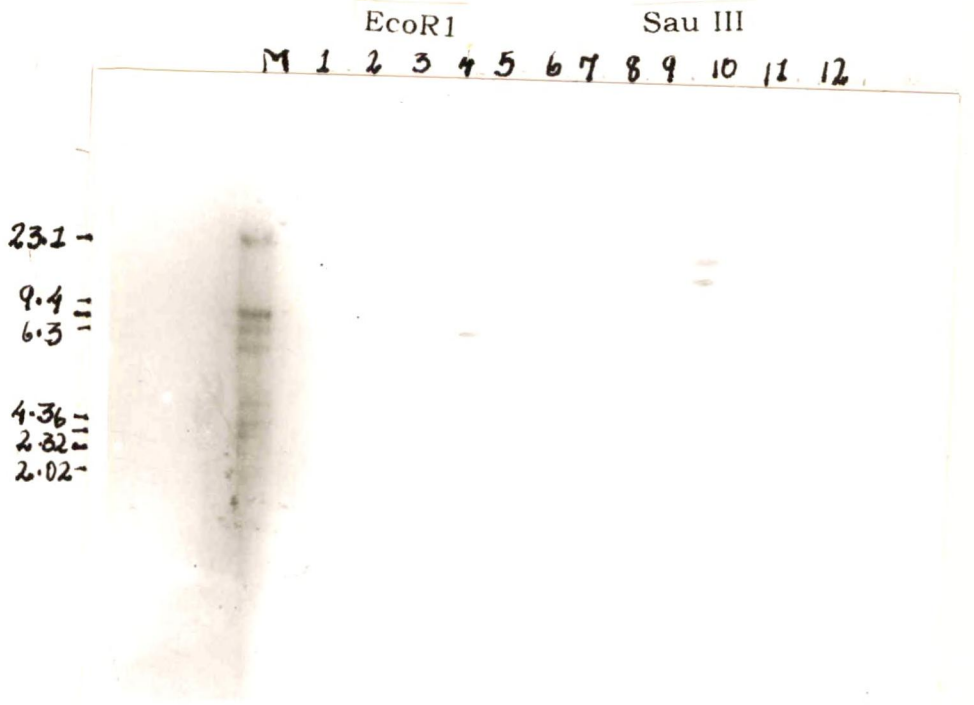


Table 12. Base sequence of primers and the number of amplification products of coconut genomic DNA

Primer Number		Sequence (5' to 3')	No of amplification products		
			Polymorphic	Monomorphic	Total
1	OPC-01	TTCGAGCCAG	0	6	6
2	OPC-02	GTGAGGCGTA	4	2	6
3	OPC-04	CCGCATCTAC	7	4	11
4	OPC-07	GTCCCGACGA	3	3	6
5.	OPC-06	GAACGGACTC	4	3	7
6	OPC-08	TGGACCGGTG	3	6	9
7	OPC-10	TGTCTGGGTG	4	2	6
8	OPC-11	AAAGCTGCGG	5	5	10
9.	OPC-14	TGCGTGCTTG	5	2	7
10	OPC-20	ACTTCGCCAC	5	4	9
11	OPC-13	AAGCCTCGTT	-	-	-
12	OPC-15	GACGGATTAG	-	-	-

Plate 16. RAPD profile for genomic DNA of coconut varieties with primers OPC 1 and OPC 20

OPC 1

M - Lambda DNA/Hind III digest

Lane 1 - West Coast Tall (WCT)

Lane 2 - East Coast Tall (ECT)

Lane 3 - Kulasekharam Green Dwarf (KGD)

Lane 4 - Chowghat Orange Dwarf (COD)

Lane 5 - Chowghat Orange Dwarf x West Coast Tall (D x T)

Lane 6 - East Coast Tall x Malayan Green Dwarf (T x D)

OPC 20

Lane 7 West Coast Tall (WCT)

Lane 8 - East Coast Tall (ECT)

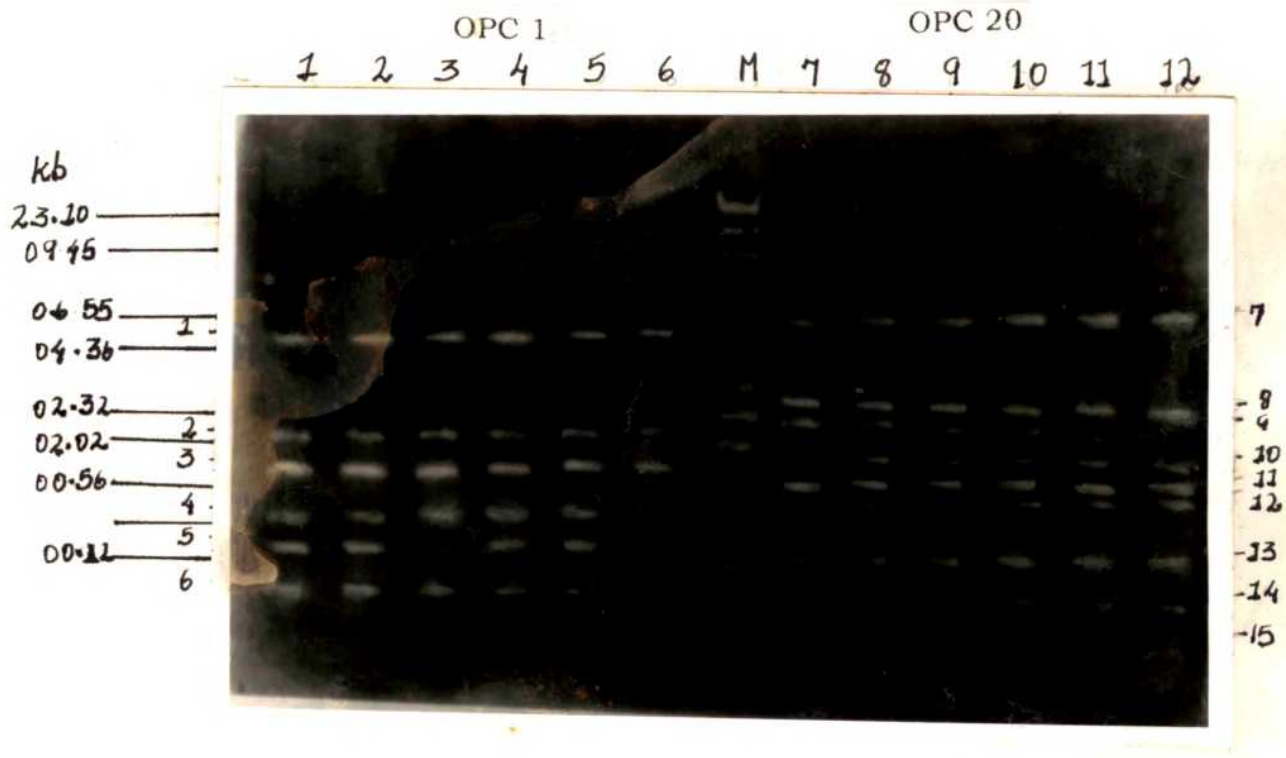
Lane 9 - Kulasekharam Green Dwarf (KGD)

Lane 10 - Chowghat Orange Dwarf (COD)

Lane 11 - Chowghat Orange Dwarf x West Coast Tall (D x T)

Lane 12 - East Coast Tall x Malayan Green Dwarf (T x D)

Plate 16


OPC 1
OPC 20
1 2 3 4 5 6 M 7 8 9 10 11 12
kb
23.10
09.45
06.55
04.36
02.32
02.02
00.56
00.11
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Band-14 was missing in the dwarf variety KGD (lane 9) Band 15 was seen only in the two dwarf varieties and hybrids (Lanes 9, 10, 11 and 12).

OPC 6 and OPC-8

Pattern of amplification produced by primer OPC-6 and OPC-8 is shown in plate 17. OPC-6 produced nine amplification products serially numbered from 116 to 24. Band-17 was absent in the KGD variety. Band 18 was missing in the tall variety ECT and in T x D hybrid (lane 2 and 6). Band 21 wass missing in the tall variety WCT (lane 1). Band 22 was missing in the KGD variety (lane 3).

OPC-8 produced 7 amplification products numbered serially from 25 to 37. Band 25 was seen only in the two tall varieties and the T x D hybrid (lane 7,8 and 12). Band 26 was seen in the tall varieties alone (lane 7 & 8) Band 29 was missing in the two tall varieties (lane 7 and 8) This primer could distinguish the tall varieties and dwarf varieties with respect to band 25, 26 and 29.

OPC-7 and OPC-14

Amplification pattern of OPC-7 and OPC-14 primers is shown in plate 18. OPC-7 produced 6 amplification products below 2 Kb Serially numbered from 32 to 37 Band 32 was seen only in the KGD variety and the D x T hybrids (lane 3 and 6).Band 35 was seen only in the two tall varieties and the T x D hybrid (lane 1, 2 and 6). Band 37 was absent in COD variety and hybrids (lane 4,5 and 6).

OPC-14 produced 7 bands serially numbered from 38 to 44. Band 38 and 39 was absent in both the tall varieties (lane 7 and 8) Band 40 was seen only in the ECT variety (lane 8). Band 43 was seen only in the COD variety and D x T hybrid. Band 44 was absent in the WCT variety.

Plate 17. RAPD profile for genomic DNA of coconut varieties with primers OPC 6 and OPC 8.

OPC 6

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

OPC 8

Lane 7		West Coast Tall (WCT)
Lane 8	-	East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 17

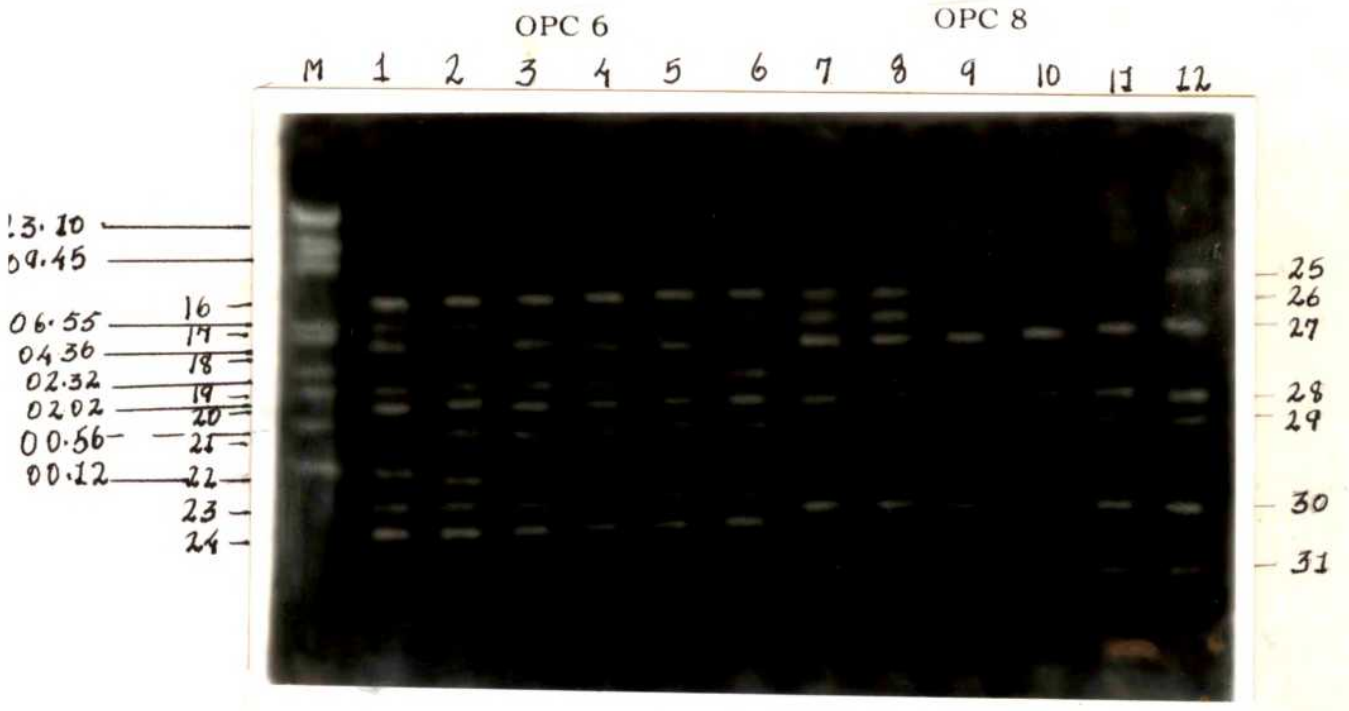

OPC 6
OPC 8
M 1 2 3 4 5 6 7 8 9 10 11 12
13.10
09.45
06.55
04.36
02.32
02.02
00.56
00.12
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

Plate 18. RAPD profile for genomic DNA of coconut varieties with primers OPC 7 and OPC 14

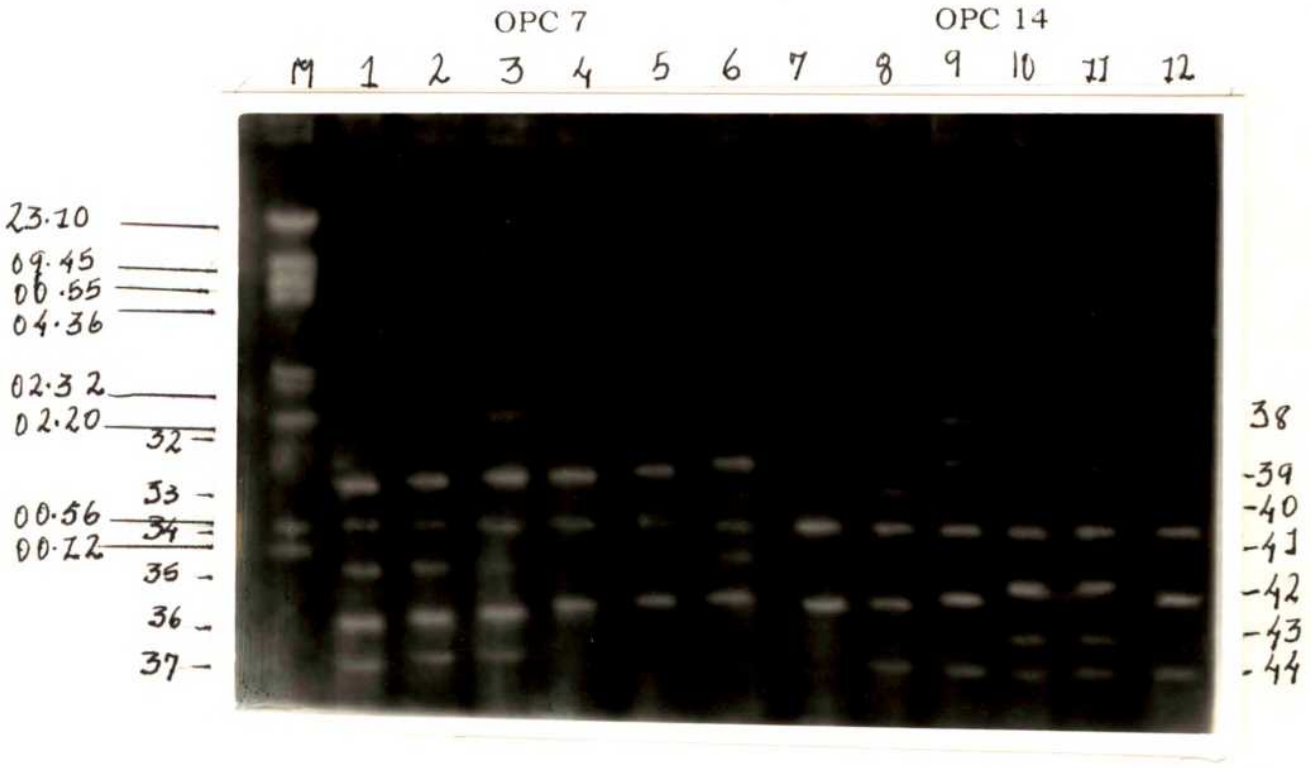
OPC 7

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

OPC 14

Lane 7	-	West Coast Tall (WCT)
Lane 8	-	East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 18


OPC 7
OPC 14
M 1 2 3 4 5 6 7 8 9 10 11 12
23.10
09.45
06.55
04.36
02.32
02.20
00.56
00.22
32
33
34
35
36
37
38
39
40
41
42
43
44

OPC-10 & OPC-11

. Pattern of amplification of DNA of the different coconut varieties with primers OPC-10 and 11 is given in plate 19. Primer OPC-10 produced only six bands (numbered from 45 to 50) but showed polymorphism in the different varieties. Band 45 was absent in the ECT variety (lane-2) and the two hybrids (lanes 7 and 8). Band 46 was absent in both the dwarf varieties (lane 3 and 4). Band 47 was absent in the varieties WCT and ECT. Band 49 was present in the KGD and COD variety alone. Band 50 was uniformly present in all the varieties.

OPC-11 produced ten amplification products serially numbered from 51 to 58. Band 51 and 52 were uniform for all the varieties Band 53 was absent in the WCT and ECT varieties (lanes 7 and 8). Band 55 was absent in the tall varieties (lane 7 and 8) and T x D hybrid also (lane 12). Band 56 was present only in the tall varieties WCT and ECT (lanes 7 and 8). Band 58 was absent in the KGD variety (lane 9). Band 60 was present only in the ECT, KGD and D x T hybrid (lanes 8, 9 and 11).

OPC-2 and OPC-4

Amplification pattern of DNA from the coconut varieties with primers OPC 2 and 4 is shown in the plate 20. OPC-2 primer produced six amplification products and was polymorphic for four products. The bands were numbered serially from 61 to 66. Band 61 and 64 were monomorphic. Band 62 was absent in the COD variety (lane 4). Band 63 was present only in tall varieties (Lanes 1 and 2), whereas band 65 was present only in the dwarf varieties. (lane 4 and 5). Band 66 was present only in the West Coast Tall Variety and the T x D hybrid (lane 6).

Plate 19. RAPD profile for genomic DNA of coconut varieties with primers OPC 10 and OPC 11

OPC 10

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

OPC 11

Lane 7	-	West Coast Tall (WCT)
Lane 8	-	East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 19

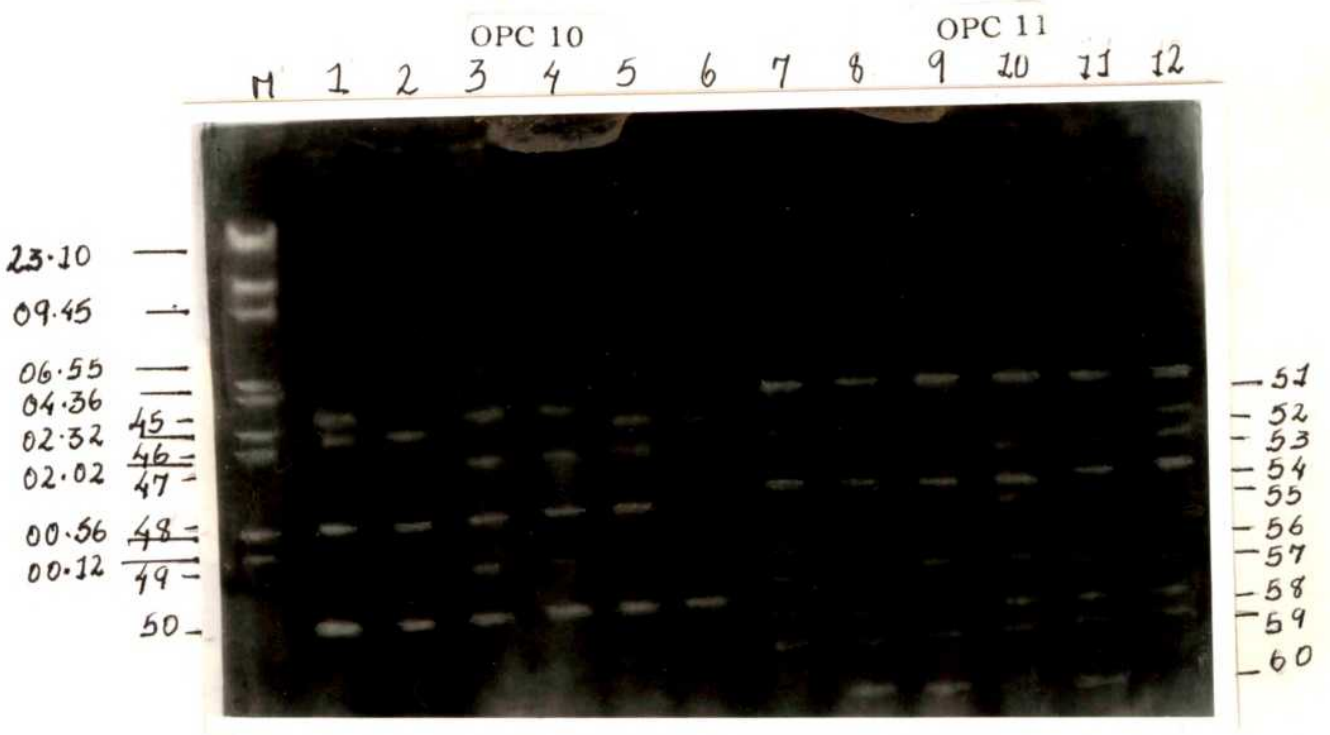

OPC 10
OPC 11
M 1 2 3 4 5 6 7 8 9 10 11 12
23·10
09·45
06·55
04·36
02·32
02·02
00·56
00·12
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Plate 20 . RAPD profile for genomic DNA of coconut varieties with primers OPC 2 and OPC 4

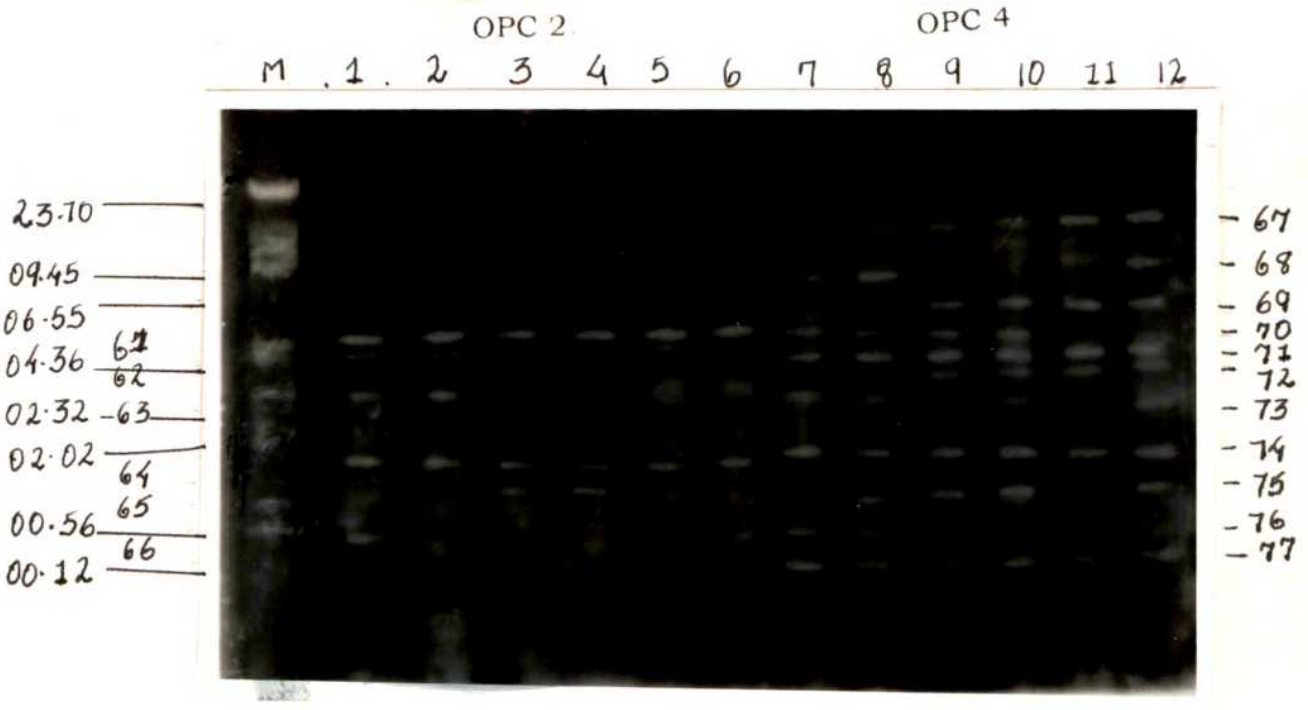
OPC 2

M	-	Lambda DNA/Hind III digest
Lane 1	-	West Coast Tall (WCT)
Lane 2	-	East Coast Tall (ECT)
Lane 3	-	Kulasekharam Green Dwarf (KGD)
Lane 4	-	Chowghat Orange Dwarf (COD)
Lane 5	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 6	-	East Coast Tall x Malayan Green Dwarf (T x D)

OPC 4

Lane 7	-	West Coast Tall (WCT)
Lane 8	-	East Coast Tall (ECT)
Lane 9	-	Kulasekharam Green Dwarf (KGD)
Lane 10	-	Chowghat Orange Dwarf (COD)
Lane 11	-	Chowghat Orange Dwarf x West Coast Tall (D x T)
Lane 12	-	East Coast Tall x Malayan Green Dwarf (T x D)

Plate 20


OPC 2
OPC 4
M 1 2 3 4 5 6 7 8 9 10 11 12
23.10
09.45
06.55
04.36
02.32
02.02
00.56
00.12
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77

Primer OPC-4 (lane 4) produced eleven amplification products of which eight products showed polymorphism. This primer could distinguish the Tall varieties, the Dwarf varieties and hybrids distinctively. Bands were serially numbered from 67 to 77. Bands 67, 71, 74 and 77 were monomorphic. Band 68 was present only in the two tall varieties (WCT and ECT) and the T x D hybrid (lane 6). Band 69 was present only in the dwarf varieties and the hybrids (lane 9, 10, 11 & 12). Band 70 was absent in hybrids. Band 72 was present in the Dwarf varieties and the hybrids (lanes 9, 10, 11 and 12). Band 73 was absent only in the D x T hybrids (lane 10). Band 75 was missing in the WCT and D x T hybrid (lane 7 and 11). Band 76 was present only in the two tall varieties.

Similarity indices among the 6 varieties based on the amplification products produced by all the primers are given Table 13. It is evident that the hybrids showed high similarity with both the groups of parents (Talls and Dwarfs). The dwarf varieties showed only around 75% similarity with the tall varieties. But the varieties within each other the two groups had more than 90% similarity among themselves.

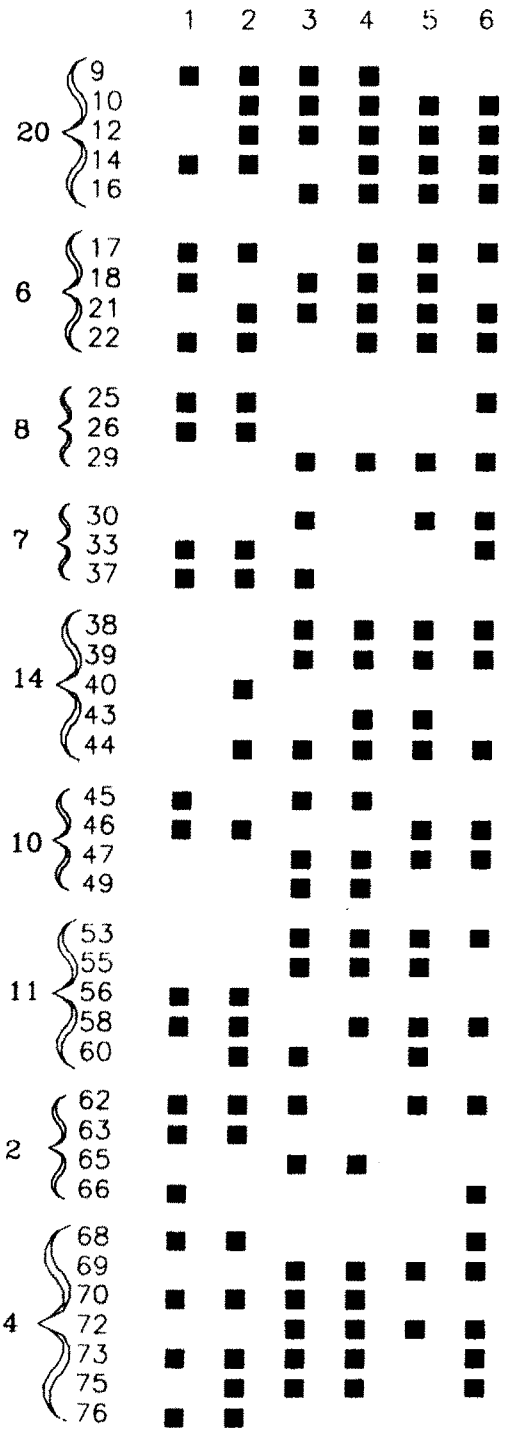
A graphical method of recording the RAPD data which allowed differences in genotypes generated by the different primers simultaneously is given in Fig 12. The distribution of product sizes after amplification with 10 primers is given in Fig 13. Dendrogram constructed based on similarity indices is given in Figure 14.

Table 13. Similarity indices for the DNA amplification products of coconut genotypes

	WCT	ECT	KGD	COD	D x T
ECT	0.96				
KGD	0.73	0.79			
COD	0.72	0.78	0.93		
D x T	0.79	0.83	0.88	0.91	
T x D	0.83	0.89	0.84	0.88	0.94

WCT - West Coast Tall
ECT - East Coast Tall
KGD - Kulasekharam Green Dwarf
COD - Chowghat Orange Dwarf
D x T - Chowghat Orange Dwarf x West Coast Tall
T x D - East Coast Tall x Malayan Green Dwarf

Fig.12. BAND MAP SHOWING POLYMORPHIC AMPLIFICATION PRODUCTS IN COCONUT GENOTYPES



PRIMER NUMBER AND THE POLYMORPHIC BAND NUMBERS INDICATED IN THE LEFT MARGIN

Fig.13. FREQUENCY DISTRIBUTION FOR THE SIZE OF GENOMIC AMPLIFICATION PRODUCTS

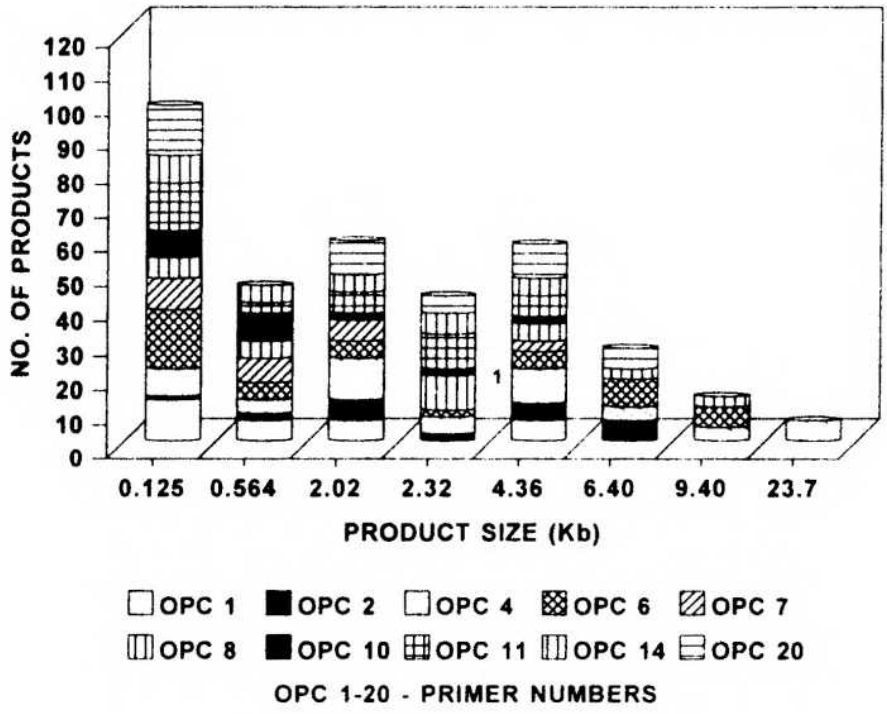
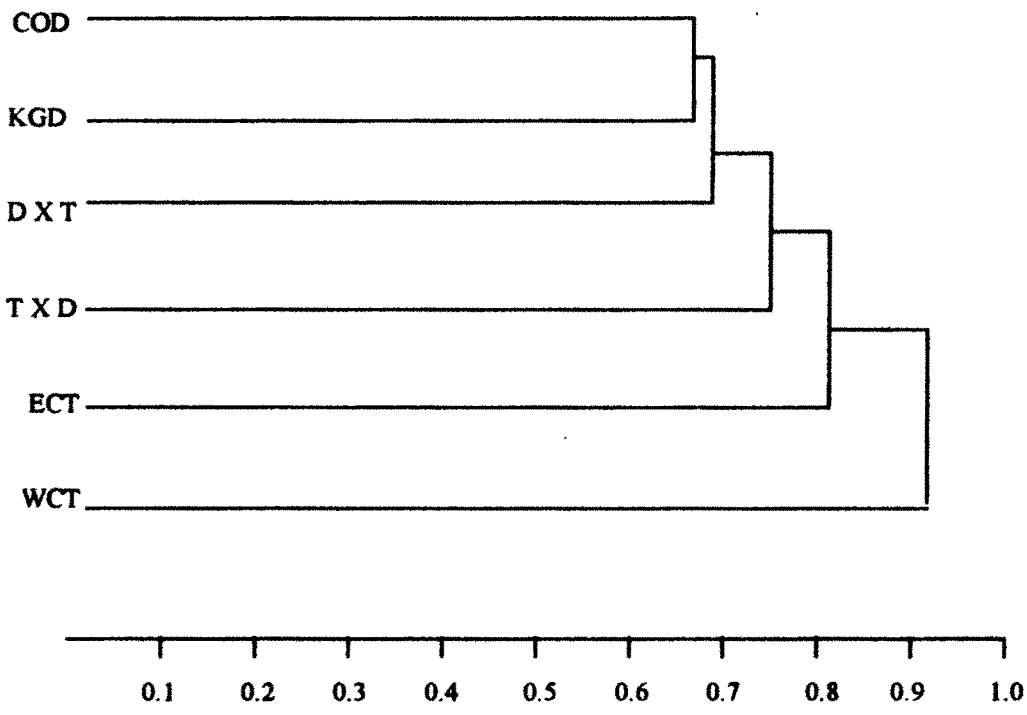


Fig 14. Dendrogram of coconut genotypes drawn using RAPD markers by UPAGMA-complete linkage maximum method



Discussion

CHAPTER V

DISCUSSION

Cocos nucifera L. is a monotypic species and tropical in distribution. Because of its highly outcrossing nature, several types widely differing from each other exist. Even though there were many attempts to differentiate the genotypes based on morphological descriptors, very few attempts have been made to assess the genetic diversity at the biochemical and molecular levels. With this objective, protein electrophoresis, isozyme analysis, RAPD and RFLP studies were conducted to assess genetic diversity in coconut, especially between the two size classes "Talls and Dwarfs" and their hybrids. Results obtained from each analysis are discussed in detail in this chapter.

5.1. Morphological markers

Coconut is a robust palm with tall slender and uniformly thick stem and massive crown with large number of leaves, bearing bunches of nuts in their axis. Variability exist among cultivars of Tall and Dwarf for many characters. To get a general awareness of the variability existing among the coconut cultivars and to have a better understanding of the coconut varieties, morphological data on 20 variables in 30 genotypes were analysed statistically.

5.1.1. Distribution of characters observed

All the variables studied can be used to distinguish the genotypes from each other. Certain characters (high F values) discriminate more than

others (low F values). Thus the variable relative to root bulb circumference (C-20) and the leaf character rachis length (RL) showed very high F values providing a clear separation of genotypes. **Root bulb** circumference giving clear separation of ecotypes in the tall population of the coconut palm was reported by N'cho **et al.** (1993). Pillai **et al.** (1991) reported that the coconut cultivars could be characterised and classified successfully based on leaf characters, stem characters, inflorescence characters and nut characters.

5.1.2. Relations between variables

The correlations between variables provided an idea of the degree of association existing between the different parameters measured. Most of the significant links existing between the characters studied are positive. These relations are particularly marked between stem characters, between leaf characters or between fruit variables. Thus for the stem a strong link can be seen (0.88) between the **Root bulb** circumference at 20 cm from the ground and the stem girth measured at 150 cm from the ground. These two characters are negatively linked to the leaf scars. Similar trends were reported by N'Cho **et al.** (1993). In tall coconuts indications are that root bulb formation determines stem sturdiness but due to this initial lateral growth, the rate of leaf emission is slowed down.

Yield (number of nuts/palm/year) was found to be positively linked to number of female flowers, point of insertion of first female flower on the spikelet and number of nuts per bunch. Theoretical leaf area had significant positive correlation with fruit weight. Earlier studies conducted in coconut palms have revealed that the length of the stem, number of leaves, length of leaves and number of flowers per bunch are correlated to yield

(Satyabalan **et al.**,1972; Abey Wardena, 1976 and Ramanathan, 1984).

5.1.3. Cluster analysis

The analysis presented herein grouped the 30 genotypes studied into 6 different clusters. All the dwarf varieties studied, were grouped in the I cluster. Tall varieties were resolved into 3 clusters III, V and VI. Hybrids were grouped along with the tall varieties in group except for the COD x WCT (which is a D x T hybrid) which was grouped along with the dwarf varieties. San Ramon and Andaman Giant formed a distinct cluster. Laccadive Micro was distinct from all the varieties and formed a separate cluster which was farther from all the remaining clusters. This study revealed that apart from the talls and dwarfs there are varieties like Laccadive micro, Andaman Giant and San Ramon which cannot be grouped in either of the two classes. Nair and Ratnambal (1994) have reported a separate group, the intermediate types which can include these varieties.

Varieties for biochemical and molecular studies were chosen from three clusters, Cluster I, III and VI. Dwarf varieties (KGD, KYD and COD) and the D x T hybrid (COD x WCT) from cluster I tall variety ECT and T x D hybrid (ECT x MGD) from cluster III and tall varieties Jamaica and WCT from cluster VI were chosen. Since the main objective was to differentiate the two size classes (Talls and Dwarfs), the intermediate types were excluded from the study.

5.2. Storage protein studies

Cocos nucifera, coconut palm is mainly cultivated for the endosperm or kernel within the nut. The main products produced from the kernel are coconut oil, copra (dried kernel) and the copra cake that remains

after oil extraction (Satyabalan, 1989). Copra has 20 percent protein by weight and is used for numerous edible products from confectioneries to milk substitutes (Satyabalan, 1989). So it is worth investigating the content of protein and protein fractions in different varieties.

5.2.1. Quantification of protein and protein fractions

Total protein content showed significant variation between the varieties. This is contrary to the finding of Sarangamath **et al.** (1983) who have reported that protein content did not show much variation among the cultivars. This may be due to the exclusion of hybrids which showed high content of protein in the particular study.

Among the different fractions, globulins (5% NaCl soluble proteins) was found to be the major protein fraction in coconut endosperm, followed by albumins, the water soluble protein. Balachandran and Arumugham 1992 also reported that 80 per cent of the protein content in coconut endosperm is contributed by albumins and globulins. There was significant variation between the cultivars for the three protein fractions globulins, albumins and glutelins. In coconut, the storage protein fraction and its variation between cultivars is yet to be investigated deeply.

5.2.2. Storage protein profile

Proteins being the primary products of genes, the variation in the protein profile in **Cocos nucifera** is worth investigating.

Total protein in coconut endosperm resolved into four major polypeptides ranging from 14 KDa to 66 KDa. The same was reported by Dem[illegible]son and Chandrasekhar (1990) and Balachandran and Arumugham

(1992). Albumins produced four major bands and globulins resolved into three major bands, both between 14 KDa and 66 KDa, this was also reported by Balachandran and Arumugham (1992).

Total storage protein profile did not show any specific variation between different cultivars with respect to the four major polypeptides. But the minor polypeptides showed variation. Sixth polypeptide was seen only in the two dwarf varieties and hybrids and was greatly diminished in the tall varieties. This polypeptide seen between 35 KDa and 45 KDa can be considered as a marker to distinguish between the Tall varieties and Dwarf varieties. But the absence of this polypeptide in one of the dwarf varieties points out the necessity to screen larger number of varieties (Talls and Dwarfs) for confirming this marker. Canto **et al.** (1992) also have reported that although dwarf and the tall varieties of coconut are closely related there are slight differences that permit a clear biochemical distinction between the Malayan Dwarf and Atlantic Tall trees.

In the albumin protein profile also major polypeptides did not differ between the varieties. But eighth polypeptide between 29 KDa and 24 KDa was very specific and seen only in the dwarf variety KYD and the D x T hybrid. In the globulin protein profile second polypeptide around 29 KDa molecular weight was present only in the tall varieties and the hybrid T x D. In the glutelin protein profile also fifth polypeptide was visible only in the tall varieties that too came within 29 KDa and 24 KDa range. Prolamine fraction of protein also showed polymorphism but the bands were very faint. Further investigation is required in the electrophoresis pattern of this fraction of protein in coconut endosperm. In the three

fractions studied (globulin, albumin and glutelin) there showed polymorphism in bands between molecular weights 29 KDa and 24 KDa. This region in the protein profile of the storage protein fractions should be studied in depth to confirm a biochemical marker for coconut cultivars. Koranyi (1989) had reported that albumin and globulin protein profiles are strongly variety dependent. Profiles of alcohol soluble seed proteins are frequently utilized for varietal description and the genetic control of these proteins is well established by Soare and Salamani (1983), and Gupta and Shepherd (1990).

5.3. Isozyme analysis

It is an established fact that most enzymes exist in multiple molecular forms which are governed by different loci. These enzymes with identical substrate specification occur within the same organism (Markert and Moller, 1959). The isozymes have been subjected to extensive investigation after the development of the zymogram technique by Hunter and Markert (1957). The large amount of variation detected in isozyme studies renders it useful as markers in a number of investigations.

In coconut, very few works have been done using isozymes as markers. Results obtained from three isozymes peroxidase, esterase and polyphenol oxidase are discussed below.

5.3.1. Peroxidase

Plant peroxidases are oxidoreductases that catalyze the oxidation of various donors by hydrogen peroxide. Most higher plants possess a number of different isozymes of peroxidases. Peroxidases in plants are involved in several physiological functions like wound healing, biosynthesis

of cell wall including lignification and sterilization of cell wall, organogenesis, growth regulation, auxin catabolism and senescence (Essen and Soost, 1974). Understanding the importance of this isozyme, its variability among coconut cultivars and hybrids were studied.

Assuming that isozymes observed in different zones are under the control of separate structural genes, gene symbol was assigned tentatively as prx A and prx B for the genes controlling the isozymes of zone 1 and 2 (Nagarajan, 1995). Isozymes in both the zones showed polymorphism between the varieties.

The prx A locus expressing two isozymes, showed polymorphism for one isozyme. Isozyme PRX 7 was seen only in the two tall varieties and the hybrids. PRX 22 was monomorphic. In the Prx B locus two isozyme PRX 42 and PRX 50 were found. PRX 42 found only in the two tall varieties and hybrids. PRX 50 isozyme also showed polymorphism. Three dwarf varieties were uniform for both loci. The T x D hybrid expressed the genes at both loci indicating codominant expressions of the isozyme locus. Such codominant expression of isozyme locus for hybrid identification was observed in **Digitaria** (Hayward and Hacker, 1980) in **Cucurbita** (Weeden and Robinson, 1986, in sesame (Parani, 1994) in green gram and black gram derivatives (Nagarajan, 1995). PRX 7 and PRX 42 could be developed as markers for confirming hybridity in coconut. But Canto **et al.** (1992) reported that peroxidase isozyme patterns were variable among the varieties and this was due to unknown factors other than genotypes of the variety.

5.3.2. Esterase

Esterases are hydrolysing enzymes which catalyse the addition or

removal of water in biological reactions. Esterases are widespread in nature and occur frequently in plants. Their variability in coconut cultivars was investigated in this study.

For esterase also two zones of activity were observed. The gene symbol was assigned tentatively as est A and est B for the genes controlling the isozymes of zone 1 and zone 2. In the locus est A, two isozymes EST 25 and EST 30 were seen. EST 25 was present in all the varieties except the two dwarf varieties. Since this isozyme had only medium activity and the other isozyme EST 30 was uniformly present. The locus est A should be considered monomorphic.

In the locus est B, there were five isozymes of which four isozymes EST 50, EST 55, EST 67 and EST 75 showed polymorphism. Neither of the loci could give a specific isozyme to distinguish the two size classes (talls and dwarfs) nor to confirm hybridity. Canto **et al.** (1992) reported esterase isozyme pattern in extracts from the inflorescence of four varieties (Atlantic tall, Malayan Green Red Yellow dwarfs) of coconut to be monomorphic.

5.3.3. Polyphenol oxidase

Polyphenol oxidase is a copper containing enzyme found in plants and in some animals. In higher plants, polyphenol oxidase seems to be associated with particular portions of the cell such as plastids and mitochondria and plays some role in the respiration of plants (Kaplan, 1968).

The isozymes could be visualised as low intense bands on the gel. This would be because when the enzyme catalyses the oxidation of catechol the rate is at first linear and during its action the enzyme becomes

inactivated by the O-quinone formed (Kahn, 1976) and hence the colour did not intensify.

In coconut, polyphenol oxidase showed only one zone of activity and the gene symbol ppoA was assigned tentatively to that zone. This locus had three isozymes PPO 35 and PPO 43. Among these PPO 43 isozyme was very specific and seen only in the dwarf varieties with high intensity. This was totally absent in the tall varieties and the T x D hybrid expressed a medium intensity of this isozyme. Shivashankar (1988) while studying the polyphenol oxidase isozymes in coconut genotypes under water stress showed a similar pattern of polyphenol oxidase isozymes when separated in poly acrylamide gel electrophoresis. PPO 43 isozyme in the locus ppo A could be taken as a marker for differentiating the Dwarf varieties and the Tall varieties and for confirming hybridity in the T x D hybrid. This locus also showed the codominant expression of the allele.

Therefore, isozyme analysis can be used for hybrid identification as they have the advantages; apart from codominant expression such as non-epistatic nature, stability in expression, ability to be assayed in a number of tissues and in a number of samples at a time with precision and free from environmental influence. In coconut, the isozymes peroxidase and polyphenol oxidase can be suggested for further investigation in confirming a marker for varietal identification and hybridity tests.

5.4. RFLP-analysis

The assessment of genetic variation at the DNA level is an essential prerequisite to the design of evolutionary breeding and genetic studies employing DNA probes. Construction of the genomic library and cDNA

probes from coconut genome is laborious and time consuming. So the genomic probes from oil palm were chosen for the study. Since both the crops belong to the family Palmae, there is a chance of getting hybridisation. Similar works had been reported by many authors. For example use of maize probes in sugarcane (Lu **et al.**, 1991) wheat and rice ribosomal probes in banana (Lanaud 1991) have been reported. About 80 percent of maize probes can be used to probe sorghum.

In the present investigation, autoradiogram developed showed faint signals. The signals confirm that the probe CL-54 could hybridise EcoR1 and Sau III digested coconut genomic DNA at one or two sites. Since hybridisation time, washing time and exposure time have not been standardised for the coconut crop, the present study can be considered as the first attempt for the RFLP analysis in coconut with heterologous probe from oil palm. Stringent washing would have washed away the loosely bound sites of hybridisation. Presence of the faint signals after the stringent washing indicates the strong hybridisation of that particular site with the probe. Due to the non-availability of radioactive material the experiment could not be repeated to give a conclusive evidence.

RFLP analysis though very efficient in assessing genetic diversity and confirming hybridity by codominant markers, the laborious nature of work and the use of hazardous radioactive material limits its wide use. So a simpler and easily repeatable RAPD analysis was adopted to trace the molecular markers.

5.5. RAPD analysis

DNA amplification based on RAPD markers were developed recently

(William **et al.**, 1990; Welsh and McCalland 1990; and CaetanoAnolles **et al.**, 1990). The random amplified polymorphic DNA (RAPD) reaction performed on genomic DNA with an arbitrary oligonucleotide primer results in the amplification of several discrete DNA products. These are usually separated on agarose gel and visualised by Ethidium bromide staining. The polymorphism between individuals result from sequence difference in one or both of the primer binding sites and are visible as presence or absence of a particular band. Such polymorphism in general behave as dominant genetic markers. The pattern differences existing between two species or varieties can be used for species or varietal identification. Also it can be used to study the pattern of introgression in hybrids.

The RAPD amplification products generated can be classified into two types: constant (monomorphic) and variable (polymorphic). These differences can be used to examine and establish systematic relationships (Hadrys **et al.**, 1992).

This study could standardise an optimum condition for the amplification of coconut DNA. Optimum composition of the cocktail, the thermocycler programme and the primer which could give amplification in the coconut DNA was standardised after repeated trials. In this study, RAPD turned to be a powerful tool to develop polymorphism in the coconut cultivars. RAPD technique in coconut pointed out many molecular markers to distinguish the two type classes (Talls and Dwarfs) and also to confirm hybridity. Ten random primers could develop a total of 77 amplification products in the coconut varieties. Of these more than 50 per cent of the products were found to be polymorphic. The band map as suggested by

Wilde **et al.** (1992), Figure 12 shows the extent of polymorphism in the different varieties.

The primers chosen for the study revealed the advantage of GC rich primers in bringing about amplification. Williams **et al.** (1990) tested a set of primers with GC content ranging from 0 to 100 per cent in the amplification of soybean genomic DNA to find that GC content of 40 per cent or more generated detectable levels of amplification products. Fukuoka **et al.** (1992) found that in rice increasing GC content in the range between 40 to 60 per cent tended to increase the number of amplification products. In this study, all the primers which gave good amplification had GC content of 60 per cent or more. The two primers OPC 13 and OPC 15 which did not give any amplification products had a GC content of 50 per cent only. But an increase in number of amplification products with an increase in GC content was not revealed.

Among the different primers studied OPC-4 and OPC-11 could generate highest number of amplification products, OPC-4 gave the highest percentage of polymorphic bands. Out of the 11 amplification products 7 were polymorphic. This primer could distinguish the tall varieties, dwarf varieties and hybrids distinctively. Presence of amplification products in one of the parents and in the hybrids confirm the dominant nature of RAPD markers. The main objective of this study was to detect markers at the molecular level which will distinguish the two type classes of coconut (Tall and Dwarf forms). Primers OPC-8 (amplification products 25, 26 and 29), OPC-14 (amplification products 38 and 39) OPC-10 (product 53), OPC-2 (products 61 and 65), OPC-4 (products 66,69,79,72 and 76) and OPC-20 (product 15) could clearly demarcate the tall varieties and dwarf varieties.

Thus out of the ten primers which gave amplification products, seven primers could give products showing polymorphism between the two type classes. This points out that there are differences between the two type classes at the molecular level. PCR generated RAPD marker have been shown to be as effective in identifying useful polymorphism in repetitive DNA as in low-copy DNA sequences (Williams **et al.**, 1990). So it should be assumed that the two type classes (Tall and Dwarf) differ in both repetitive as well as low copy DNA sequences. Nambiar and Swaminathan (1961) and Thankamma Pillai **et al.** (1982) observed significant differences in the meiotic behaviour between different cultivars of Tall and Dwarf forms.

Regarding the hypothesis advanced to explain the origin of dwarf coconut type no clear evidence could be obtained from this study. Comparative studies on the meiosis of Talls and Dwarfs (Sharma and Sarkar, 1956; Nambiar and Swaminathan, 1961; Ninan **et al.**, 1961; Abraham **et al.**, 1961) reported that the meiosis in the tall varieties was normal. It was therefore assumed that the talls were ancestral forms. If the dwarf varieties have originated from the tall varieties by continuous inbreeding then all the dominant RAPD markers should have been present in both the size classes. But in this study many amplification products were missing in the dwarf varieties and also many new amplification products were seen only in the dwarf varieties.

Williams **et al.** (1990) attributed polymorphism between genotypes to (1) nucleotide changes that prevent amplification by introducing a mismatch at one primary site. (2) deletion of a priming site, (3) insertions

that render priming sites too distant to support amplification and (4) insertions or deletions that change the size of the amplified product. Therefore these differences between the Tall and Dwarf forms would lead to the polymorphism in the RAPD analysis. Such polymorphism make RAPD markers well suited for studies on genetic diversity and genetic relationships. Many workers could use RAPDs in estimation of genetic variability in crop plants. Applications include finger printing of a genotype, identification of duplicate accessions and analysis of genetic diversity in a collection. This technique would, therefore be of high value for germ plasm characterization and genetic resource maintenance in coconut. Even today there is a lot of confusion among experienced coconut breeders in classifying certain cultivars/types. The classical example is Gangabondham variety. This is a typical dwarf cultivar which has been classed by some as semitall (Pillai **et al.**,1991). RAPD markers will be helpful to clarify the confusion in such cases.

Size of the amplification products varied with the primer but highest number of amplified products was seen in the 0 to 0.125 Kb range. Above 9 Kb only primer OPC-4 gave amplification products. In cocoa (**Theobroma cacao**) Wilde **et al.** (1992) reported that thirteen primers studied produced amplification products of different sizes upto 3 Kb in length.

Similarity indices and the dendrogram constructed based on RAPD markers shows similar results as in cluster analysis based on morphological markers. The D x T hybrid was placed near the dwarf varieties in both the studies and the ECT near the T x D hybrid which were grouped in the same clusters in cluster analysis. But the tall varieties, WCT and ECT which

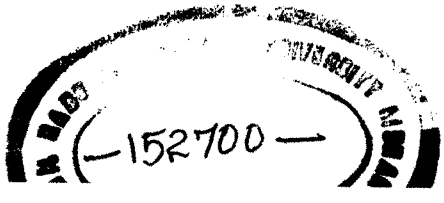
showed highest similarity index as per the RAPD analysis were grouped in two clusters in morphological analysis. This may be because of the high environmental influence on the morphological characters. However, the distance between the clusters was low.

RAPD proved to be an excellent technique to assess the genetic diversity in coconut. Since RAPD markers are dominant in inheritance, they will be efficient to screen the open pollinated seedlings for superior mother palm markers. For a perennial crop like coconut, screening of seedlings based on molecular markers will be advantageous as they are least influenced by environment. Use of RAPD markers for genotype discrimination, clonal stability, environmental stability and experimental reproducibility were studied in many perennial crops like oil palm (Shah **et al.**, 1994), apple (Koller **et al.**, 1993) and coffee (Lashermes **et al.**, 1993).

5.6. Comparative study of biochemical and molecular markers

The results obtained from protein electrophoresis, isozyme analysis, RFLP analysis and RAPD analysis indicate that variation exist among the varieties of coconut at the biochemical and molecular level. Molecular markers are essential for both identification purposes and diversity studies in **Cocos nucifera** especially considering the long duration of the crop by providing early selection markers. These markers yield quicker results from a few micrograms of leaf tissue which can be taken from one month old seedlings thereby considerably reducing the long duration of the breeding schemes and thus saving time, energy and resources. RAPD markers appear more striking than that obtained by isozymes. As suggested by Williams

et al. (1991), RAPD by virtue of the ability to study a large number of loci, can cover more of the genome thus leading to a better representation of the structuring of germ plasm diversity. Molecular markers will particularly assist coconut breeding programmes and basic genetic research and they will help to throw light into molecular biology and genome organization in coconut.



Summary

CHAPTER VI

SUMMARY

This investigation was undertaken to evaluate the variability existing among the coconut genotypes at the molecular level and to search for unique molecular / biochemical markers to distinguish the two type classes in coconut "Talls and Dwarfs" and to identify markers to assess hybridity.

As a basic step, the genetic variability existing was assessed in the different coconut genotypes. Morphological data on twenty characters for thirty genotypes were analysed statistically. The analysis revealed that all the genotypes differed significantly with respect to the twenty characters assessed. But the characters, collar girth at 20 cm from the ground level and rachis length showed very high F values providing a clear separation of the genotypes.Correlation studies showed that yield was positively correlated with number of female flowers, distance between the point of spikelet insertion on the axis to the first female and number of nuts/bunch. Plant height did not have significant correlation with any of the characters but had negative correlation with number of leaf scars.

The Cluster analysis resolved the thirty genotypes into six clusters. All the dwarf varieties were grouped in one cluster. Tall varieties were resolved into three clusters, cluster III, V and VI. Hybrids were grouped along with tall varieties except for the COD x WCT (D x T hybrid) which was grouped with the dwarf varieties. Laccadive micro formed a separate cluster farthest from all the clusters. Andaman Giant and San Ramon formed another cluster.

Quantitative assessment of protein and protein fractions revealed that all the genotypes studied differed with respect to the total protein content. Globulins and albumins constituted 80 per cent of the coconut endosperm proteins. Protein profile studies showed that total protein profile constituted with four major polypeptides ranging from 14 KDa to 66 KDa and eleven minor polypeptides. Albumins produced four major polypeptides and 7 minor polypeptides and globulins resolved into three major polypeptides both between 14 KDa and 66 KDa.

Total storage protein profile did not show variation between cultivars with respect to the four major polypeptides. But the minor polypeptides, showed variation. Sixth polypeptide between 35 KDa and 45 KDa was very much diminished in the tall varieties but was present in dwarf varieties. In the albumin and globulin protein profiles also, major polypeptides did not differ between the varieties. But the eighth polypeptide between 29 KDa and 24 KDa was seen only in the dwarf variety KYD and the D x T hybrid. In the globulin protein profile second polypeptide around 29 KDa molecular weight was present only in the tall varieties and the hybrid T X D. In the glutelin protein profile also fifth polypeptide was visible only in the tall varieties that too came within 29 KDa and 24 KDa range. In the three fractions studied (globulins, albumins and glutelin) there was polymorphism in the bands between molecular weights 29 KDa and 24 KDa.

Three isozymes peroxidase, esterase and polyphenol oxidase were studied. Results showed that peroxidase isozyme analysis detected two isozymes PRX 7 and PRX 42 only in the tall varieties and hybrids. They

could be developed as markers for confirming hybridity. Esterase isozyme analysis did not show much variation between the cultivars. Polyphenol oxidase analysis detected one isozyme PPO 43 present only in the dwarf varieties with high intensity. PPO 43 is a suitable marker for differentiating the tall varieties and dwarf varieties.

RFLP analysis revealed that the enzymes EcoRI and Sau III in combination with the oil palm genomic probe CL 54 can produce hybridization in the enzyme digested genomic DNA of coconut. Further investigation on this side is suggested to develop RFLP markers with suitable probes to differentiate genotypes in coconut.

RAPD analysis turned out to be an efficient tool for developing molecular markers in coconut genotypes. Optimum conditions for the amplification of coconut genomic DNA were standardised in this study. Ten primers could produce 77 amplification products of which 40 amplification products were polymorphic. All the primers which produced amplification products had more than 50 per cent GC content.

Among the different primers studied, OPC-4 and OPC-11 could generate highest number of amplification products, OPC-4 gave the highest percentage of polymorphic bands and out of the eleven amplification products seven were polymorphic. This primer could distinguish the tall varieties, dwarf varieties and hybrids distinctly. Primers OPC-8 (amplification products 25, 26 and 29) OPC-14 (amplification products 38 and 39), OPC-10 (amplification product 46) OPC-11 (amplification product 15), OPC-4 (amplification products 66, 69, 79, 72 and 76) and OPC-20 (amplification product 15) could clearly demarcate the tall varieties and

dwarf varieties. Thus out of the ten primers which gave amplification products seven could give amplification products, showing polymorphism between the two type classes, Tall and Dwarf. This points out that there is difference between the two type classes at molecular level.

Size of the amplification products varied with the primer and the highest number of amplification products was seen in the 0 to 0.125 Kb range. Similarity indices based on RAPD markers between varieties indicated that the hybrids showed high similarity with both the group of parents. The dwarfs showed 75% similarity with tall varieties. Within each group of tall and dwarf varieties, 90% similarity was evidenced. The dendrogram constructed based on the RAPD markers shows results similar to the outcome as in cluster analysis based on morphological markers. In both the studies D x T hybrid was placed near the dwarf varieties and the tall variety ECT near the T x D hybrid. RAPD markers are suggested as efficient means to screen the open pollinated seedlings for superior mother palm characters.

The results obtained from the protein electrophoresis, isozyme analysis, RFLP analysis and RAPD analysis indicate that variation exist among the varieties of coconut at the biochemical and molecular level. Molecular markers are essential for both identification purpose and diversity studies in **Cocos nucifera**, especially considering the long duration of the crop by providing early selection markers. These markers yield quicker results from a few microgram leaf tissue which can be taken even from one month old seedlings thereby considerably reducing the long duration of the breeding schemes and thus saving time, energy and resources.

RAPD markers appeared more efficient than the other markers (protein, isozymes and RFLP markers) in developing polymorphism. By virtue of its easiness, stability and repeatability and ability to study a large number of loci they can cover more of the genome thus leading to a better representation of the structuring of germ plasm diversity. Molecular markers are particularly assisting coconut breeding programmes and basic genetic research and they will help to throw light into the molecular biology and genome organization in coconut.

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