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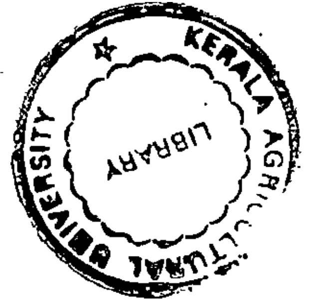
# ISOZYME VARIATION IN *Areca catechu* L. AND ALLIED SPECIES




By

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**THESIS**

*Submitted in partial fulfilment of the requirement for the degree of*

## Master of Science in Horticulture

*Faculty of Agriculture*
*Kerala Agricultural University*

Department of Plantation Crops and Spices
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680656
KERALA, INDIA

**2002**

# DECLARATION

I hereby declare that this thesis entitled "**Isozyme variation in *Areca catechu* L. and allied species**" is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
10. 12. 2002

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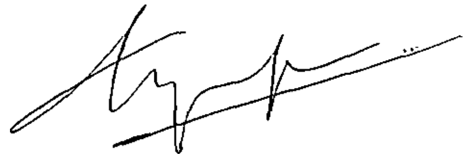
# CERTIFICATE

Certified that this thesis entitled "**Isozyme variation in *Areca catechu* L. and allied species**" is a record of research work done independently by **Mr. A. Arul Swaminathan** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to him.



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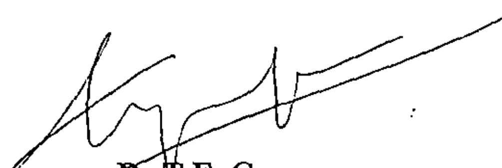
We, the undersigned members of the Advisory Committee of **MR. A. Arul Swaminathan**, a candidate for the degree of Master of Science in Horticulture with major in Plantation Crops and Spices agree, that the thesis entitled **"Isozyme variation in *Areca catechu* L. and allied species"** may be submitted by **MR. A. Arul Swaminathan**, in partial fulfilment of the requirement for the degree.



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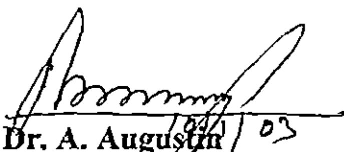

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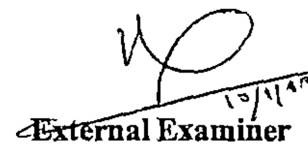

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*A. Arul Swaminathan.*

**Dedicated to**

MY BELOVED FATHER

*(Late) Head Master Mr. K. Angamuthu*

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# *Introduction*

# 1. INTRODUCTION

Arecanut palm (*Areca catechu* L.) is the source of common masticatory nut, popularly known as arecanut, betelnut or suparinut. Its nuts are extensively used in various forms in India by all sections of people as a masticatory and also find use as an essential component of several socio-religious ceremonies. Consequently, the arecanut palm occupies a prominent place among the cultivated crops in the states of Karnataka, Assam, Kerala, Maharashtra, Goa, Tamilnadu, Meghalaya and Andaman & Nicobar Islands.

Arecanut is indigenous to Philippines. In India, arecanut palm occupies an area of 2.22 lakh hectares with an annual production of 3.34 lakh tonnes. Karnataka stands first in area and production followed by Kerala and Assam. India is the largest producer as well as consumer of arecanut in the world.

Byproducts like tannin from immature nuts, fat from matured nuts, products made from husk and biodegradable cups and plates made from leaf sheath etc., fetch good market price. Biodegradable products from arecanut palm have excellent export potential owing to its eco-friendly nature. In addition to these, the trunk also has got good demand in furniture industry.

The genus *Areca* comprises of 76 species including the arecanut palm. Taxonomic history of the genus gives ample evidence of several anomalies and discrepancies with respect to classification, typification, identification and keying out the species. Tremendous variation exists with respect to growth parameters and yield attributes among varieties / cultivars / types of *Areca catechu* which has been morphologically, anatomically, cytologically and biometrically characterized (Bavappa and Ramachander, 1967a). However, information on biochemical characterization in general and isozyme variation in particular of these genotypes is lacking at present.

Martius (1850) was the first to attempt to restrict the limits of the genus *Areca.* However, this attempt was not satisfactory, as the limitations were not based on real affinities. The genera now recognized as *Dictyosperma, Dypsis, Acanthophoenix* etc., were retained in the genus *Areca,* while the closely related palms were excluded from it.

The yellow leaf disease (YLD) remains today as the most serious malady affecting the crop. This disease known as *"Manjalippu" in* Malayalam was reported from several parts of Kerala about a century back (Nambiar and Sreenivasan, 1951). The exact cause of the disease is yet to be known. However, reports indicate the association of fungi, bacteria, virus, MLO, mites, nematodes and also soil and nutrient factors with the disease. The screening trials revealed the tolerance of Hirehalli Dwarf and the field tolerance of Indonesia 2 and British Solomon Islands 1 to YLD.

Studies on phylogeny and evolution are essential pre-requisites for scientific crop improvement. Isozyme variations are used as a powerful tool to complement and supplement the conventional phylogenetic studies (Rick *et al.* 1977 and Gottileb, 1977).

The advantages of isozyme analysis over phenotypic characterization are manifold. Isozymes are basic biochemical constituents which are not affected either by direct selection pressure during the course of domestication and evolution of a taxon or by environmental factors and hence provide a more accurate picture of the original variation present in the population. Further, the relative ease of electrophoretic analysis makes isozyme studies a potent tool in phylogenetic investigations.

Isozyme variations are being used for complementing and supplementing the conventional biological studies. Isozymes are generally direct products of single locus and relating of phenotypic variations with genotypic characters is relatively easier. The zymograms pertaining to various genotypes will divulge the exact genomic

position of different varieties and hence screening of genotypes through isozyme markers will be more reliable for disease resistance/tolerance.

In this backdrop, a research project titled " Isozyme variation in *Areca catechu* L. and allied species" was undertaken in order to supplement and verify the information of the inter-relationship among the species of *Areca* and varieties / cultivars / types of *Areca catechu*. The study has also attempted to bring out the possible association of isozyme banding patterns with the tolerance reaction to yellow leaf disease in the tolerant types.

# Review of Literature

## 2. REVIEW OF LITERATURE

Arecanut (*Areca catechu* L.), the source of common masticatory nut is grown largely in the states of Karnataka, Assam, Kerala, Maharashtra, Goa, Tamilnadu, Meghalaya and Andaman & Nicobar Islands. India is the largest producer and consumer of arecanut and it continues to dominate the world scenario in area under cultivation, production and productivity. Eventhough the government policies discourage area expansion, the area under arecanut has substantially increased because of the good market for the produce (Ananda, 2002).

Despite being grouped under the major plantation crops, arecanut is yet to get its due importance in the research front as compared to other plantation crops regarding the characterization of the genus especially in the biochemical aspects like isozymes. There has been some work done by Bavappa and Ramachander (1967a) to characterize the cultivars, but only morphologically.

Yellow leaf disease of arecanut has been a threat to arecanut industry since 1951, as reported by Nambiar and Sreenivasan. There were several reports regarding the causal factors for this disease viz., fungi, bacteria, virus and MLO and also soil nutrient status. Research conducted for the protein profile of the genus revealed ample variation revealing the scope to go for the early diagnosis of the disease (Menon, 1960a).

The literature has been reviewed from taxonomy to evolutionary significance of the genus *Areca*. Since there is not much literature available on isozyme studies in arecanut, the literature pertaining to other members of the family Palmae has been reviewed extensively. Pertinent literature on isozymes in other horticultural crops as well as some major field crops is also included.

In addition to this, the literature related to yellow leaf disease has also been incorporated in this chapter.

## 2.1 TAXONOMY

Arecanut palm (*Areca catechu* L.), also known as betel nut palm is a widely cultivated commercial crop of tropical Asia. Linnaeus (1753) classified *Areca* as a monospecific genus and *Areca catechu* L. was the only species included in this genus.

Benthem and Hooker (1883) in their treatise Genera Plantarum described the genus as the first one in the family Palmae under the tribe Areceae. The genus expanded rapidly from its monospecific status and is at present believed to contain about 76 species. Among these, *Areca catechu* is the only cultivated species, the nuts of which are chewed as a mild stimulant though nuts of a few other species such as *Areca triandra* Roxb. are also used as a masticatory.

Bavappa (1963) studied the distribution pattern of *Areca* species and observed that the contiguous areas of Malaya, Borneo and Celebes having a maximum of 24 species could well be the area under the greatest diversity where wealth of forms of *Areca* are concentrated. Thus, the East Indies group of islands may be taken as the centre of maximum variation.

The range of variation in flowers and size and shape of fruits in different cultivars of *Areca catechu* L. occurring in Assam was described by Raghavan and Baruah (1956). Murthy and Bavappa (1962) identified the cultivars from Kerala, Karnataka and Maharashtra based on fruit size and discussed the pattern of variation in relation to the topography of the tract.

Based on variation in number and size of nuts and stomatal characters pertaining to four cultivars of *A. catechu*, Bavappa (1966) concluded that cultivars could be identified on the basis of number of stomata per unit area.

Bavappa and Pillai (1976) found highly significant differences in respect of number of leaves shed, spadices and female flowers produced, nut set, number of nuts harvested and weight and size of nuts among 13 cultivars of *A. catechu* from eight

countries. Apart from *Areca catechu*, there are 43 other species noted in the monograph of arecanut. In *Areca*, two subgenera are there namely *Burmeoareca* and *Beccariareca*. *Burmeoareca* contains three sections and 31 species and *Beccariareca* contains two sections and 12 species.

There has been some speculation regarding the origin of the genetic name *Areca*. The hypothesis of De-Candole (1886) with regard to its origin from "Telenga name" did not receive much supporting evidence in literature (Bavappa, 1964). The possibility of the generic name coined by Linnaeus based on popular Malayalam name 'Adeka' or a variant Kannada name was indicated by Bavappa (1964). According to Mc Curraach (1960), the name *Areca* was derived from a Malayan word meaning "cluster of nuts".

### 2.1.1 Limits of Genus *Areca*

Martius (1850) was the first to restrict the limits of the genus *Areca*. However, the attempt was not satisfactory, as the limitations were not based on real affinities. The genera now recognized as *Dictyosperma, Oncosperma, Dypsis and Acanthophoenix* etc., were retained in the genus *Areca*, while the closely related palms were excluded from it.

Blume (1836) separated various species hitherto grouped under *Areca* into different genera, based on the nature of albumen, the position of ovule, the distribution of male and female flowers on the spadix and limited the genus to close relatives of the type *Areca catechu*.

Blume's arrangements however were not accepted by Martius (1850), Griffith (1850) and Miquel (1868, cited by Furtado, 1933) among others. Benthem and Hooker (1883) listed 24 species under *Areca* and disagreed as to the limits placed by Scheffer (1871, cited by Furtado 1933) and Beccari (1919). In ascribing only 14 species to the genus *Areca*, Drude (1889, cited by Furtado, 1933) followed Benthem and Hooker

(1883) in excluding *Mischophloeus* from *Areca*, but included only those species, which Scheffer (1871, cited by Furtado 1933) and Beccari (1919) had retained under the genus.

The views of Benthem and Hooker (1883) and Drude (1889) have been followed by Blatter (1926). But Furtado (1933) found it impossible to maintain *Mischophloeus* as a genus and amalgamated it with *Areca*. For the sake of convenience and for the purpose of bringing out better affinities he divided the reconstituted genus *Areca* into two subgenera *Blumeoareca* and *Beccarioareca*. The character that distinguishes these two subgenera is the arrangement and glomerules of the male flowers being unilateral or distichous in *Blumeoareca* and spiral in *Beccarioareca*.

Furtado (1933) again subdivided *Blumeoareca* into three sections *Arecella* Wendl.et Drude, *Oeotheanthe* Scheff. and *Axonianthe* Scheff. The subgenus *Beccarioareca* was also divided into two-sections, the first section was called *Microareca* Furtado, consisting of small plants known to occur only in Malay Peninsula, Lingga Island and Borneo. The other section *Mischophloeus* (Schef.) Becc. included massive palms known only from region between Celebes and the Solomon islands.

### 2.1.2 Cultivars of *A. catechu*

Rau (1915) described a cultivar of arecanut from Mysore based on the sweet kernels of mature fruits and designated it as *A. catechu* var. *deliciosa*. Beccari (1919) recognized four cultivars of arecanut from the Philippines and termed them as *A. catechu* var. *communis.*, *A. catechu* var. *silvatica.*, *A. catechu* var. *batanensis* and *A. catechu* var. *longicarpa* based on the size and shape of the fruits and kernel. Cultivars available in Malaya, Sri Lanka and South India have been designated by local names (Sands, 1926, Grist 1926, Molegode 1944, Nambiar 1954, Aiyer 1966). According to Kanangara (1941), there are apparently no distinct varieties in arecanut in Mysore though some palms are bearing yellow and green fruits.

The occurrence of a dwarf arecanut palm was reported by Naidu (1963) from Hirehalli (Karnataka). According to the description, the 40-year-old mutant palm had attained a height of only 4.57 m and had suppressed internodal spaces so that the annular scars appeared as superimposed. The inflorescence and floral characters were similar to *A. catechu*. The nuts were of medium size and slightly elongated.

## 2.2 CYTOGENETICS

### 2.2.1 Chromosome Number

The chromosome number of *A. catechu* L. was first determined and reported by Venkatasubban (1945) as 2n= 32. The chromosome number of the species was later confirmed by Sharma and Sarkar (1956), Raghavan and Baruah (1958), Abraham *et al.* (1961) and Bavappa and Raman (1965).

A chromosome number of 2n=32 reported by Darlington and Jananki Ammal (1945) for *A. triandra* Roxb. and was later confirmed by Sharma and Sarkar (1956) and Bavappa and Raman (1965). Nair and Ratnambal (1978) determined the meiotic chromosome number of *A. macrocalyx* Becc. as n=16.

### 2.2.2 Meiosis

Meiotic abnormalities such as non-disjunction lagging chromosomes, univalents and pentads were reported in *A .catechu* by Sharma and Sarkar (1956). Bavappa and Raman (1965) observed in the meiosis of four ecotypes of *A. catechu,* abnormalities like univalents at diakinesis and metaphase I, non-synchronoisation of orientation, clumping, delayed disjunction, chromosome bridges and laggards at anaphase I and II, chromosome mosaics and supernumerary spores.

Sharma and Sarkar (1956) found the meiotic division quite normal in *A. triandra* except for the presence of 14 and 18 chromosomes occasionally at metaphase II.

Bavappa and Raman (1965) also reported regular meiotic division in the types of *A. triandra* studied by them.

Intra cultivar variation in meiotic behavior of *A. catechu* was reported by Bavappa (1974) and Bavappa and Nair (1978). While normal bivalent formation was observed in some palms, others had maximum association of hexavalents, octavalents and even decavalents. Abnormalities like bridges, laggards and disorientation of chromosomes at anaphase I and anaphase II were also reported in this species.

Intra palm variation in chromosome numbers in the pollen mother cells of *A. catechu*, *A. triandra* and their hybrids was reported by Bavappa and Nair (1978) and cytomixis to the extent of 39 per cent seemed to have contributed to this abnormality. In spite of high degree of multivalents in *A. catechu*, pollen fertility was very high. The possibility of the frequency of multivalent formation and disjunction being under genotypic control and being subjected to selection was suggested by Bavappa and Nair (1978).

Nair and Ratnambal (1978) reported chromosome association in *A. macrocalyx* during microsporogenesis. While 16 bivalents were of the highest frequency at diakinesis and metaphase I, the maximum configuration observed was one hexavalent at both stages of division. The chromosome association in *A. macrocalyx* indicated the possibility of autopolyploid origin with restricted multivalent formation as in the case of *A. catechu* and *A. triandra.*

### 2.2.3 Karyotype

Venkatasubban (1945) observed two pairs of short satellite chromosomes in the somatic chromosome complement of *A. catechu*. Three pairs of long chromosomes, six pairs of medium sized chromosomes and seven pairs of short chromosomes were observed by Sharma and Sarkar (1956) in *A. catechu*. They categorized the chromosomes into seven groups based on their morphology and relative length. Two pairs of long chromosomes next to the longest were found to have secondary

constrictions. They also observed that the chromosomes of *A. triandra* were longer than those of *A. catechu.*

Bavappa and Raman (1965) found the chromosomes of *A. catechu* and *A. triandra* differing in size, total chromatin length, position of primary and secondary constrictions and number and position of satellites. Based on the assumption of Sharma and Sarkar (1956) that gradual reduction in chromatin matter had taken place in the evolution from primitive to advanced from different genera and tribe of *Palmae*, Bavappa and Raman (1965) considered *A. catechu* as more advanced than *A. triandra.*

The chromosome morphology of a few cultivars of *A. catechu* from Assam was reported by Raghavan (1957). Minor variation in structure and length of individual chromosomes, total length of the complement and position of constrictions among the types was noticed by him. On the basis of morphology, he recognized nine groups in the somatic chromosomes of the cultivars.

Studies on the karyotypes of eight cultivars of *A. catechu* and four ecotypes of *A. triandra* (Bavappa, 1974) revealed considerable differences in their gross morphological characteristics. The karyotypes of the *A. triandra* ecotypes showed a higher frequency of submedian and median chromosomes as compared to *A. catechu.* A classification of the karyotype of the two species according to the degree of their asymmetry which recognized three grades of size differences and four grades of asymmetry in centromere position (Stebbins, 1958) showed that karyotypes 1B, 2A, 2B and 3B were represented in *A. catechu* and only 1A, 2A and 2B were represented in the ecotypes of *A. triandra.* Even within the same cultivar of *A. catechu* two different types of asymmetry in karyotypes were observed while there was no such variation in *A. triandra* ecotypes. Evidently, *A. triandra* had a more symmetrical karyotype than *A. catechu.* It was concluded that delineating the cultivars of *A. catechu* on the basis of standard karyotype seemed to be rather difficult. The fact that *A. catechu* had lesser chromatin matter and asymmetrical karyotype compared to *A. triandra* showed that the latter was more primitive.

### 2.2.4 Basic Number

Based on the cytogenetical studies on different genera of Palmae, Venkatasubban (1945) suggested a basic number of x=7 and 9 derived from x=8 by the fusion and fragmentation respectively. Darlington and Janaki Ammal (1945) proposed x=16 as the basic number for *Areca*. Sharma and Sarkar (1956) stressed the role of amphidiploidy in the initial stages of evolution of the tribe *Areceae* and deduced a basic number of x=8 for the tribe.

A basic number of x=7 was assumed for *Areca* by Bavappa and Raman (1965) based on the secondary association and karyomorphological data. They could recognize seven groups in the chromosomes complement of *A. catechu* as distinguished by the length and morphology of somatic as well as pachytene chromosomes and concluded that *A. catechu* is a secondary allotetraploid.

## 2.3 BIOMETRICAL STUDIES

### 2.3.1 Correlation and Heritability

In an attempt to establish relationship between vigour of the seedlings and subsequent yield of arecanut palm, Bavappa and Ramachander (1967a) worked out phenotypic and genotypic correlation between some of the morphological characters of the seedlings at the time of planting as well as one and two years after, with the yield in the first four years of bearing. It is observed that morphological characters like number of leaves, girth at collar and height at the time of planting were phenotypically correlated with the yield during the first year of bearing only. Characters recorded one and two years after planting had significant positive correlation with yield in all the four years except for the girth at last exposed node for the second year.

Genotypic correlation worked out with yield during the first four years of bearing showed that the number of leaves at the time of planting, girth at collar, one

year after planting and number of nodes two years after planting had significant positive correlation with yield during all the four years.

Bavappa and Ramachander (1967b) observed very low heritability for yield (0.20) in arecanut and hence, practically no improvement in yield could be achieved by direct selection. They tried to identify characters having high heritability as well as correlation with yield. Among the 11 characters considered, age at first bearing alone had high heritablity and correlation with yield. The percentage of inflorescences to leaves shed and number of inflorescence produced also had high heritability. But in view of genotypic correlation of these characters with yield, selection based on these two characters would not help in improvement of yield. Percentage of nut set was highly correlated with yield but the heritability was relatively low. Eventhough the mean weight of nut was negatively correlated with yield, in the absence of a threshold value the total weight of nuts produced increased with the number of nuts and this negative correlation did not set a limit to the possible yield improvement (Bavappa and Ramachander, 1967b).

## 2.4 GENETIC DIVERGENCE

Bavappa (1974) recorded morphological, anatomical and yield characters for 13 cultivars of *Areca catechu* L. and four ecotypes of *A. triandra* for the years 1963,1966 and 1972. The analysis of variance obtained in 1963 showed that the differences between the cultivars were highly significant for all the six morphological characters. A combined analysis of the data for two years for 24 common characters recorded during 1967 and 1972 also revealed significant difference between cultivars for all characters.

A significant interaction between years and cultivars was seen in the case of height, girth, internodal distance, number of bunches and inflorescence on the palm, length and breadth of leaf sheath, length and volume of the nut and length, breadth, weight and volume of kernel.

## 2.5 $D^2$ ANALYSIS

Bavappa (1974) worked out 136 $D^2$ values between cultivars, the number of characters being unequal in different years. The magnitude of $D^2$ values indicated that considerable divergence existed between many of the cultivars in all the years. He grouped 13 cultivars and four ecotypes from nine countries into six clusters for the independent years 1963,1966 and 1972 and found that though the number of clusters was the same, constituents in the different clusters were slightly different in different years. The number of clusters and pattern of clustering were more or less similar for the years 1966 and 1972. In the pooled analysis the number of clusters got reduced from six to five. However, the pattern of clustering was more or less in conformity with the groups obtained for the individual years.

All the four ecotypes of *A. triandra* were in one cluster in the pooled analysis and this cluster continued to show maximum divergence from the rest. The divergence between clusters IV and V was due to the differences in nut and kernel characters, breadth of leaf sheath, breadth of leaflets and number of leaflets. Bavappa (1974) based on the analysis concluded that detection of the genetic divergence in the early years of the productive phase is of considerable advantage in formulating breeding programme in a perennial crop like arecanut.

The ranking obtained by the different characters during 1966 for their contribution towards overall genetic divergence showed that the mean volume of nut and breadth of the kernel were the characters of primary importance. For divergence between *A. triandra* and *A. catechu*, mean length of fruit was found to be second in importance next only to volume of nut. The results of characters from 1972 and the pooled data also revealed the importance of nut and kernel characters in differentiation within *A. catechu* cultivars and between *A. catechu* and *A. triandra* types. The results obtained from canonical analysis were also in broad agreement with the clustering pattern found from $D^2$ analysis. However, Bavappa (1974) concluded that the

canonical analysis could be of only limited utility in view of the fact that the first two canonical roots accounted for only 85 per cent or less of the total variation.

The grouping obtained by $D^2$ analysis revealed that the three cultivars each from Saigon and British Solomon Islands and the two ecotypes of *A. triandra* from Indonesia were invariably in one cluster each. As against this, close similarity between the cultivars from different countries has also been observed. The cultivar from Singapore got grouped with the three cultivars from Saigon in one cluster. A similar affinity between the two geographically distant cultivars was shown by Ceylon-1 and Indonesia-6 both always coming within the same cluster. Of the two cultivars of *A. catechu* from Ceylon, 'Ceylon-2' was always forming a separate cluster indicating its distinct nature of divergence. The clustering pattern of cultivars and ecotypes revealed that geographic diversity need not always be related to genetic diversity (Bavappa, 1974).

## 2.6 EVOLUTIONARY SIGNIFICANCE

Based on the clustering pattern of certain cultivars of *A. catechu* from countries such as India, Sri lanka, Singapore, Indonesia and Saigon, Bavappa (1974) deduced that probably both *A. catechu* and *A. triandra* had their origin in group of islands in Indonesia as concluded earlier (Bavappa 1963, Corner 1966). He presumed that probably these species moved to west through Malaysia to India, Srilanka and Mauritius all through maintaining their specific identity while *A. catechu* found its way to North Saigon as well.

Bavappa (1974) also deduced the evolutionary course of *A. catechu, A. triandra* and *A. concinna* on the evidences of their distribution similarities of synthetic hybrid between *A. catechu* and *A. triandra* to *A. concinna* and also the natural occurrence of *A. catechu* and *A. triandra* hybrids. Based on the available information he concluded that probably *A. catechu* and *A. triandra* were the putative parents of *A. concinna*.

## 2.7 CHARACTERS OF THE SPECIES/VARIETIES//TRADITIONAL CULTIVARS USED IN THE STUDY

### 2.7.1 *Areca* Species (Murthy and Pillai, 1982)

#### *2.7.1.1 A. catechu*

Stem erect, smooth green in upper portion, annulate. Leaves pinnate, base of the petiole expanding into smooth green amplexicaul sheath. Leaflets thin, often confluent, with several midribs attached to the rachis in a vertical line. Spadix androgynous, below the leaves, branched, bearing numerous closely set spikes, spathes several. Male flowers many, minute, occupying the upper portion of the spikes. Sepals small, petals much longer, obliquely lanceolate, valvate, stamens 3-6. Filaments short. Anthers basified, erect. Female flowers much larger, few at the base of the spikes, perianth accrescent, sepals and petals orbicular, imbricate, the petals with acute valvate tips. Ovary one celled. Stigma 3, sessile. Ovule1, basal, erect. Fruit ovoid or oblong supported by persistent perianth, mesocarp fibrous. Seeds with a truncate base. Endosperm deeply ruminate. Embryo basilar. Trunk is solitary, quite straight, 12-30m high, usually about 20 inches in circumference. Uniformly thick leaves of 1.2-1.8m, Leaflets numerous, 30-60 cm, upper confluent, glabrous. Spathe double, compressed, glabrous. Spadix much broader bearing male and female flowers. Rachis stout, compressed, branches with filiform tips. Male flowers very numerous, sessile, without bracts. Calyx 1- leaved, small, 3- cornered, 3 parted, petals 3 oblong, rigid straiated. Stamens 6, anthers sagittate. Female flowers solitary or 2 or 3 at or near the base of each ramification of the spadix, sessile, without bracts. Sepals 3, cordate, rigid, fleshy, permenent. Petals 3, like the sepals permanent. Staminodes 6, connate, styles scarcely any. Stigmas 3, short, triangular. Fruit 3.8 – 5.0 cm long, smooth orange or scarlet.

#### *2.7.1.2 A. normanbyii*

The trees are semi tall in growth habit. Stem is thicker. Leaves are short. The pulp is sweet in nature.

*2.7.1.3 A. triandra*

Found in India and Malaysia. Clustered palm, with bamboo like slender trunks, attractive leaves and bunches of red seeds. Medium in height, prefers shade and moisture. More female flowers are there in their inflorescence. The fruit set percentage is very high. It is a source for mite resistance. The fruits can be used as a masticatory. Useful as an indoor decorative plant (Graf, 1985).

*2.7.1.4 A. lutescens (Synonyms: Dypsis lutescens, Chrysalidocarpus lutescens)*

This *Areca* species is grown for ornamental purpose. They are popularly known as Butterfly Palm, *Areca* Palm and Golden Cane Palm. They will grow up to 25 feet tall (7.5 m). This species is originated from Madagascar. They can be grown in full sun to shady area. This will produce yellow male and female flowers on the same inflorescence and bear yellow to purple coloured oval fruits of 0.75 inches (Graf, 1985).

**2.7.2 Released Varieties** (Ananda, 2002)

*2.7.2.1 Mangala*

This is a selection made from VTL-3. It was introduced from China and released in India during 1973. It is a semi tall tree with partially drooping crown. It has well spaced leaves with dark green characteristic crinkling at the tip. It is early bearing in nature and is having high nut set. Ripe nut is dark yellow to orange in colour and the medium sized nut is round to oval in shape. It has good chewing and market quality. This variety is recommended for Coastal Kerala and Karnataka. The average yield of ripe nuts is 20 t $ha^{-1}$.

*2.7.2.2 Sumangala*

It is a selection made from VTL-17. This was introduced from Singapore and it was released during 1985 in India. They are tall palms with partially drooping habit and flowers in 4-5 years. Ripe nuts are deep yellow to orange in colour. They are having oblong, round shaped and bold nuts. This variety is recommended for the areas of coastal Kerala and Karnataka. The average yield of ripe nuts is 23.6 t $ha^{-1}$.

*2.7.2.3 Sreemangala*

It is a selection from VTL 11. This was introduced from Indonesia and in India, it was released in the year 1985. They are tall palms with partially drooping habit and flowers in about 5 years. The ripe nuts are deep yellow in colour and are oblong to round in shape and of medium size. This variety is recommended for the areas of Karnataka and Kerala. The average yield of ripe nuts is 21.4 t $ha^{-1}$.

*2.7.2.4 Mohitnagar*

It is a selection from Indonesian cultivar from West Bengal and was released in 1991. They are tall palms with well spaced bunches. The nuts are loosely arranged in the bunches. They are oval to round in shape and medium in size. The yield is stabilized early and it is a consistent high yielder. It is recommended for the Northern parts of West Bengal, Coastal Karnataka and Kerala. The average yield of ripe nuts is 20.7 t $ha^{-1}$.

*2.7.2.5 Samruthi*

It is a selection from CAL-7 at CARI, Andaman and Nicobar Islands. They are tall palms with longer internodes. This variety is a consistent yielder and high yielding in nature with well spaced bunches. The nuts are round and bold. It is recommended

for the areas of Andaman and Nicobar Islands. The average yield of ripe nuts is 239 q / ha.

### 2.7.3 Traditional Cultivars (Ananda, 2002)

#### *2.7.3.1 South Kanara / Kasaragod Local*

This variety is traditionally grown in Dakshina Kannada district of Karnataka and northern parts of Kerala. The variety exhibits tall growth habit with partially drooping crown and hard stem. It is characterized by large sized nuts with uniform bearing. Average chali yield is about 2.0 kg per palm per year. Ripe nuts of this cultivar are mainly used for making chali.

#### *2.7.3.2 Thirthahalli*

The variety is predominantly grown in Malnad areas of Chikmagalur and Shimoga districts of Karnataka. It is a tall cultivar with longer internodes and possesses drooping crown. Nuts are smaller in size and oblong in shape. Green nuts (6-7 months old) of this cultivar are preferred for tender nut processing. Its yield is 2.6 kg chali per palm per year.

## 2.8 ISOZYME ANALYSIS

Since the discovery by Hunter and Market in 1957, isozymes have played an essential role in many branches of biology like taxonomy, host pathogen interaction analysis and evolutionary studies. Today it has become the most widely recognized link between the organism and molecular approach to our science. Isozymes are different variants of the same enzymes having identical or similar functions and present in the same individual (Market and Moller, 1959).

Isozyme variations are used as a powerful tool to complement conventional biochemical and genetic studies (Yndgard and Hoskuldson, 1989).

### 2.8.1 Isozyme Studies in Palms

Isozymes were used as genetic indicators in date palms. Analyses of seven gene-enzyme systems in parents and progenies of *Phoenix dactylifera* were carried out by starch-gel electrophoresis in mature leaflets. Alcohol dehydrogenase, two esterases, two glutamate oxaloacetate transaminases, phosphoglucose isomerase and phosphoglucose mutase were each controlled by a single gene with two alleles. Isozyme variations were related to the allelic state of the genes. Genotypes of 45 female and 20 male cultivars and 9 hybrids were tabulated showing single gene markers (Tisseral and Torres, 1979).

A breeding programme initiated at Indio, California, in 1948, had given progeny populations of known parentage of date palms (*Phoenix dactylifera* L.). Extracts from leaflets of parents and progeny were used to genetically analyse the isozymes of alcohol dehydrogenase, esterase, glutamate oxaloacetate transaminase, phosphoglucose isomerase and phosphoglucomutase by starch gel electrophoresis. The five enzyme systems were governed by eight genes, seven of which were polymorphic with 14 alleles (Torres and Tisseral, 1979).

Isozyme analysis was used to identify the oil palm (*Elaeis guineensis* Jacq.) species. Six enzyme systems were analysed by gel electrophoresis in *Elaeis guineensis, E. oleifera* and their $F_1$ interspecific hybrids. Esterases and Acid phosphatases were found to be useful genetic markers for identifying the different fruit types. Certain bands were species specific, while others were found only in the hybrids (Rahman *et al.*, 1981).

Enzyme polymorphism in oil palm (*Elaeis guineensis* Jacq.) and the genetic control of nine enzyme systems were analyzed. Starch gel or polyacrylamide gel electrophoresis of pollen extracts showed that 38 alleles at 14 polymorphic loci controlled the 9 enzyme systems in a study of 252 trees in 40 progenies from 4 regions, namely (1) Indonesia and Malaysia, (2) Angola, (3) Zaire and (4) the Ivory

Coast. Endopeptidase and shikimate dehydrogenase were each controlled by one locus with 3 and 2 alleles respectively, glutamate oxaloacetate transaminase (aspartate aminotransferase) by two loci with 4 and 3 alleles respectively, isocitrate dehydrogenase by one locus with 3 alleles, malate dehydrogenase by 3 loci with 2 alleles each, cathodal acid phosphatase by one locus with 3 alleles, anodal acid phosphatase by one locus with 2 alleles, phosphoglucose isomerase by one locus with 3 alleles and phosphoglucose mutase by 2 loci with 3 alleles each. The best interpretation of the banding patterns of 6-phosphogluconate dehydrogenase appeared to be controlled by 2 loci, one with 3 alleles (one null) and the other with one allele (Ghesquiere, 1984).

Isozyme and chromosomal analyses were done in tissue culture derived date palms. Regenerated plants of four *Phoenix dactylifera* cultivars derived from callus cultures were analysed for isoenzyme banding patterns using PAGE and for chromosome numbers. Similar banding patterns within cultivars for esterase, glutamate oxaloacetate transaminase [aspartate aminotransferase] and leucine (cytosol) aminopeptidase suggested a high genetic uniformity, with regenerants being true to type. Variation in PAGE patterns was observed between cultivars. All plants had chromosome numbers of 2n = 36 except one plant of cv. Maktoom where polyploidization (2n = 70) had occurred (Salman *et al.*, 1988).

Isozyme analysis was carried out in the pollen of the oil palms. Electrophoresis of proteins and isoenzymes from pollen of seven accessions of *E. oleifera* and a hybrid between *E. oleifera* and *E. guineensis* was carried out. The banding patterns produced from each genotype showed a low degree of homology. Esterase isoenzyme banding patterns differed between the two species (Ataga and Fatokun, 1989).

Coconut leaves were used to analyse the catalase and peroxidase isozymes activity. Results of research on the relationship between the activities of catalase and peroxidase and yield of the crop were reported. Low-yielding, medium-yielding and high-yielding coconut palms cv. West Coast Tall were selected and the activities of

the two enzymes were estimated in fresh leaf samples. No overall relationship between yield and activities of these enzymes was detected (Narayanankutty and Gopalakrishnan, 1990).

Isozyme analysis was done in oil palms (*Elaeis guineensis* Jacq.) derived from leaf tissue culture to detect the genetic variability. Leaf tissue from a tenera variety of oil palm propagated by both tissue culture and seed was analysed for electrophoretic variability in proteins and esterase and peroxidase isoenzymes. Patterns derived from the tissue-cultured plants proved to be less variable than those from the seed-derived material, indicating greater genetic uniformity in the former (Budiani and Tahardi, 1991).

Isoenzyme analysis was carried out in oil palm (*Elaeis guineensis* Jacq.) for genetic studies. Isoenzyme analysis was used to study genetic variation among oil palm families and their origins. Isoenzymes were assayed by gel electrophoresis with vertical (Cyanogum gel) and horizontal (Starch gel) migration. From 21 enzyme systems studied, individuals could be described and compared for 23 loci with 56 alleles. The preliminary results showed that isoenzyme polymorphism existed that could be used to identify genetic variability and as important molecular markers for genetic studies in oil palm (Hutomo and Subronto, 1991).

Isozyme variation was studied in coconut at varied stages in development and differentiation. Isoenzyme expression was analysed in different organs and at different developmental stages to find the best organ/developmental stage for isoenzyme analysis as a genetic marker in coconut breeding. Esterase, peroxidase and glutamate oxaloacetate transaminase [aspartate aminotransferase] were assayed by starch gel electrophoresis. As enzyme activity levels differed at different developmental stages except in the leaves, coconut palm leaves were recommended for use in isoenzyme analysis (Hengky and Hartana, 1994).

Genetic variation was studied in the Zairean oil palm (*Elaeis guineensis* Jacq.) germplasm collection by employing native PAGE of isoenzymes. Three isoenzymes extracted from leaves were investigated, namely superoxide dismutase (SOD), peroxidase (PRX) and glutamate dehydrogenase (GDH). Polymorphism was detected in SOD and PRX systems but there was no genetic variation in GDH system. Polymorphic indices obtained from SOD and PRX systems revealed that the degree of polymorphism increased from the family to the ecotype level. This study also showed that the degree of polymorphism was influenced climatically and geographically (Choong *et al.*, 1996).

Isozyme analysis was carried out in coconut (*Cocos nucifera*) leaf proteins. Electrophoretic patterns of leaf peroxidases, endopeptidases and coomassie blue stained proteins were analysed in four cultivars (West African Tall, Rennell Tall, Malayan Yellow Dwarf, Cameroon Red Dwarf) of coconut (*Cocos nucifera*), and in the hybrids PB121 (Malayan Yellow Dwarf X West African Tall) and PB111 (Cameroon Red Dwarf X West African Tall). Polymorphisms indicated the expression of two alleles of a dimeric peroxidase, two alleles of a monomeric endopeptidase, and a pair of active and null alleles of a coomassie blue stained protein. Four distinctive genotypes were identified one for each of the tall cultivars, another for both of the dwarf cultivars, and one for both hybrids (Cardena *et al.*, 1998).

### 2.8.2 Isozyme Studies in Other Horticultural Crops

Isozyme pattern was used to identify the cultivars of Cherimoya (*Annona cherimola*) and Atemoya (*A. cherimola* x *A. squamosa*). Each of the cultivars of Cherimoya and Atemoya showed distinct patterns. The isozyme variation was studied at 15 loci resolved from eight enzyme systems for 15 varieties of Cherimoya and one variety of Atemoya (Ellstrand and Lee, 1986).

Studies conducted by Bashan *et al.* (1987) on the relation of enzymes and resistance against *Pseudomonas syringae* pv. tomato revealed presence of four-

dibased peroxidase isozymes in extracts from diseased plants, while only one was present in healthy plants.

Isozyme banding pattern was used as genetic marker in peach (*Prunus persica* L). It was investigated using starch gel electrophoresis. Leaf samples were taken from both juvenile and mature plants. A survey of 38-enzyme activity stain and five electrophoretic buffer systems were conducted. Only 12 staining systems produced well-resolved banding patterns; of these, nine were monomorphic among all genotypes surveyed and three showed some variation (Durham *et al.*, 1987).

Ganguly and Dasgupta (1988) studied the polyphenol oxdase isozymes from healthy roots of tomato variety Pusa Ruby infected by *Meloidogyne incognita*. They reported the absence of a band with Rm value of 0.520 in healthy or appaaarently healthy tissues.

Peroxidase activity was used as a biochemical marker for the resistance of musk melon (*Cucumis melo*) to *Pseudoperonospora cubensis*. The activity of the infected plants was higher than that of the uninfected plants. There were about 257 plants including cultivars, breeding lines and crosses of susceptible and resistant plants, which were used to predict the resistance and susceptibility (Reuveni *et al.*, 1991).

Peroxidase isozyme banding patterns were determined in 20 germplasm accessions of *Zizania latifolia*. The patterns were stable between years and could be used for the early identification of varieties and for varietal classification (Cao *et al.*, 1993).

Isozyme was used as genetic marker to characterize seven Spanish Cherimoya (*Annona cherimola*) cultivars. Fifteen enzyme systems were analyzed. Two cultivars only had identical banding pattern for all enzymes tested (Pascual *et al.*, 1993).

Based on the peroxidase isoenzyme patterns obtained in 41 *Malus* accessions the systematic positions of the 41 genotypes in the genus *Malus* were examined (Li *et al.*, 1995).

Twenty-eight ginger cultivars (*Zingiber officinalis*) were compared for peroxidase isoenzyme patterns by fuzzy cluster analysis in Fujian. The cultivars differed in isoenzyme pattern activity and intensity. They were divided into three types viz., da-fei-jiang, huang and zhu-zi-ziang (He *et al.*, 1995).

Sebastian *et al.* (1996) reported the ideal part for analysis of peroxidase was root or mature leaf and immature leaf for glutamate oxaloacetate transaminase (GOT) and esterase in pepper.

Isoenzyme analysis carried out in 26 varieties and 11 species of Pepper showed considerable variation at inter specific and intra specific level. They were grouped based on peroxidase, esterase and glutamate oxaloacetate transaminase (GOT) banding pattern (Sebastian *et al.*, 1996).

Satrabhandhu *et al.* (1996) reported that esterase isoenzyme could be used to discriminate lime (*C. aurantifolia*) cultivars and peroxidase isoenzyme showed no difference among genotypes.

Isozyme markers were used to identify several cultivars of Purple loose strife (*Lathyrum* sp) and interspecific hybrids. There were two zones of activity for phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), and two zones for malate dehydrogenase (MDH) in Purple loosestrife (Strefeler *et al.*, 1996).

The intra and inter population variation in the reaction of adaptation of individuals of *Elytrigia repens* collected at different altitudes in Eastern Germany showed variability for esterase and peroxidase isoenzyme patterns (Guttel and Hartenstein, 1996).

Kuriakose (1998) used peroxidase and esterase isozyme banding patterns as genetic markers of resistance for mosaic virus in pumpkin.

Isozyme banding pattern was used to characterize the rootstocks of *Citrus aurantium* and *C. taiwanica.* Here isozyme-banding pattern was also used to distinguish the nucellar seedlings from the hybrid seedlings in *C. taiwanica.* The isozymes used were glutamate oxaloacetate transaminase (GOT), superoxide dimutase (SOD) and malate dehydrogenase (MDH) (Protopapadakis and Papanikolaou, 1998).

Isozyme polymorphism was used to study the variation in turmeric (*Curcuma longa*) accessions. Seedling progenies showed maximum similarity and differed distinctly from the clonally propagated accessions. Fifteen accessions of *C. longa* collected from different geographical areas in India along a few seedling progenies were studied for variation based on polymorphism on isozyme (Shamina *et al.*, 1998).

Bose (1999) reported that peroxidase and polyphenol oxidase isozyme banding patterns could be used as genetic markers of resistance in tomato for bacterial wilt disease.

Joseph (1999) used the isozyme banding patterns of peroxidase, esterase and glutamate oxaloacetate transaminase (GOT) for working out the inter and intra specific relationships in curcuma.

Isozyme variation was used to differentiate the sex in Papaya. Among thirty-six isozymes studied about nine isozymes were useful to differentiate the sex in papaya. Of these, peroxidase, leucine aminopeptidase (LAP) and esterase were able to distinguish male from female plants in the Australian cultivar Richter and peroxidase and phosphoglucoisomerase (PGI) were able to distinguish hermaphrodite from female plants in the Hawaiin cultivar Sunset (Somsri, 1999).

In lemon and lemon like citrus cultivars, four isozymatic systems were used to detect the genetic diversity. Leaf tissues were used to analyze the isozymes of *Citrus limon, Citrus aurantiflolia, C. latifolia, C. mayeri* and 2 cultivars of local selections. The variation was analyzed in the isozymes namely malate dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), tetrazolium oxidase (TO) and esterase. The GOT enzyme difference was found to be adequate for distinction at the species level. Zymograms of esterase were very much useful as a diagnostic tool for cultivar identification in view of the extensive polymorphism of this enzyme (Protopapadakis and Papanikolaou, 1999).

Peroxidase isoenzyme pattern has been used to identify the teak (*Tactona grandis)* clones, collected from different regions of Karnataka. Under this there were about 49 clones collected from 9 locations and the variation was observed in the banding pattern (Padmanabha and Somashekar, 1999).

### 2.8.3 Isozyme Studies in Field Crops

Farkas and Stahmann (1966) reported the presence of two new peroxidase isozymes I and II in peroxidase zymogram pattern of bean leaves infected with Southern bean mosaic virus. Uninfected leaves exhibited peroxidase isozymes IV and I.

Hwang *et al.* (1982) classified barley cultivars into highly resistant, moderately resistant and highly susceptible to powdery mildew based on esterase zymograms.

Hussain *et al.* (1987) reported the usefulness of esterase banding patterns for identifying the cultivars of cassava using the extracts of the viable roots of the cultivars for analysis.

Liu *et al.* (1988) reported that shoots and upper leaves of smut resistant millet cultivars showed more number of peroxidase and polyphenol oxidase bands compared

to susceptible cultivars. They suggested the possible use of above observations as a marker for selecting smut resistant cultivar in Maize.

Peroxidase activity was positively correlated with induced resistance against TMV and Tobacco blue mould (*Peronospora tabaciana*) in cultivar KY 14 in which the stem was injected with *Peronospora tabacina* and the leaf was inoculated with tobacco mosaic virus. The increase was evident in cytosol, intercellular fluid and cell wall fractions. The isozyme pattern of peroxidases on isoelectric focusing gels showed an increase of two anionic peroxidases. Both peroxidases were positively correlated with induced resistance (Ye *et al.*, 1990).

A specific peroxidase isozyme (rf = 0.47) was identified from *Hordeum bulbosum* roots by Deyu *et al.* (1995). They related this band to BaYMV resistance and suggested the use of it as a marker in barley disease resistance breeding.

Gupta *et al.* (1995) studied the levels of total phenol, polyphenoloxidase and peroxidase in leaves of *Alternaria* leaf blight resistant and susceptible cultivars of *Brassica* spp. They reported an increased level of total phenol and more number of bands for polyphenol oxidase in resistant cultivars.

Genetic variation based on isozymes was studied in 43 landraces and cultivbars of *Brassica campestris* from China, four cultivars of *B. campestris* from Sweden and one from India and five cultivars of *B. oleracea* from Sweden and one from China (*B. alboglabra*). A total of 17 isozyme loci were studied, 10 of these were polymorphic in *B. campestris* and six were polymorphic in *B. oleracea* (Simonsen and Heneen, 1995).

Soybean rust resistant cultivars had four additional bands for peroxidase isozymes than susceptible cultivars as reported by Fei *et al.*, (1997).

Peroxidase activity was used to understand the susceptible and resistant interactions between Cassava (*Manihot esculenta*) and *Xanthomonas axonopodis*

pv.manihotis and *Xanthomonas cassavae*. It was found that the cell wall bound peroxidase activity in the resistant interactions was two fold higher than that of the control or the susceptible interactions, which may be related to lignin deposition (Pereira *et al.*, 2000).

Genetic relationship among *Saccharum* species clones was assessed using six isozyme systems. Species affinities as revealed through dendrogram constructed based on phylogenetic analysis using parsimony indicated the closeness between *S. barberi* and *S. sinense* and between *S. officinarum* and *S. robustum* clones (Hemeprabha and Rangasamy, 2001).

## 2.9 YELLOW LEAF DISEASE

The yellow leaf disease remains today, as the most serious malady affecting the Arecanut crop. This disease, which is known as "*Manjalippu*" in Malayalam was reported from Muvattupuzha, Meenachil and Chalakudi areas in Kerala about a century back (Nambiar and Sreenivasan, 1951).

In earlier years, it was felt that YLD was more or less similar to the leaf and root disease, which is known as *chandiroga* (Dastagir, 1963,1965). The malady does not kill the palm outright but is only debilitating in nature.

Thorough and systematic observation was made with respect to the pattern and spread of the disease at the CPCRI Research Centre, Palode located in a predominantly affected area. Seedlings planted in 1961 in virgin soil manifested symptoms in 1968 and there after within a period of four years 80 per cent of the palms contracted the disease (Rawther and Abraham, 1972) as the spread was rapid and it did not follow any definite pattern.

### 2.9.1 Etiology

A number of fungi like *Cercospora arecae, Exosporium arecae, Leptosphaera sp., Diplodia sp., Phyllosticta sp.* and *Trametes corrugata* were isolated from the diseased leaves (Menon, 1959).

Menon (1960a) suggested the possibility of a virus or virus like organisms being involved in the disease.

Paper chromatographic studies (Menon 1961) indicated that some proteins or their subunits were present in diseased areca palms, which were absent in healthy ones. Serological investigation with crude arecanut antigen and disease specific rabbit antiserum showed that there were precipitation reactions indicating antibody formation.

Khandige *et al.* (1957) reported the association of mites with the yellow leaf. Menon (1960 b) distinguished the yellowing caused by mite from the foliar yellowing due to yellow leaf disease.

Menon and Kalyankutty (1961) reported a reduction in the intensity of foliar yellowing when sprayed with salts of magnesium and manganese.

Nair (1964) observed the presence of nematodes *Meloidogyne javanica, Helicotylenchus* sp. and *Tylenchorhynchus* sp. in the root zone of the yellow leaf affected palms at Palode.

Weischer (1967) recorded seven genera of plant parasitic nematodes from a few soil samples collected from the root zone of the healthy and diseased palms.

Water logging was considered to be one of the predisposing factors for the disease incidence (Rawther *et al.*, 1982).

Velappan (1969) observed that deficiencies of nitrogen, phosphorous and magnesium had some relationship with the disease.

Srivastava *et al.* (1970) reported bacterial streaming associated with yellow leaf disease affected roots. Out of the two distinct forms of bacteria, one was tentatively identified as *Pseudomonas* sp.

### 2.9.2 Symptomatology

Nambiar (1949) observed the symptoms of the disease as yellowing of the leaves and shedding of both mature and immature nuts. The endosperm of the diseased nuts had a blackish appearance and was soft to touch, which rendered it unsuitable for consumption and hence fetched only very low prices in the market.

According to Menon (1963), the first visible signs were translucent spots of 1-3 mm in diameter on the growing spindle. Brown necrotic streaks running parallel to the lamina were present in the unfolding leaves. As the leaves developed yellowing started from the tip of leaflets gradually extending to the middle of the lamina. This chlorosis could be distinguished from the physiological yellowing by the abrupt demarcation between the green and yellow regions in the diseased leaves. Subsequent studies had shown that the first visible symptom was the yellowing at the tips of leaflets in two or three leaves of the outermost whorl (Rawther, 1976).

### 2.9.3 Biochemical Studies

Earlier studies gave strong indications for the presence of nucleoproteins in diseased arecanut leaves and its absence in healthy leaves (Menon, 1960a). The observations envisaged detailed studies to characterize the associated proteins. The difference observed in the electrophoretic pattern and paper chromatograms between healthy and diseased leaf extracts offered good scope for developing diagnostic tests before the onset of the visible symptoms.

Nair (1969) studied the amino acid make up in different parts of healthy and diseased palms. The amino acid content of the diseased leaves (Cystine, Aspartic acid and Threonine) showed a fall in the early and middle stage of the disease and an accumulation in the advanced stages. On the other hand, Lysine and Arginine contents of leaves progressively increased with advancement of disease. The amino acid serine and glutamic acid were absent in leaves but present in large quantities in the inflorescence tissues.

### 2.9.4 Source of Resistance

The available germplasm accessions at CPCRI- RS, Vittal were screened against yellow leaf disease of arecanut for locating sources of tolerance / resistance. Thirteen different experiments are in progress at Palode, Kannara and Vittal involving all the accessions to identify the tolerant genotypes. Among the exotic types screened so far, only two palms Indonesia 2, British Solomon Islands 1 remained free from the disease (Nair and Ratnambal, 1994).

Fifty accessions of indigenous and exotic collections of arecanut were planted from 1961 onwards at the CPCRI- Research Centre, Palode in order to assess their field reaction against yellow leaf. All of them except *Areca triandra* began to exhibit disease symptoms after about eight years of planting. Only few instances of doubtful cases of disease incidence have been noticed in *A. triandra* (Rawther *et al.*, 1982).

A dwarf mutant of *A. catechu*, Hirehalli Dwarf is the probable donor for the tolerance to the yellow leaf disease in the hybridization programmes. (Bavappa and Nair, 1982).

Inter varietal crosses were carried out at Vittal among Mangala, VTL 11, 13, and 17, Mohitnagar, Thirthahalli and the dwarf mutant during 1975. The seedlings raised from these crosses were planted in a field trial at Palode, South Kanara during 1976

with a view to study the disease reaction to the yellow leaf disease of arecanut. Observations recorded till 1981 indicated that hybrid seedlings derived from crosses involving dwarf mutant have some degree of tolerance (Bavappa and Nair, 1982).

# Materials and Methods

# 3. MATERIALS AND METHODS

The present investigations were conducted at the Department of Plantation Crops and Spices and Biochemistry laboratory of College of Horticulture, Vellanikkara, Thrissur from December 2000 to October 2002.

## 3.1 MATERIALS

Materials analyzed included four species of genus *Areca* including *Areca catechu* L. The taxon was also studied in detail for analyzing within species variation as well. The materials included five released varieties of arecanut in addition to traditional and exotic cultivars. Three of the yellow leaf tolerant lines of arecanut were collected from CPCRI-Regional Station, Vittal and analyzed for variation. The materials were collected from the germplasm maintained at College of Horticulture, Vellanikkara, CPCRI-Regional Station, Vittal, Agricultural Research Station-Arallam, State Seed Farm-Mannuthy, Tamilnadu Agricultural University-Coimbatore and also from some of the farmers' fields (Table 1 and Plates 1 – 8).

## 3.2 METHODS

### 3.2.1 Isozyme analysis

All the twenty-four genotypes / lines / cultivars (Table 1) were used for isozyme studies. Peroxidase, esterase and polyphenoloxidase were the enzymes analyzed.

#### *3.2.1.1 Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) using Hoefer Mighty Small$^{TM}$ II system was used for the purpose. Acrylamide monomers were polymerized with N-N methylene bis acrylamide [($CH_2$(NH CONH = $CH_2$)$_2$ bis] to obtain the gel.

**Table 1. Arecanut accessions used in the study**

| Sl. no | Accession no. | Name of the Varieties/Cultivars/Species | Source |
|---|---|---|---|
| 1 | V1 | Mangala | CoH, Vellanikkara |
| 2 | V2 | Sumangala | CoH, Vellanikkara |
| 3 | V3 | Sreemangala | CoH, Vellanikkara |
| 4 | V4 | Mohitnagar | CoH, Vellanikkara |
| 5 | V5 | Samruthi | TNAU, Coimbatore |
| 6 | TC1 | South Kanara | CoH, Vellanikkara |
| 7 | TC2 | Thirthahalli | CPCRI-RS, Vittal |
| 8 | TC3 | Mettupalayam Local | TNAU, Coimbatore |
| 9 | TC4 | Coimbatore Local | Coimbatore |
| 10 | TC5 | Attur Local | Attur |
| 11 | TC6 | Kecheri Local | Kecheri |
| 12 | TC7 | Kunnamkulam Local | Kunnamkulam |
| 13 | TC8 | Kannara Local | SSF, Mannuthy |
| 14 | TC9 | Kasaragod Local | SSF, Mannuthy |
| 15 | TC10 | Thrissur Local | SSF, Mannuthy |
| 16 | TC11 | Peechi Local | CoH, Vellanikkara |
| 17 | TC12 | Hirehalli Dwarf | CoH, Vellanikkara |
| 18 | EC1 | Saigon 2 | ARS, Arallam |
| 19 | EC2 | British Solomon Islands 1 | CPCRI-RS, Vittal |
| 20 | EC3 | Indonesia 2 | CPCRI-RS, Vittal |
| 21 | S1 | *A. catechu* | CoH, Vellanikkara |
| 22 | S2 | *A. triandra* | CoH, Vellanikkara |
| 23 | S3 | *A. lutescens* | CoH, Vellanikkara |
| 24 | S4 | *A. normanbyii* | CPCRI-RS, Vittal |

**Plate 1.** ***Areca catechu***

**Plate 2.** ***Areca triandra***

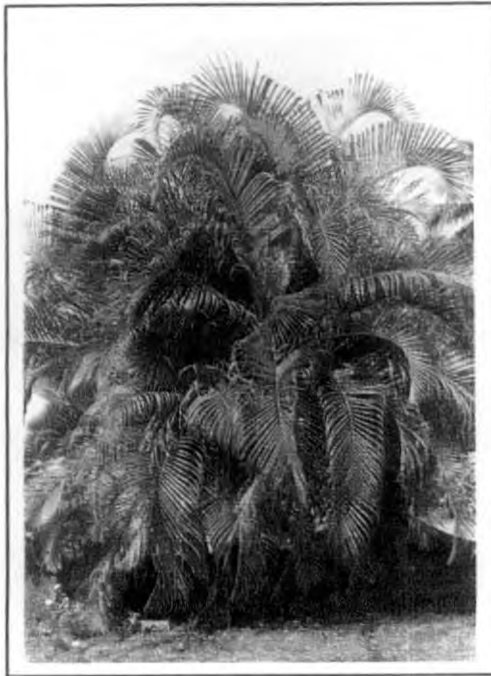


**Plate 3.** ***Areca lutescens***

**Plate 4. Hirehalli Dwarf**

**Plate 5. Mangala**

**Plate 6. Sumangala**

**Plate 7. Sreemangala**

**Plate 8. Mohitnagar**

N, N, N', N'- tetramethyl ethylene diamine (TEMED) was acted as formative and preparative agent and freshly prepared ammonium persulphate acted as catalyst. Polyacrylamidegel was preferred because of its chemical inertness, high resolution, easiness in handling, transparency of the gel and easiness in preparation.

Preparation of the sample

To select the ideal leaf for analysis from the tree, various leaves viz., first, third, fourth and sixth fully opened leaves from the top of the crown were taken and analyzed for the isozyme variation. As all the leaves have shown similar banding pattern for all the enzymes, fourth leaf has been selected for the study. The leaves were brought from the selected gardens in icebox during morning hours. The leaf samples were washed and rinsed with distilled water. The rinsed material was gently pressed with blotting paper to remove traces of water.

Samples were cut with sharp scissors and weighed two grams in the electronic balance and ground in a pre-cooled mortar along with extraction buffer (0.1*M* Tris-HCl, pH 7.6). The extraction buffer was added to the samples in suitable proportion around 5-10$^0$ C by keeping in an ice tray. From the different proportions tried, it was found that a sample buffer ratio of 1:1 to 1:3 was ideal to get the required concentration of sample for the study. During rainy season, the quantity of buffer was reduced due to high moisture content of the leaves. The homogenized sample was centrifuged at 15000 rpm for 15 minutes in a Remi Cool Centrifuge at 5$^0$ C. The supernatant was taken into vials and stored at subzero temperature.

Preparation of the gel

Reagents

The enzyme study was carried out in anionic system. The following stock solutions were prepared.

Solution A

Tris-------- 36.6 g

TEMED-- 0.23 ml

Adjusted the pH to 8.9 with 1*N* HCl

Volume made upto 100ml with distilled water

Solution B

Acrylamide ---------------------- 28.0 g

N'N' methylene bisacrylamide- 0.735 g

Volume made upto 100 ml with distilled water

Solution C (freshly prepared)

Ammonium presulphate – 0.14 g

Volume made upto 100 ml with distilled water

Preparation of the gel column

The Hoefer Mighty Small™ II System of Pharmacia Biotech Inc, California was used. The size of the gel was 8.0 cm x 9.4 cm. The gel preparation was carried out as follows:

| | Acrylamide concentration | | |
|---|---|---|---|
| | 7.5% | 8.5% | 10.5% |
| Solution A (ml) | 2 | 2 | 2.5 |
| Solution B (ml) | 4.285 | 4.860 | 7.497 |
| Solution C (ml) | 9.715 | 9.150 | 10.003 |

Mixed the stock solutions A, B and C in the above quantities to get the required gel recipes. Solution A and B were stored in amber coloured bottles. Of the above, 7.5% gel was observed as the best for the samples. The quantities of various stocks given for 7.5% gel strength were mixed serially. They were stirred and injected after removing the air into the gel caster with the help of guard bulb pipette. The combs

were pushed in between the caster plate for making wells and allowed to polymerize for about 45 - 90 min. Stacking gel to a height of 1-1.5 cm was also used for better resolution of bands for all enzymes. Care was taken to remove air bubbles.

Stacking gel solution

Stacking gel solution was prepared by using the following solutions:

1. *Monomer stock solution* (30% acry., 2.7% bis.)

Acrylamide ------30.0 g

Bis acrylamide -- 0.8 g

Volume made upto 100 ml with distilled water

Stored at $4^0$ C away from light

2. 4X stacking gel buffer (0.5*M* Tris-HCl, pH 6.8)

Tris base- 0.6 g

Adjust the pH to 6.8 with 1*N* HCl

Volume made upto 100 ml with distilled water

3. Initiator (10% APS, Prepared freshly)

Ammonium persulphate – 0.1g

Volume made upto 1 ml with distilled water

4. N, N, N', N'- tetramethyl ethylene diamine (TEMED)

Stacking gel was prepared by using the above solutions and the quantity of the solutions used were,

30 % monomer solution --------- 0.7 ml

Stacking gel buffer --------- 1.25 ml

TEMED---------------------------- 10 µl

APS-------------------------------- 50 µl

Distilled water--------------------- 3.0 ml

Electrophoretic run

*The following two solutions were prepared*

Electrode buffer

Stock solution

Tris 6 g, Glycine 28.8 g

Volume made upto one litre with distilled water keeping the pH at 8.3. The stock buffer was diluted 1:9 before use.

Tracking dye

Bromophenol blue - 25 mg

Volume made up to 10 ml with Tris – HCl buffer, pH 6.7

Stored at $5^0$ C in the refrigerator up to two - three weeks for use.

Preparation of Tris – HCl buffer solution of pH - 6.7 was done by using,

Tris ------- 5.98 g

TEMED -- 0.46 ml

pH adjusted to 6.7 with 1*N* HCl

Volume made up to *100 ml with distilled water.*

After polymerization, the gels were transferred to elrectrophoretic apparatus. The upper and lower tanks were filled with the pre-chilled electrode buffer. The ratio of the sample extract and the tracking dye used was 9:1. Fifteen μl of the sample: dye mixture was added to the wells after removing the combs by guard bulb pipette. Upper tank was connected to cathode and the lower one to anode. The enzyme extracts were subjected to electrophoresis under alkaline system of Davis (1964).

The running was carried out at $5^0$ C for the enzymes Peroxidase and Esterase while it was in the room temperature for Polyphenol oxidase. A current of 10 mA was maintained per plate and it took 45-90 min for completion of the run.

### *3.2.1.2 Peroxidase*

Gel concentration of 7.5 % acrylamide was found best for the Peroxidase enzyme separation in *Areca spp.*

Gel buffer ----------- Tris-HCl, pH 8.9

Electrode buffer----- Tris glycine, pH 8.3

Staining solution (Conkling and Smith, 1971)

Solution A 0.05 g O-dianisidine in 1 ml of 1*N* HCl

0.05 *M* Sodium acetate buffer pH 5.4 - 3ml

Distilled water - 26 ml

Solution B 0.01 % $H_2O_2$

After running, the gel was incubated in Solution A for 30 minutes at $37^0$ C. After half an hour, solution A was removed and solution B was added into the gel. Orange red colour bands of peroxidase developed. The reaction was arrested by adding destaining solution of 7 % acetic acid and the bands faded on standing for long time. Hence, photographs were taken on the same day of staining.

### *3.2.1.3 Esterase*

Gel concentration: Same as that of Peroxidase

Gel buffer and electrode buffer: Same as that of Peroxidase

Staining solution (modified from Shaw and Koen, 1968)

100ml of staining solution contained,

| | |
|---|---|
| Phos A - $Na_2HPO_4$ (0.2 *M*), pH 8.8------- | 10 ml |
| Phos B - $NaH_2PO_4$ (0.2 *M*), pH 4.16 ----- | 50ml |
| Fast blue RR -------------------------------- | 100mg |
| α-napthyl acetate 60 mg in 50% acetone- | 2ml |
| Distilled water ------------------------------ | 38ml |

After the running, the gel was taken for incubation in the staining solution at 37$^0$ C for 45–60 min until grey coloured bands appeared. The gels were destained in 7 % acetic acid. The bands remained stable for 2-3 days.

### *3.2.1.4 Polyphenol oxidase*

Gel concentration: same as that of the Peroxidase

Gel buffer and electrode buffer: same as that of the Peroxidase

Staining solution

The staining solution composed of the following;

0.1 *M* potassium phosphate buffer pH 7.0-------- 200 ml

ρ-phenylene diamine ------------------------------- 0.2 g

Catechol---------------------------------------------- 600 mg

Equilibrated the gel in the staining solution for 30-60 min until brown bands appear. The reaction was arrested by adding destaining solution of 7% acetic acid and the photographs were taken.

### *3.2.1.5 Nomenclature of Isozymes*

The enzymes were designated by the following abbreviations

1. Peroxidase ----- PRX. 2. Esterase -------- EST. 3. Polyphenol oxidase ----- PPO

### *3.2.1.6 Numbering*

All the isoenzymes in the species were pooled for numbering of the enzymes. The fast moving anodal band was numbered 1 (e.g. PRX1). The slower were given subsequent numbers. Relative mobilities (Rm) of bands were calculated as per the formula,

$$Rm = \frac{\text{Distance migrated by the band}}{\text{Distance migrated by the dye}}$$

### 3.2.2 Measurement of Similarity

The electrophoretic similarity among varieties and morphotypes of *Areca catechu* and other species were calculated by using the Jaccard (1908) coefficient. The calculation was performed using NT-SYSpc (version 2.01) software of M/S Exeter. Average of similarity indices for all enzymes were computed and pooled data were compared.

### 3.2.3 Cluster Analysis

#### *3.2.3.1 Data Scoring and Analysis*

For each isozyme, presence of band on the gel was scored as 1 and its absence as 0. Pooled data from three isozymes viz., peroxidase, esterase and polyphenol oxidase were taken for the cluster analysis. The analysis was performed using NT-SYSpc (version 2.01) software of M/S Exeter. SAHN clustering (Sequential Agglomerative Hierarchical Nested) based on UPGMA (Unweighted Pair Group Method using Arithmetic Mean) was performed as briefed below:

To start with, the degree of genetic similarity between pair of accessions estimated using Jaccard's coefficient (Jaccard, 1908). Then all other accessions were sequentially amalgamated with this pair following agglomerative algorithm. The clusters thus obtained were hierarchically nested and presented in the form of dendrogram.

### 3.2.4 Protein Estimation

The protein content of the accessions was estimated using the method described by Lowry *et al.* (1951). The procedure is described below:

Preparation of the sample

Two grams of the sample were extracted in 6 ml of 0.1 *M* Tris-HCL buffer of pH 7.6. Ground well in the ice tray at $4^0C$ centrifuged at 15000 rpm for 15 minutes in Remi Cool centrifuge. The supernatant was used for the estimation. The following reagents were prepared.

Reagent A. 100 ml of 0.1 *N* NaOH + 2 g $Na_2 CO_3$
Reagent B. 1 g sodium potassium tartrate in 100ml of DW + 0.5 g $CuSo_4$
Reagent C. 50 ml of Reagent A + 1 ml of Reagent B (freshly prepared).
Reagent D. Folincio calteau's phenol reagent at 1:1 dilution

The reagents were prepared and mixed well with the sample supernatant as follows:

In a clean test tube, 25 µl of the supernatant was taken and made into 400 µl with distilled water. 2 ml of reagent C was added to this mix and kept for 10 minutes. After 10 minutes, 0.2 ml Reagent D was added to this mixture. Then it was incubated for half an hour in the dark for the formation of the colour. After the incubation was over, the absorbance of the solution was measured at 660 nm. The protein content of the accessions was estimated by plotting these values in the standard graph.

The standard graph was prepared using the values obtained from the above same analysis done with the standard chemical Bovine Serum Albumin powder (v). The stock solution of the standard was prepared by dissolving 20 mg of BSA in 10 ml distilled water.

**3.2.5 Enzyme Activity**

***3.2.5.1 Peroxidase***

The procedure given by Sadasivam and Manickam (1991) was followed. Two grams of the leaf sample were extracted in 4 ml of the phosphate buffer of 0.1 *M* of pH

6.5. Ground well in the ice tray at $4^0$ C, centrifuged at 15000 rpm in the Remi cool centrifuge for 15 minutes. The supernatent was taken for the assay.

The analysis was done with the following.

1. Phosphate buffer of pH 6.5 of 0.1 *M* --- 3.5 ml
2. Plant tissue supernatent ------------------- 0.2 ml
3. 0-dianisidine solution---------------------- 0.1 ml
4. 0.2 *M* $H_2O_2$ -------------------------------- 0.2 ml (to prepare 0.2*M* of $H_2O_2$ 1.5 ml made to 100 ml)
5. Distilled water ------------------------------ 0.2 ml

Blanks:

1. 5 ml $PO_4$ buffer (6.5 pH) –(absorbance had been set at '0' with this)
2. 3.5 ml $PO_4$ buffer + 0.1 ml dianisidine +0.2 ml of 0.2 *M* $H_2O_2$ + 0.2 ml distilled water (reading recorded)
3. Exclude only $H_2O_2$ from the above mixture 2 and added with 0.2 ml of the plant sample (reading recorded)

The readings were taken at 430 nanometer for every thirty seconds upto 3 min.

### *3.2.5.2 Polyphenol oxidase*

The procedure given by Sadasivam and Manickam (1991) was followed. Leaf sample of 200 mg was homogenized in one ml 0.1 *M* Sodium Phosphate buffer (pH 6.5), centrifuged at 15000 rpm for 15 min and the supernatant was used. To a clean cuvette, added 3 ml bufferd catechol solution (0.01 *M* catechol freshly prepared in 0.1 *M* $PO_4$ buffer at pH 6.0) and the absorbance was set as zero at 495 nm. To this 1 ml of enzyme extract was added, mixed gently and placed in the chamber. Recorded the absorbance for every 30 sec upto 5 min.

Preparation of the Catechol 0.01 $M$

For 1 $M$ Catechol----------- 110.11 g / lit

0.01 $M$ --------------------- 1.1 g / lit or 0.11 g / 100 ml

Blanks

1.Phosphate buffer of 6.5 pH ----- 4ml

2.Buffered Catechol 3 ml + 1 ml distilled water

3.Buffered Catechol 3ml + Enzyme extract 1ml

# Results

# 4. RESULTS

The results of the present study titled "Isozyme variation in *Areca catechu* L. and allied species" are given under the following headings:

4.1 Isozyme variation
- 4.1.1 Standardization of leaf sampling
- 4.1.2 Isozyme variation in *Areca catechu* L.
- 4.1.3 Isozyme variation in *Areca* species

4.2 Genetic similarity index

4.3 Cluster analysis

4.4 Protein estimation

4.5 Enzyme activity studies
- 4.5.1 Peroxidase
- 4.5.2 Polyphenol oxidase

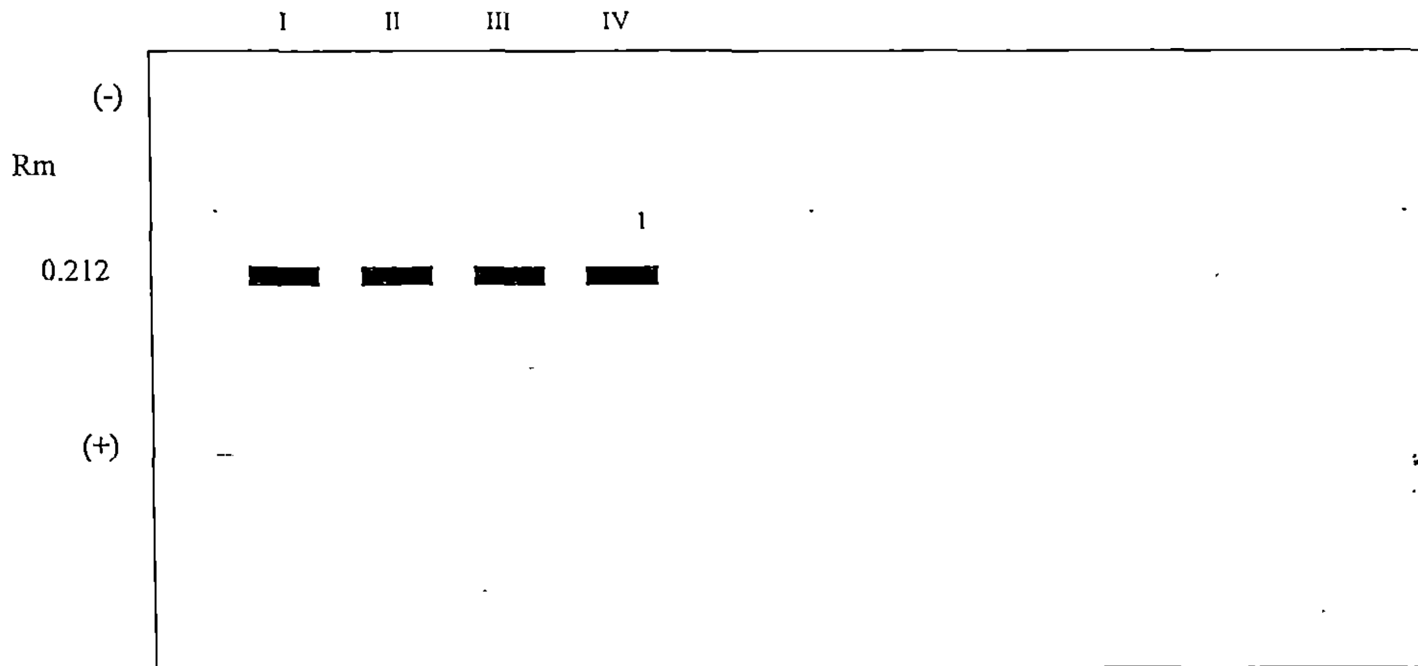
## 4.1 ISOZYME VARIATION

### 4.1.1 Standardization of Leaf Sampling

For standardizing the sample leaf for each isozyme analysis, variety Mangala has been selected as standard. The results are furnished below:

#### *4.1.1.1 Peroxidase*

In all the samples viz., first, third, fourth and sixth fully opened leaf from the crown, only one band was observed for peroxidase (Rm 0.212). The banding position also was same for all samples. The band was deep, thick and numbered as PRX 1. The numbering was later changed when traditional cultivars, varieties and species were compared. The fourth leaf was selected as ideal for the analysis (Fig. 1a and Plate 9a).




I. First leaf, II. Third leaf, III. Fourth leaf, IV. Sixth leaf

**Fig. 1a. Standardization of leaf sample for peroxidase**

#### *4.1.1.2 Esterase*

For esterase also the leaf samples were taken as that of peroxidase. Three bands were observed for all samples (Rm 0.240, 0.266, and 0.466). The first two bands were deep and the third band was feeble in nature. The banding positions also were same for all samples. The bands were numbered as EST 1, EST 2 and EST 3. The numbering was later changed when traditional cultivars, varieties and species were compared. There was no variation in the banding pattern among the samples. So again, the fourth leaf was selected as ideal for the analysis (Fig. 1b and Plate 9b).

#### *4.1.1.3 Polyphenol oxidase*

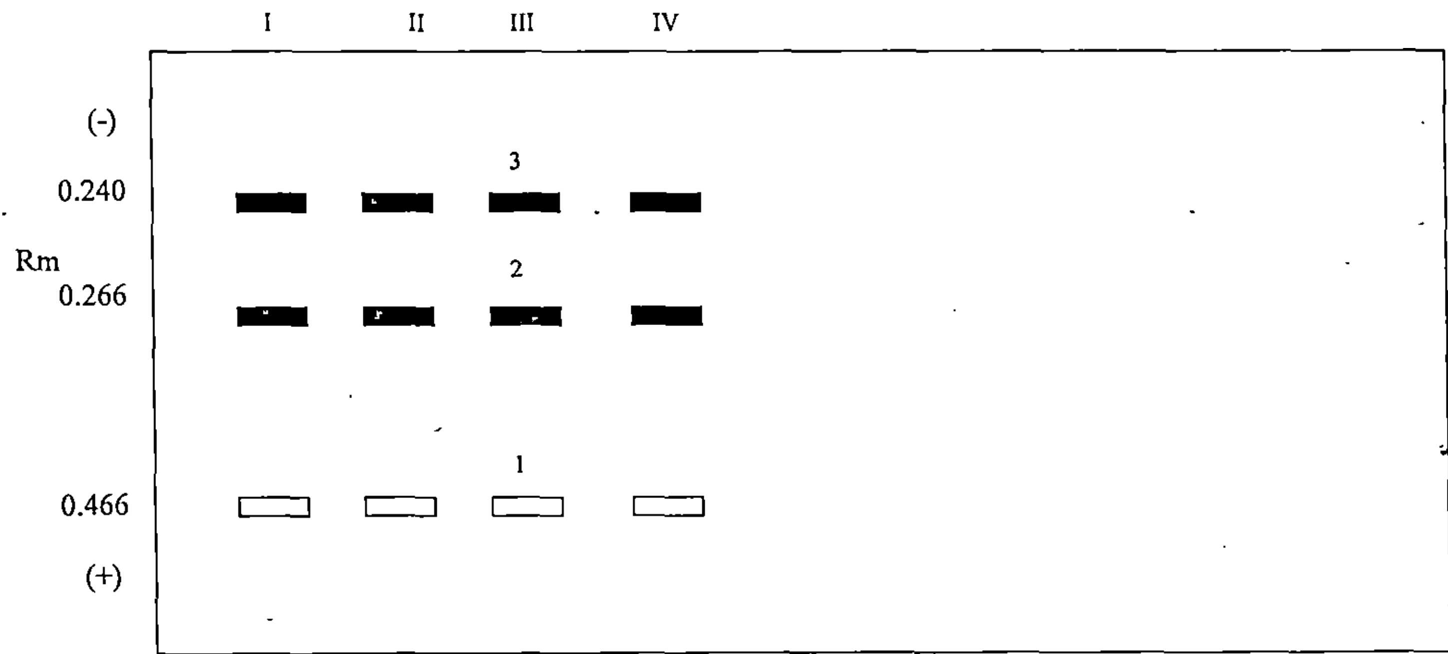
In polyphenol oxidase, only one band was observed in all samples. Here also the fourth leaf was selected as the ideal leaf for the analysis. The band was deep, thick and numbered as PPO1. The numbering was later changed when traditional cultivars, varieties and species were compared (Fig. 1c and Plate 9c).

### 4.1.2 Isozyme Variation in *Areca* Species

Four species of the genus *Areca* were compared for all the three isozyme systems. All these four species differed significantly in morphological aspects and in isozyme pattern also they had unique variation.

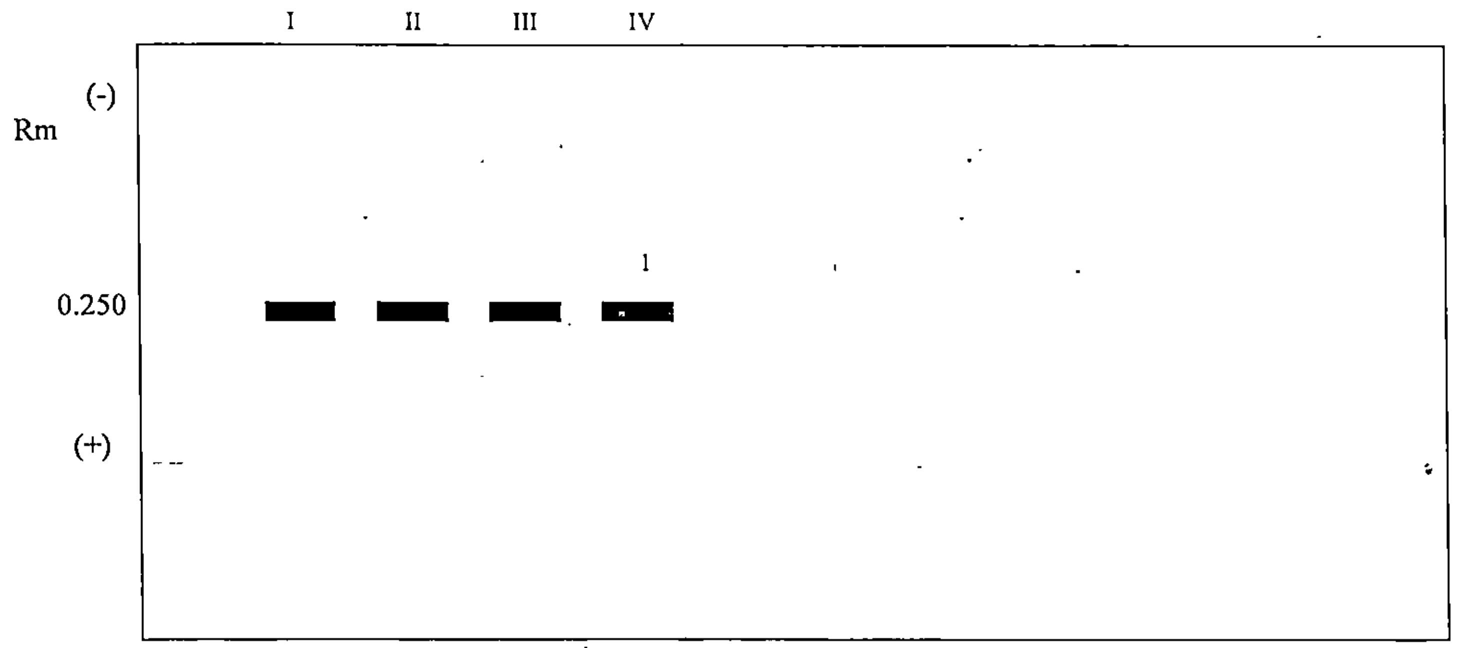
#### *4.1.2.1 Peroxidase*

Out of the two bands in the genus *Areca*, the second band (Rm 0.212) was common for *A. catechu* and *A. triandra*. The first band (Rm 0.282) was specific for *A. lutescens* and it missed the second band (Rm 0.212). The species *A. normanbyii* did not express any pattern in this elctrophoretic run as per this method. Based on this variation the species were grouped in to two.



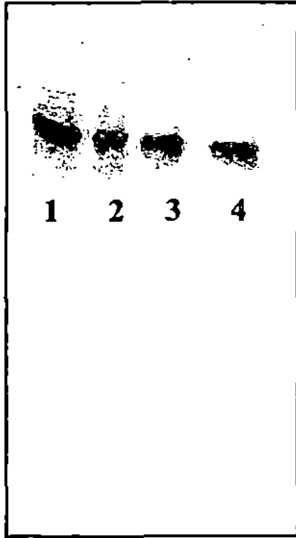

I. First leaf, II. Third leaf, III. Fourth leaf, IV. Sixth leaf

**Fig. 1b. Standardization of leaf sample for esterase**

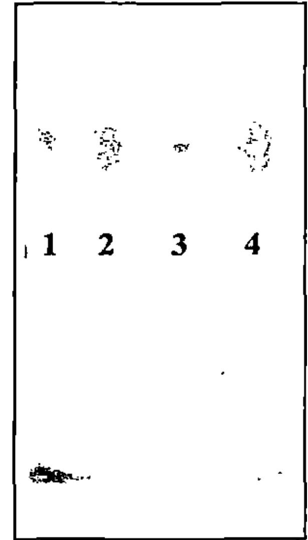



I. First leaf, II. Third leaf, III. Fourth leaf, IV. Sixth leaf

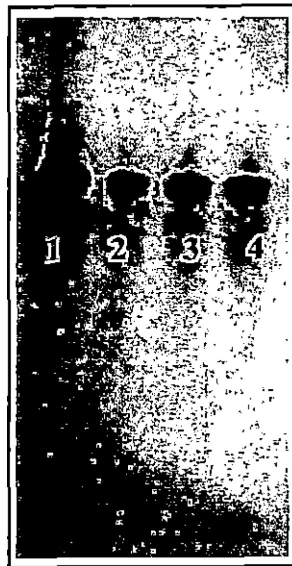
**Fig. 1c. Standardization of leaf sample for polyphenol oxidase**




A




B




C

1. First leaf 2. Third leaf 3. Fourth leaf 4. Sixth leaf

**Plate 9. Standardising the leaf for isozyme analysis: (A) Peroxidase (B) Esterase (C) Polyphenol oxidase**

#### *4.1.2.2 Esterase*

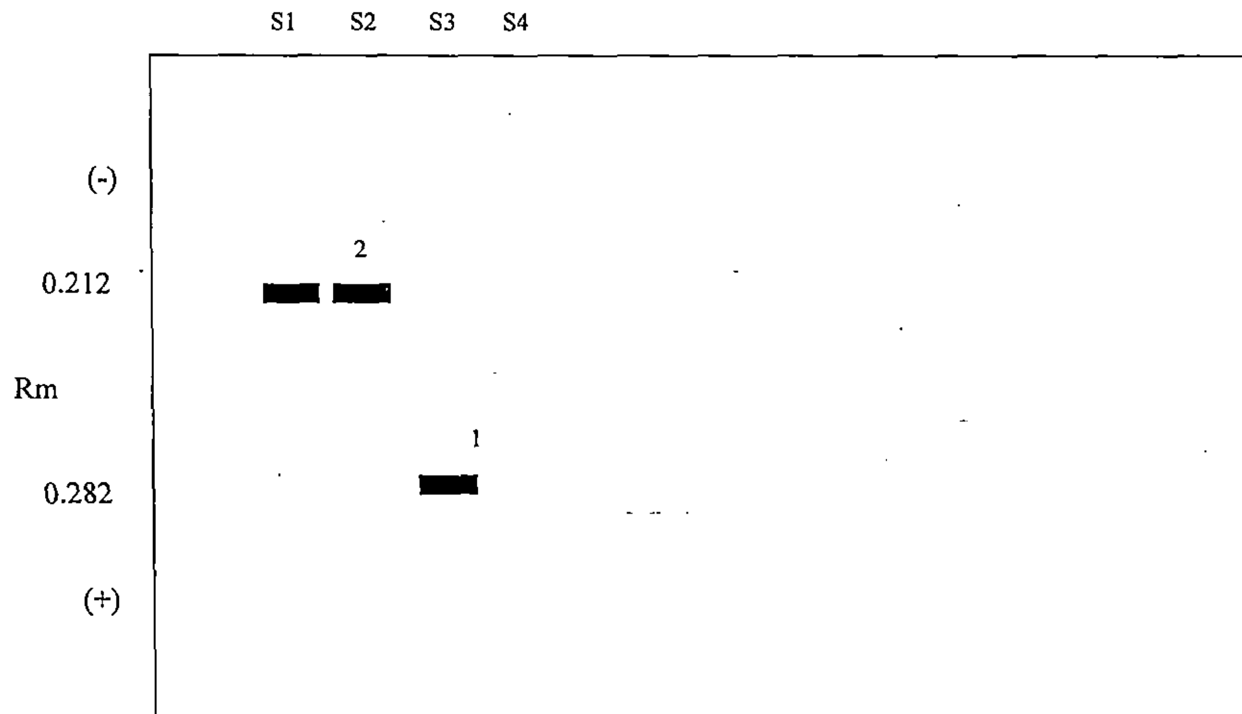
Out of six bands observed in the genus *Areca* for esterase isozyme, three bands (Rm 0.240, 0.266 and 0.466) were present in *A. catechu*. The species *A. triandra* missed the second band (Rm 0.466). The species *A. lutescens* had three bands of Rm values 0.173, 0.200 and 0.693. The species *A. normanbyii* again did not express any banding pattern in this method. Based on this variation the species are grouped in to three.

#### *4.1.2.3 Polyphenol oxidase*

Out of four bands observed in PPO, the second band (Rm value 0.250) was present only in *A. catechu*. The third band (Rm 0.243) was present only in *A. triandra*. The first and fourth bands (Rm 0.200 and 0.341) were present only in *A. lutescens*. The first band (Rm 0.341) was present in *A. normanbyii* also. Based on this variation the species were grouped in to four.(Figs. 2 a, b, c and Plates 10 a, b, c).

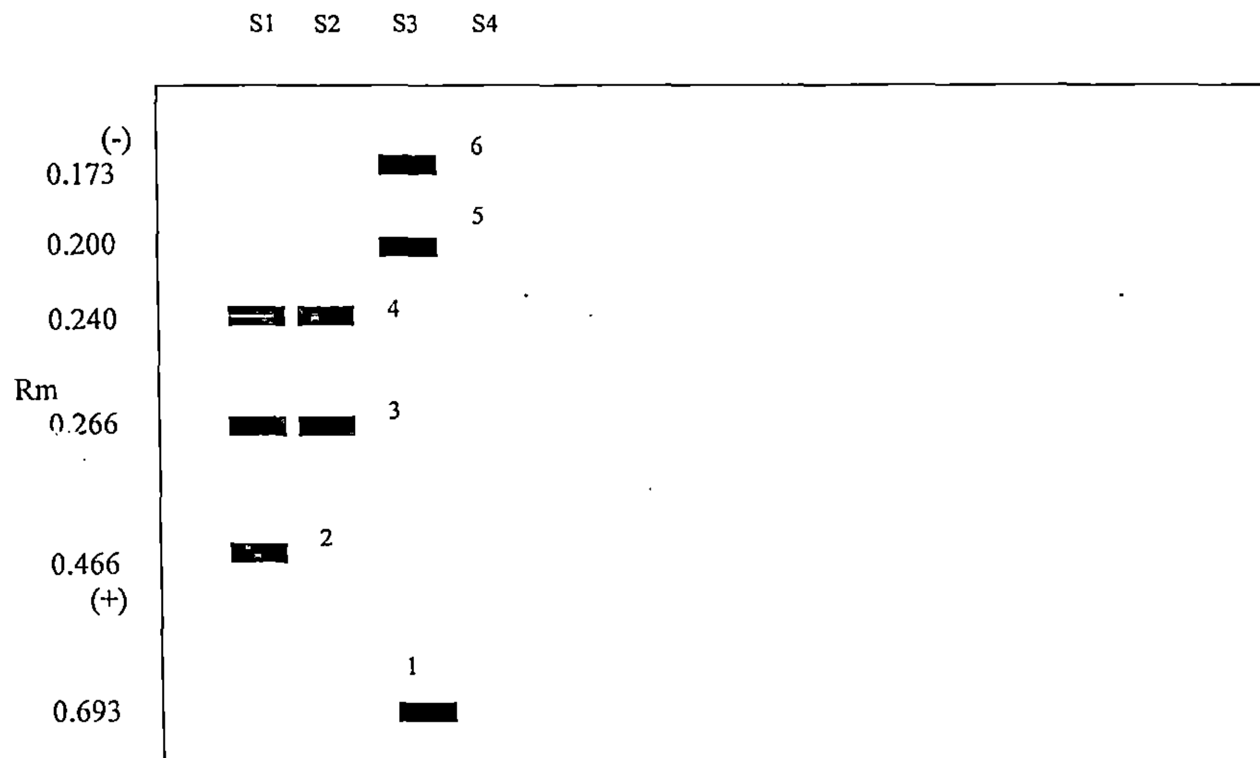
### 4.1.3 Isozyme Variation in *Areca catechu* L.

Nineteen accessions of the species *A. catechu* including five released varieties, twelve traditional cultivars and two exotic collections were analyzed for all the three isozymes namely, peroxidase, esterase and polyphenol oxidase. These nineteen accessions include two of the yellow leaf disease tolerant lines also (Hirehalli Dwarf and British Solomon Islands 1). For convenience in comparison, another YLD tolerant line Indonesia 2 was also added to this list despite it was a cultivar from *A. triandra*. It was added under exotic cultivars. Eventhough this was a cultivar from the species *A. triandra*, it has shown a different banding pattern from *A. triandra* in both esterase and polyphenol oxidase. This had a similar banding pattern with *A. triandra* in the case of peroxidase only.



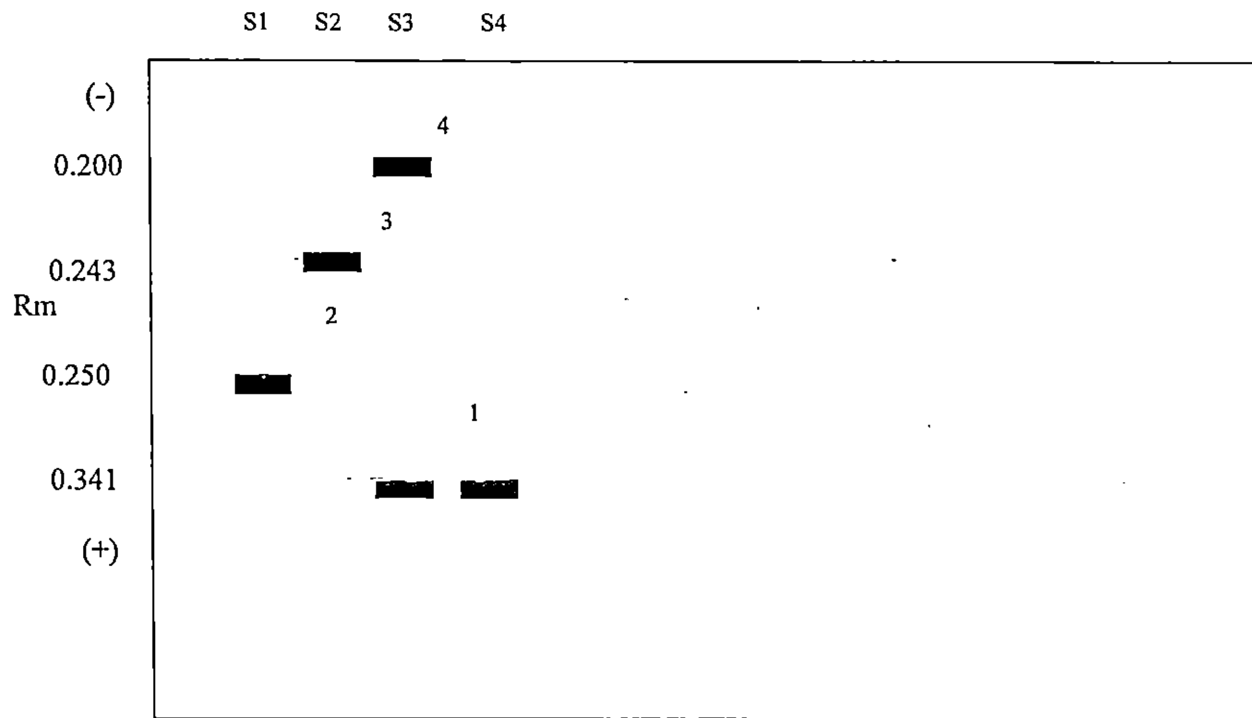

S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*

**Fig. 2a. Zymogram of peroxidase for *Areca* species**



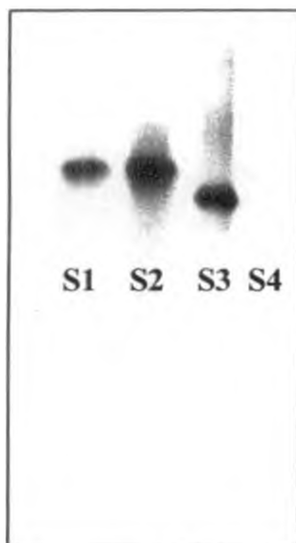

S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*

**Fig. 2b. Zymogram of esterase for *Areca* species**

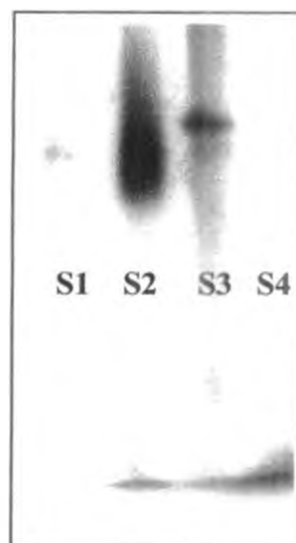



S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*

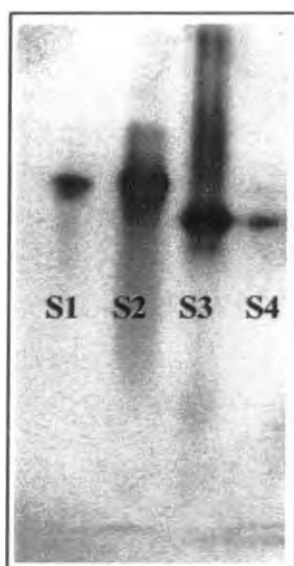
**Fig. 2c. Zymogram of polyphenol oxidase for *Areca* species**




A




B




C

S1. *Areca catechu* S2. *Areca triandra* S3. *Areca lutescens* S4. *Areca normanbyii*

**Plate 10. Banding pattern of isozymes in *Areca* species: (A) Peroxidase (B) Esterase (C) Polyphenol oxidase**

Accessions were selected to represent the different states of South India. They were also selected based on the disease reaction to the YLD of arecanut. The exotic collections and the *Areca* species collected were reported by Bavappa (1982) and Graf (1985), whereas the traditional cultivars and released varieties were reported by Ananda (2002). Some of the non-documented local cultivars from different parts of the country were also included under the traditional cultivars category for understanding the isozyme banding pattern pertaining to the genotypes of those geographical areas.

***4.1.3.1 Peroxidase***

Released varieties

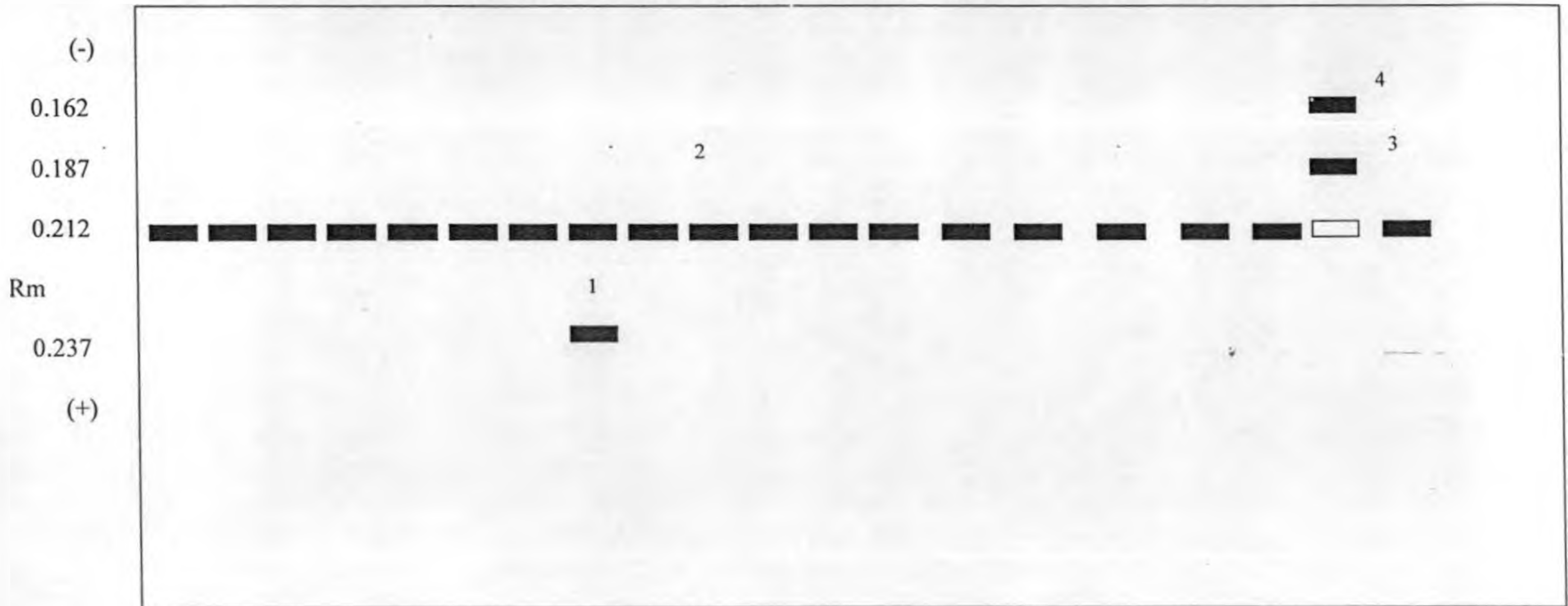
All the five released varieties showed a similar banding pattern for peroxidase and fell into one group. They all had only one band (Rm 0.212) and there was no variation in the peroxidase banding pattern among them.

Traditional cultivars

Traditional cultivars have shown very less variation with respect to the banding pattern for peroxidase. Except TC3 which had an extra band (Rm 0.237) below the common band (Rm 0.212), present in other cultivars. The traditional cultivars were divided into two groups based on the peroxidase isozyme.

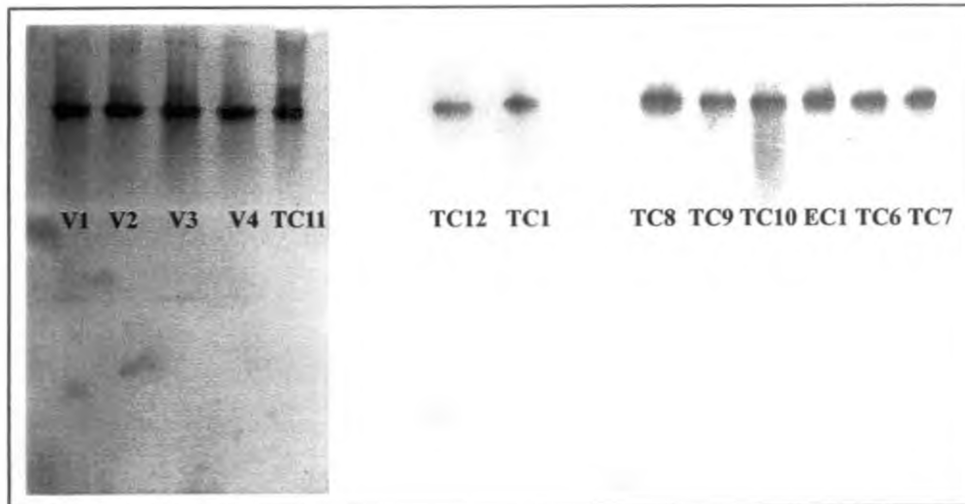
Exotic cultivars

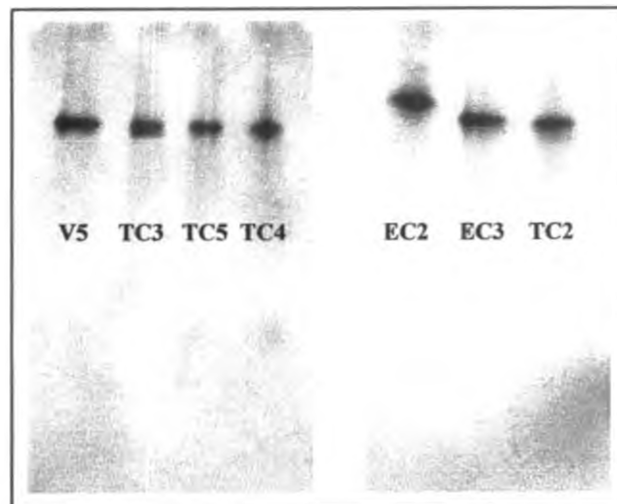
In addition to the only band (Rm 0.212) for the cultivars of *A. catechu* in peroxidase, the accession EC2 had two extra bands (Rm 0.162 and 0.187). The other two accessions EC1 and EC 3 were having the only band (Rm 0.212). Based on this variation in peroxidase banding pattern, the exotic collections were divided into two groups (Fig. 3a and Plate11).




V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South Kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. T C6.KecheriLocal. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. EC1.Saigon2. EC2.Bri.Sol. Is.1. EC3. Indonesia2.

**Fig. 3a. Zymogram of Peroxidase for *A. catechu* accessions**




V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11. Peechi Local. TC12. Hirehalli Dwarf. EC1. Saigon 2. EC2. British Solomon Islands 1. EC3. Indonesia 2.

**Plate 11. Peroxidase banding pattern in *Areca catechu* L. accessions**

*4.1.3.2 Esterase*

Released varieties

All the five released varieties exhibited three bands (Rm 0.240, 0.266 and 0.466). All the varieties were having no variation in the banding pattern of the esterase.

Traditional cultivars

All the traditional cultivars had three bands each similar to that of the varieties except for the accession TC3. Accession TC3 lacked one common band (Rm 0.466). Based on this variation the traditional cultivars were grouped into two.

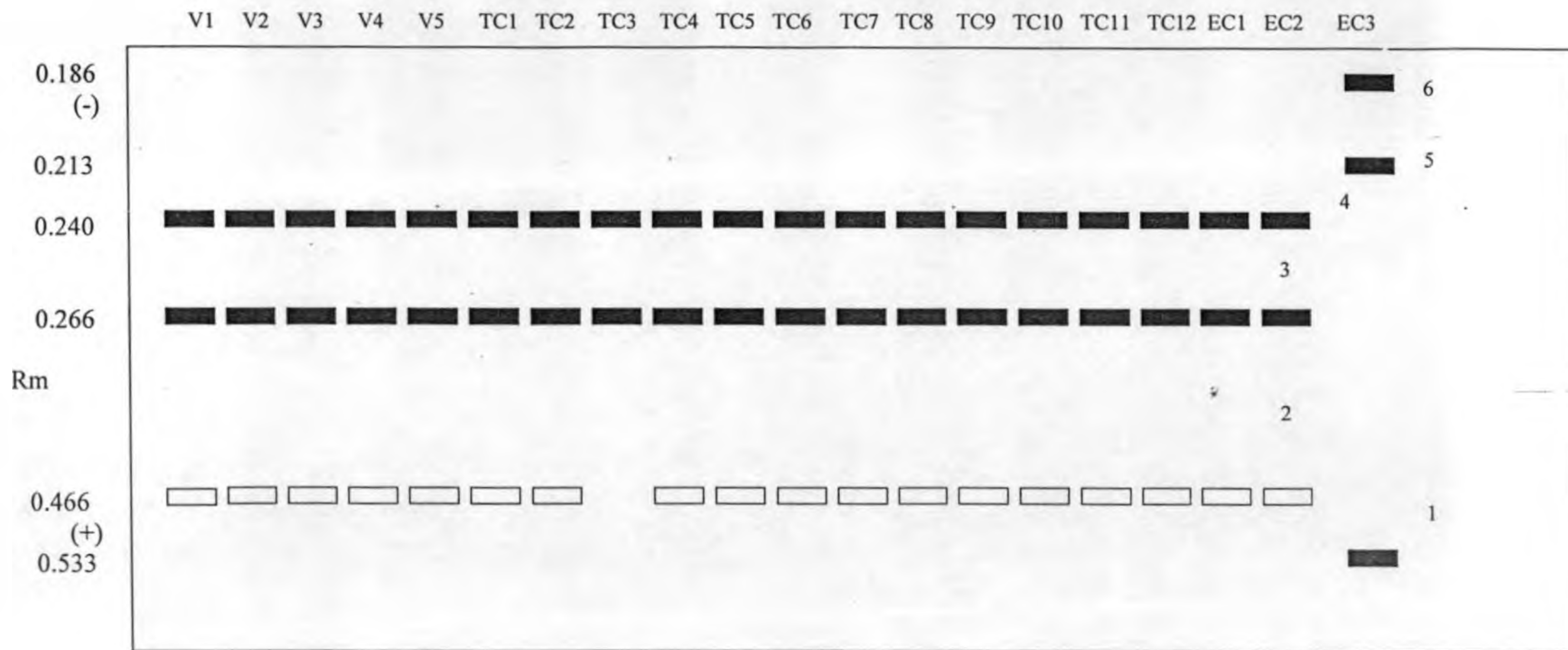
Exotic cultivars

The accessions ECI and EC2 revealed three bands in common to that of the other accessions and EC3 was entirely different in the banding pattern by having the three bands in different positions (Rm 0.186, 0.213 and 0.533). Based on this variation the exotic collections were grouped into two (Fig. 3b and Plate 12).

***4.1.3.3 Polyphenol oxidase***

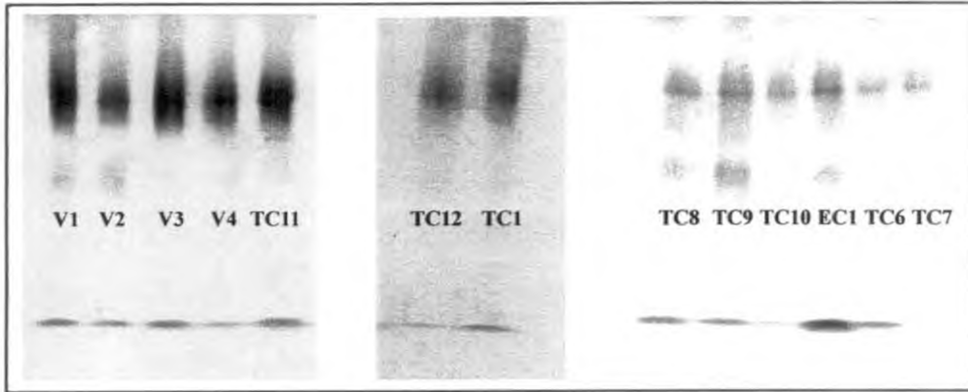
Released varieties

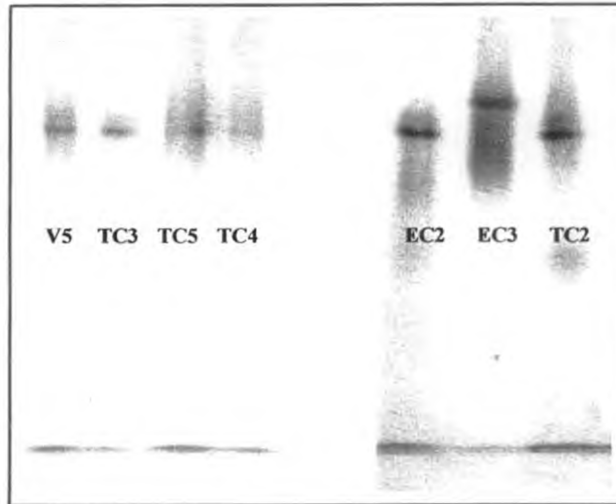
All the five released varieties produced only one band (Rm 0.250) and it was common for all the varieties. All the released varieties fell into one group based on the polyphenol oxidase isozyme banding pattern.




V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South Kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. T C6.KecheriLocal. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. EC1.Saigon2. EC2.Bri.Sol. Is.1. EC3. Indonesia2.

**Fig. 3b. Zymogram of esterase for *A. catechu* accessions**




V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11. Peechi Local. TC12. Hirehalli Dwarf. EC1. Saigon 2. EC2. British Solomon Islands 1. EC3. Indonesia 2.

**Plate 12. Esterase banding pattern in *Areca catechu* L. accessions**

Traditional cultivars

All the traditional cultivars had only one and common band (Rm 0.250) except the cultivar TC10 that had an extra band (Rm 0.350). Based on this the traditional cultivars could be grouped into two.

Exotic cultivars

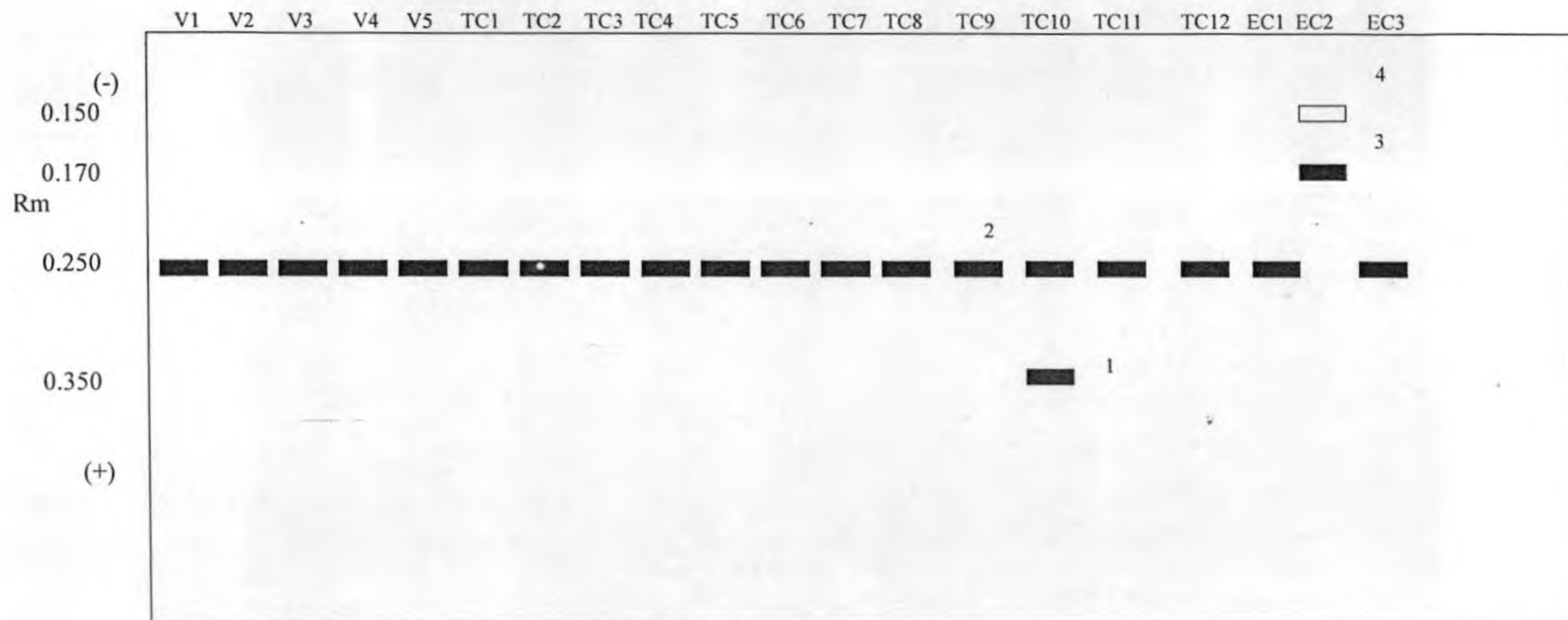
A common band (Rm 0.250) was observed for cultivars EC1 and EC3 for polyphenol oxidase. EC2 had two bands (Rm0.150 and 0.180) and missed the common band (Rm 0.250). Based on this variation the exotic cultivars were grouped in to two (Fig. 3c and Plate 13).

Based on the banding patterns for the three isozymes, all the accessions including the varieties, traditional cultivars and the species were divided into eight groups (Tables 2, 3, 4, 5 and Figs. 4 a, b, c).

## 4.2 GENETIC SIMILARITY INDEX

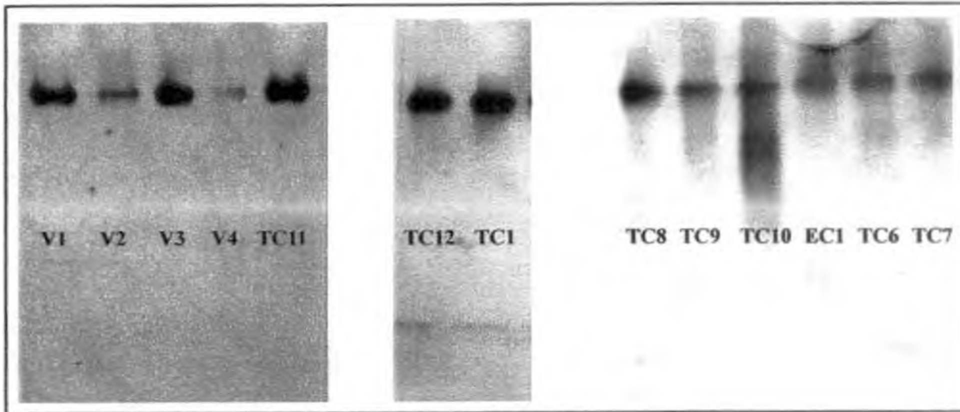
Similarity index of the accessions was calculated using Jaccard (1908) coefficient based on the isozyme banding pattern of the accessions (Table 6). The accessions V1, V2, V3, V4, V5, TC1, TC2, TC4, TC5, TC6, TC7, TC8, TC9, TC11, TC12, EC1 and S1 had the maximum similarity (1.00) among them. Both the accessions TC3 and TC10 had shown different values (0.66 and 0.83).

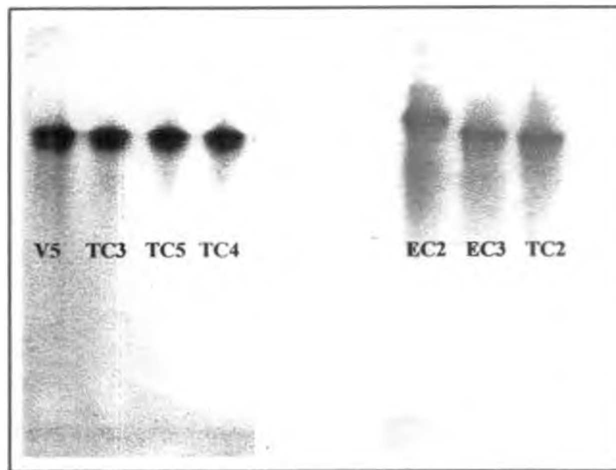
The accessions EC2 and EC3 also had shown different values (0.44 and 0.25). The accession S2 (*A. triandra*) had shown a similarity index value of 0.50 with the other accessions of *A. catechu*. The accession S3 and S4 had no similarity with the other accessions and had a very less similarity between themselves (0.16).




V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South Kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. TC6.KecheriLocal. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. EC1.Saigon2. EC2.Bri.Sol. Is.1. EC3. Indonesia2.

**Fig. 3c. Zymogram of polyphenol oxidase for *A. catechu* accessions**




V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11. Peechi Local. TC12. Hirehalli Dwarf. EC1. Saigon 2. EC2. British Solomon Islands 1. EC3. Indonesia 2.

**Plate 13. Polyphenol oxidase banding pattern in *Areca catechu* L. accessions**

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**Table 2. Groups based on pooled peroxidase zymogram**

| Groups | Accessions |
| --- | --- |
| I | S3 |
| II | TC3 |
| III | V1, V2, V3, V4, V5, TC1, TC2, TC4, TC5, TC6, TC7, TC8, TC9, TC10, TC11, TC12, S1, S2, EC1, EC3 |
| IV | EC2 |
| V | S4 |

**Table 3. Groups based on pooled esterase zymogram**

| Groups | Accessions |
|---|---|
| I | S3 |
| II | EC3 |
| III | V1, V2, V3, V4, V5, TC1, TC2, TC4, TC5, TC6, TC7, TC8, TC9, TC10, TC11, TC12, EC1, EC2, S1 |
| IV | TC3 |
| V | S2 |
| VI | S4 |

**Table 4. Groups based on pooled polyphenol oxidase zymogram**

| Groups | Accessions |
|---|---|
| I | TC10 |
| II | S3 |
| III | S4 |
| IV | V1, V2, V3, V4, V5, TC1, TC2, TC3, TC4, TC5, TC6, TC7, TC8, TC9, TC11, TC12, EC1, EC3, S1 |
| V | S2 |
| VI | EC2 |

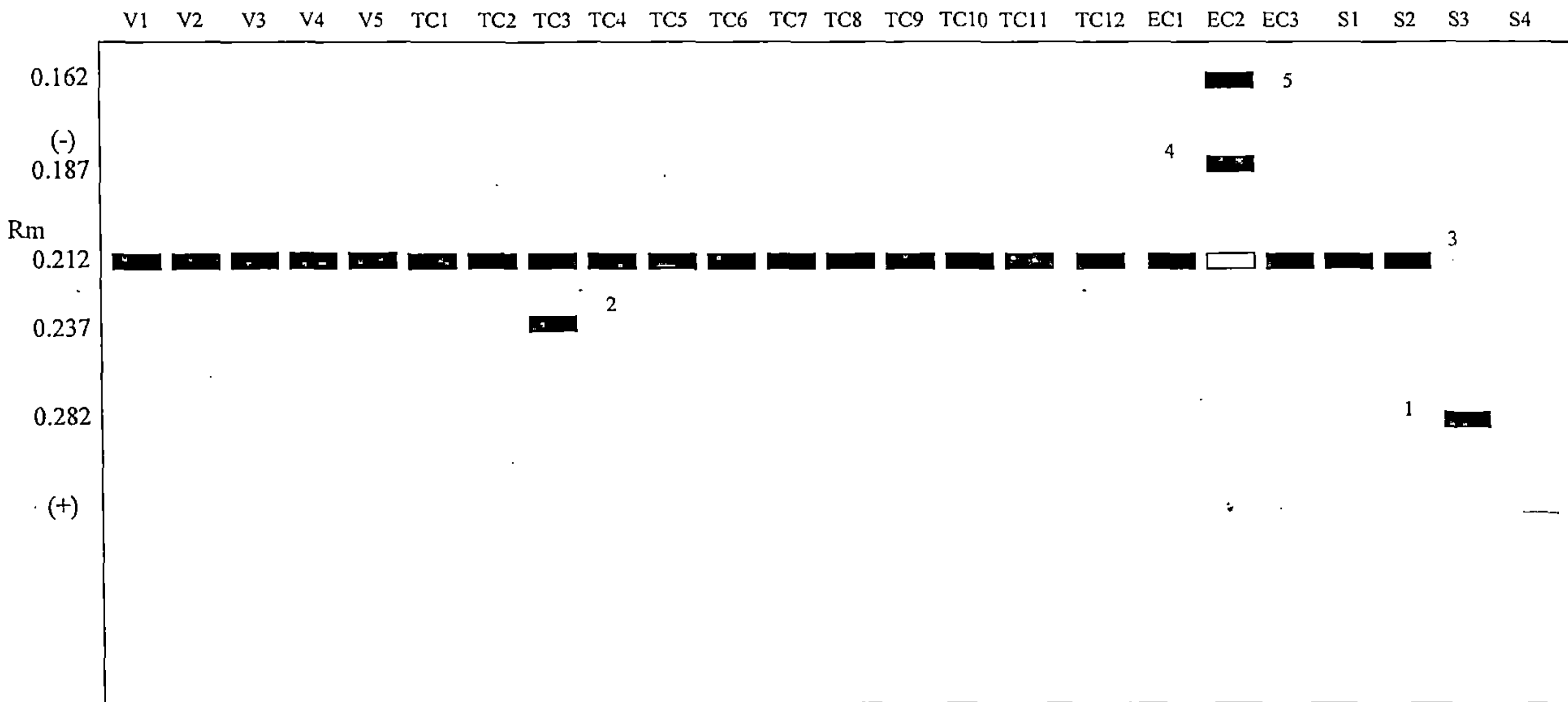
**Table 5. Groups based on pooled zymograms of peroxidase, esterase and polyphenol oxidase**

| Groups | Accessions |
|---|---|
| I | S3 |
| II | EC3 |
| III | TC10 |
| IV | TC3 |
| V | S4 |
| VI | V1, V2, V3, V4, V5, TC1, TC2, TC4, TC5, TC6, TC7, TC8, TC9, TC11, TC12, EC1, S1 |
| VII | S2 |
| VIII | EC2 |

**Table 6. Genetic similarity indices based on isozyme banding pattern in arecanut species/ varieties/ cultivars**

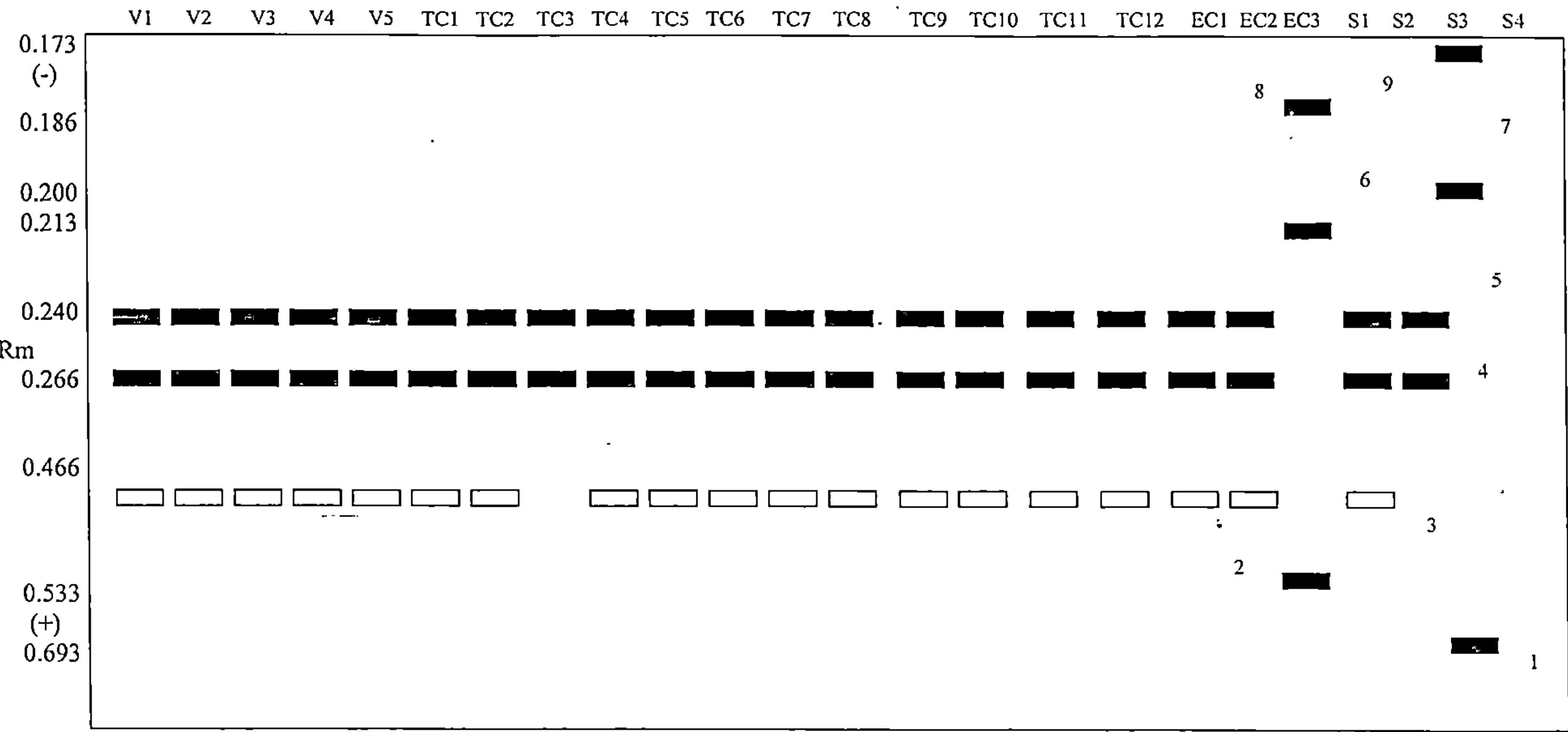
| Accessions | V1 | V2 | V3 | V4 | V5 | TC1 | TC2 | TC3 | TC4 | TC5 | TC6 | TC7 | TC8 | TC9 | TC10 | TC11 | TC12 | EC1 | EC2 | EC3 | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V1 | 1.00 | | | | | | | | | | | | | | | | | | | | | | | |
| V2 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | | | | | | |
| V3 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | | | | | |
| V4 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | | | | |
| V5 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | | | |
| TC1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | | |
| TC2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | | | | | |
| TC3 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 1.00 | | | | | | | | | | | | | | | | |
| TC4 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | | | | | | | | | | | | | | | |
| TC5 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | | | | | | | | | | | | | | |
| TC6 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | | |
| TC7 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | | |
| TC8 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | | |
| TC9 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | | | | | | | | |
| TC10 | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 0.57 | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 0.83 | 1.00 | | | | | | | | | |
| TC11 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.83 | 1.00 | | | | | | | | |
| TC12 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.83 | 1.00 | 1.00 | | | | | | | |
| EC1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.83 | 1.00 | 1.00 | 1.00 | | | | | | |
| EC2 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 | 0.30 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 | 0.40 | 0.44 | 0.44 | 0.44 | 1.00 | | | | | |
| EC3 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.22 | 0.25 | 0.25 | 0.25 | 0.08 | 1.00 | | | | |
| S1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.66 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.83 | 1.00 | 1.00 | 1.00 | 0.44 | 0.25 | 1.00 | | | |
| S2 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.42 | 0.50 | 0.50 | 0.50 | 0.33 | 0.12 | 050 | 1.00 | | |
| S3 | 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 | 0.00 | 0.00 | 1.00 | |
| S4 | 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 | 0.00 | 0.00 | 0.16 | 1.00 |

V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South Kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Covai Local. TC5.Attur Local. TC6.Kecheri Local. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasargod Local. TC10.Thrissur Local. TC11.Pecchi Local. TC12.Hirehalli Dwarf. EC1.Saigon2. EC2.Bri.Sol. Is.1.EC3. Indonesia2 S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. nornmanbyii*.



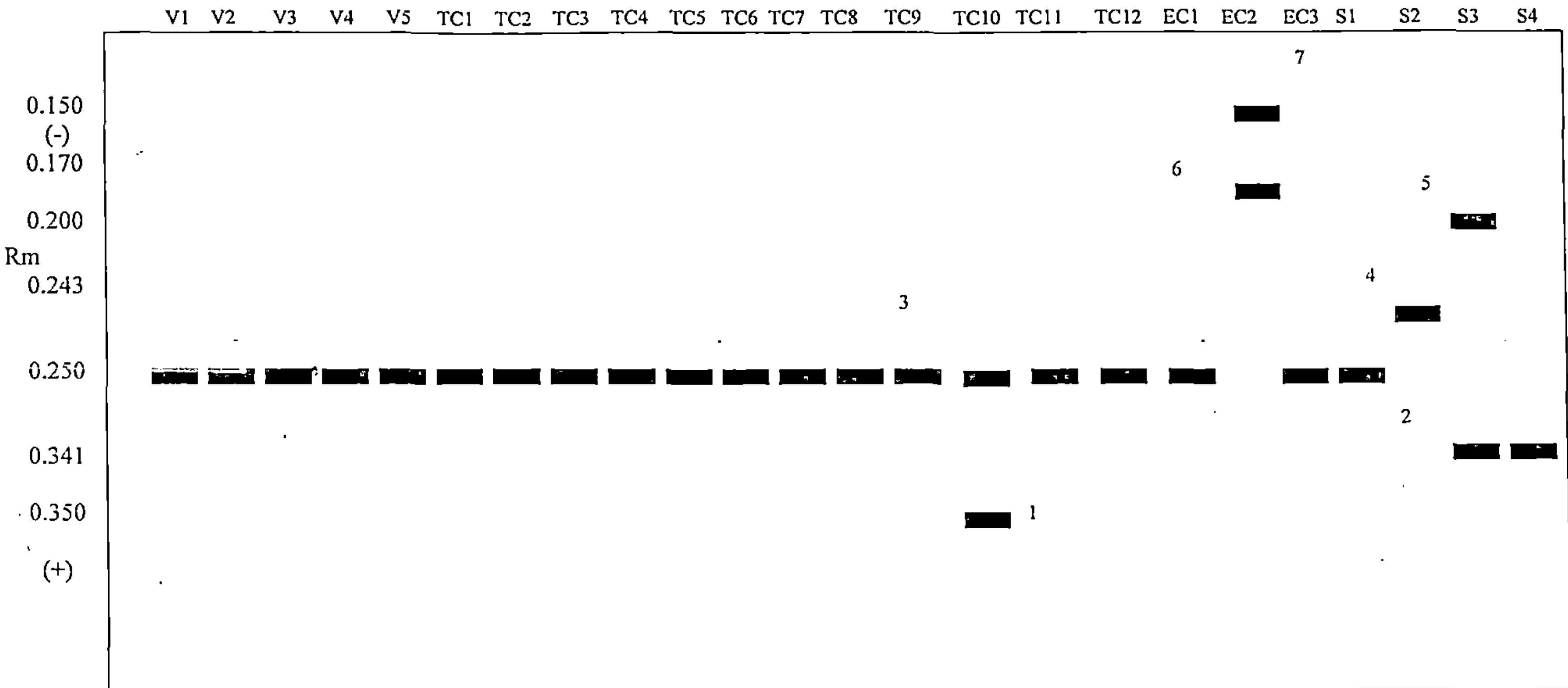

V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. TC6. Kecheri Local. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. 18.Saigon2. 19.Bri.Sol. Is.1. 20. Indonesia2. S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*.

**Fig. 4a. Pooled zymogram of peroxidase for arecanut accessions**




V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South Kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. TC6. Kecheri Local. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. 18.Saigon2. 19.Bri.Sol. Is.1. 20. Indonesia2. S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*.

**Fig.4b. Pooled zymogram of esterase for arecanut accessions**




V1.Mangala. V2.Sumangala. V3.Sreemangala. V4.Mohitnagar. V5.Samruthi. TC1.South kanara. TC2.Thirthahalli. TC3.Mettupalayam Local TC4.Coimbatore Local. TC5.Attur Local. TC6. Kecheri Local. TC7.Kunnamkulam Local. TC8.Kannara Local. TC9.Kasaragod Local. TC10.Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. 18.Saigon2. 19.Bri.Sol. Is.1. 20. Indonesia2. S1. *A. catechu*. S2. *A. triandra*. S3. *A. lutescens*. S4. *A. normanbyii*.

**Fig.4c. Pooled zymogram of polyphenol oxidase for arecanut accessions**

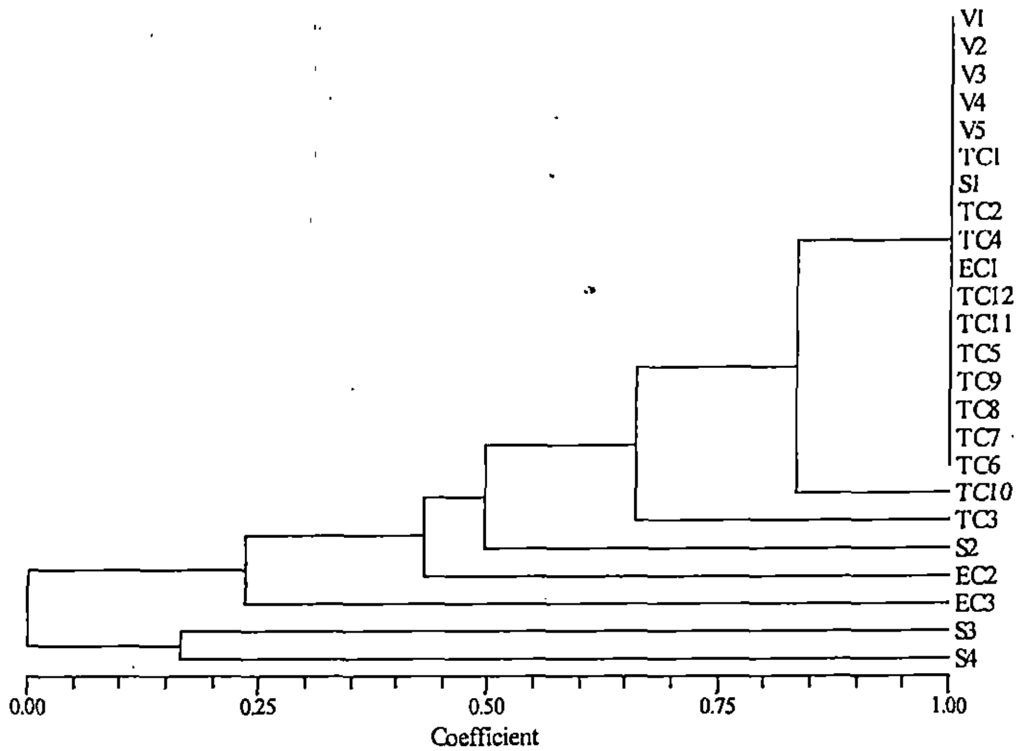
## 4.3 CLUSTER ANALYSIS

Cluster analysis was carried out using the values derived from the isozyme analysis. The results were presented in the form of dendrogram for showing the genetic relationships among the accessions analyzed using the isozymes (Fig. 5).

Dendrogram plotted from these data showed six clusters. Accession TC10 along with "*tie*" accessions such as V1, V2, V3, V4, V5, TC1, TC2, TC6, TC4, TC5 TC7, TC8, TC9, TC11, TC12, ECI, S1 comprised cluster I. Clusters II, III, IV and V were solitary clusters each of which had only one accession viz., TC3, S2, EC2 and EC3 respectively. Species *A. lutescens* and *A. normanbyii* grouped under one cluster i.e. cluster VI. *A. triandra* showed 50 per cent similarity with its closely related species *A. catechu*. Clusters containing EC2 and EC3 were distinct and they had very high dissimilarity over their counterparts.

## 4.4 PROTEIN ESTIMATION

The protein content of the leaf samples of all accessions were estimated by Lowry's method and expressed in mg/ml extract (Table 7). Much variation was observed among the accessions. It ranged from 3.4 to 8.8 mg/ml extract. The lower value was recorded for variety Samruthi and the traditional cultivar Mettupalayam local (3.4 mg/ml extract) and the maximum was recorded for *A. triandra* (8.8 mg/ml extract). All the released varieties of arecanut had lower concentrations of proteins including the traditional cultivars while the YLD tolerant lines recorded higher concentrations as evident from the table viz., British Solomon Islands 1 (7.6 mg/ml extract), Indonesia 2 (7.6 mg/ml extract), and Hirehalli Dwarf (8.20 mg/ml extract).




V1. Mangala V2. Sumangala V3. Sreemangala V4. Mohitnagar V5. Samruthi TC1. South Kanara TC2. Thirthahalli TC3. Mettupalayam Local TC4. Coimbatore Local TC5. Attur Local TC6. Kecheri Local TC7. Kunnamkulam Local TC8. Kannara Local TC9. Kasaragod Local TC10. Thrissur Local TC11. Peechi Local TC12. Hirchalli Dwarf EC1. Saigon2 EC2. Bri.Sol. Is.1 EC3. Indonesia2 S1. *A catechu* S2. *A. triandra* S3. *A. lutescens* S4. *A. nornmanbyii*

**Fig. 5. Dendrogram based on isozyme banding pattern of *Areca* species/varieties/cultivars**

**Table 7. Protein content of the accessions**

| Sl.no. | Name of the accessions | Protein Content* (mg/ml extract) |
|---|---|---|
| 1 | Mangala | 5.54 |
| 2 | Sumangala | 4.80 |
| 3 | Sreemangala | 5.20 |
| 4 | Mohitnagar | 5.80 |
| 5 | Samruthi | 3.40 |
| 6 | South Kanara | 6.60 |
| 7 | Thirthahalli | 5.80 |
| 8 | Mettupalayam Local | 3.40 |
| 9 | Coimbatore Local | 4.20 |
| 10 | Attur Local | 4.0 |
| 11 | Kecheri Local | 4.60 |
| 12 | Kunnamkulam Local | 3.80 |
| 13 | Kannara Local | 4.00 |
| 14 | Kasaragod Local | 6.20 |
| 15 | Thrissur Local | 6.0 |
| 16 | Peechi Local | 5.80 |
| 17 | Hirehalli Dwarf | 8.20 |
| 18 | Saigon 2 | 6.20 |
| 19 | British Solomon islands 1 | 7.60 |
| 20 | Indonesia 2 | 7.60 |
| 21 | *A. catechu* | 5.54 |
| 22 | *A.triandra* | 8.80 |
| 23 | *A.lutescens* | 4.00 |
| 24 | *A.normanbyii* | 5.20 |

(* Values are average of duplicate samples)

## 4.5 ENZYME ACTIVITY STUDIES

### 4.5.1 Peroxidase

The activity of peroxidase enzyme showed much variation (Table 8). Based on the peroxidase activity study, the recorded values were analyzed for various phases viz., activity at initial phase (up to 30 sec.), activity at 90 sec and activity at final phase (after 150 sec.). The accessions were divided into two groups viz., low and high activity groups based on the activity at different phases (Table 9). The YLD tolerant lines, British Solomon Islands 1, Indonesia 2 and Hirehalli Dwarf and species *A. triandra* had recorded the lowest values for all the phases while the other accessions expressed a different activity pattern.

The specific activity of peroxidase for the genus *Areca* was constant and it was found to be 1.16 units/mg protein/min. at $30^0$ C.

### 4.5.2 Polyphenol oxidase

The activity of the enzyme polyphenol oxidase also showed a great variation for the accessions (Table 10). Based on the activity study, the recorded values were analyzed for various phases viz., activity at initial phase (up to 30 sec.), activity at 180 sec and the average activity for 180-240 sec. The accessions were divided into two groups viz., low and high activity groups based on the values recorded for the said intervals (Table 11). The results revealed lower values for the YLD tolerant accessions and for the species *A. triandra.*

The specific activity of polyphenol oxidase for the genus *Areca* was constant and it was found to be 1.16 units/mg protein/min. at $30^0$ C.

## Table 8. Peroxidase activity values (OD at 430 nm)

| Accessions / Time(sec.) | V1 | V2 | V3 | V4 | V5 | TC1 | TC2 | TC3 | TC4 | TC5 | TC6 | TC7 | TC8 | TC9 | TC10 | TC11 | TC12 | EC1 | EC2 | EC3 | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 0.986 | 1.204 | 1.518 | 2.89 | 1.101 | 0.784 | 1.092 | 1.482 | 1.71 | 1.466 | 0.952 | 0.34 | 0.71 | 0.412 | 0.721 | 1.412 | 0.778 | 0.332 | 0.735 | 1.448 | 0.986 | 0.935 | 0.182 | 0.093 |
| 60 | 0.999 | 1.248 | 1.529 | 2.892 | 1.177 | 0.813 | 1.104 | 1.513 | 1.712 | 1.528 | 0.963 | 0.351 | 0.723 | 0.43 | 0.728 | 1.444 | 0.797 | 0.351 | 0.768 | 1.451 | 0.999 | 0.938 | 0.207 | 0.106 |
| 90 | 1.006 | 1.263 | 1.539 | 2.897 | 1.228 | 0.827 | 1.114 | 1.516 | 1.722 | 1.545 | 0.972 | 0.359 | 0.741 | 0.442 | 0.735 | 1.456 | 0.804 | 0.368 | 0.773 | 1.452 | 1.006 | 0.941 | 0.228 | 0.127 |
| 120 | 1.011 | 1.272 | 1.546 | 2.912 | 1.251 | 0.837 | 1.122 | 1.518 | 1.727 | 1.55 | 0.98 | 0.365 | 0.753 | 0.451 | 0.742 | 1.463 | 0.811 | 0.379 | 0.778 | 1.453 | 1.011 | 0.942 | 0.247 | 1.149 |
| 150 | 1.015 | 1.277 | 1.551 | 2.928 | 1.26 | 0.845 | 1.129 | 1.521 | 1.731 | 1.554 | 0.985 | 0.372 | 0.761 | 0.458 | 0.744 | 1.466 | 0.817 | 0.392 | 0.782 | 1.461 | 1.015 | 0.947 | 0.267 | 1.169 |
| 180 | 1.017 | 1.281 | 1.554 | 2.944 | 1.266 | 0.85 | 1.322 | 1.526 | 1.733 | 1.556 | 0.987 | 0.375 | 0.766 | 0.464 | 0.745 | 1.47 | 0.821 | 0.399 | 0.784 | 1.466 | 1.017 | 0.95 | 0.274 | 1.179 |

V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11. Peechi Local. TC12. Hirehalli Dwarf. EC1. Saigon 2. EC2. British Solomon Islands 1. EC3. Indonesia 2. S1. *Areca catechu* S2. *Areca triandra* S3. *Areca lutescens* S4. *Areca normanbyii*

**Table 9. Groups based on peroxidase activity studies**

| Sl.no | Name of the accessions | A* Initial phase (activity upto 30 sec.). | B* Activity at 90 sec. | C* Activity after 150 sec. | D* Activity / min. |
|---|---|---|---|---|---|
| 1 | Mangala | Low | Low | Low | Low |
| 2 | Sumangala | Low | High | Low | Low |
| 3 | Sreemangala | Low | Low | Low | Low |
| 4 | Mohitnagar | Low | Low | High | Low |
| 5 | Samruthi | High | High | High | High |
| 6 | South kanara | High | High | Low | Low |
| 7 | Thirthahalli | Low | Low | Low | Low |
| 8 | Mettupalayam Local | High | Low | Low | Low |
| 9 | Coimbatore Local | High | Low | Low | Low |
| 10 | Attur Local | High | High | Low | Low |
| 11 | Kecheri Local | Low | Low | Low | Low |
| 12 | Kunnamkulam Local | Low | Low | Low | Low |
| 13 | Kannara Local | High | High | Low | High |
| 14 | Kasaragod Local | Low | High | High | Low |
| 15 | Thrissur Local | Low | Low | Low | Low |
| 16 | Peechi Local | Low | High | Low | Low |
| 17 | Hirehalli dwarf | Low | Low | Low | Low |
| 18 | Saigon 2 | Low | High | High | High |
| 19 | Bri.sol.islands 1 | Low | Low | Low | Low |
| 20 | Indonesia 2 | Low | Low | Low | Low |
| 21 | *A. catechu* | Low | Low | Low | Low |
| 21 | *A. triandra* | Low | Low | Low | Low |
| 22 | *A. lutescens* | Low | High | High | High |
| 23 | *A. normanbyii* | Low | High | High | High |

**A.** 1. Low upto 250 2. High 250<
**B.** 1. Low upto 50 2. High 50<
**C.** 1. Low upto 25 2. High 25<
**D.** 1. Low upto 125 2. High 125<

*(Values are expressed in units/ml)

**Table 10. Polyphenol oxidase activity values (OD at 495 nm)**

| Accessions / Time(sec.) | V1 | V2 | V3 | V4 | V5 | TC1 | TC2 | TC3 | TC4 | TC5 | TC6 | TC7 | TC8 | TC9 | TC10 | TC11 | TC12 | EC1 | EC2 | EC3 | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 0.07 | 0.046 | 0.142 | 0.049 | 0.231 | 0.199 | 0.061 | 0.082 | 0.086 | 0.093 | 0.212 | 0.199 | 0.091 | 0.137 | 0.14 | 0.131 | 0.063 | 0.048 | 0.082 | 0.038 | 0.07 | 0.051 | 0.043 | NA |
| 60 | 0.078 | 0.053 | 0.166 | 0.057 | 0.277 | 0.236 | 0.07 | 0.092 | 0.106 | 0.125 | 0.22 | 0.209 | 0.096 | 0.143 | 0.146 | 0.137 | 0.066 | 0.053 | 0.093 | 0.048 | 0.078 | 0.054 | 0.044 | NA |
| 90 | 0.09 | 0.06 | 0.179 | 0.063 | 0.308 | 0.267 | 0.082 | 0.112 | 0.121 | 0.157 | 0.228 | 0.217 | 0.099 | 0.15 | 0.153 | 0.142 | 0.074 | 0.058 | 0.107 | 0.054 | 0.09 | 0.059 | 0.045 | NA |
| 120 | 0.104 | 0.067 | 0.191 | 0.071 | 0.331 | 0.29 | 0.089 | 0.121 | 0.145 | 0.188 | 0.228 | 0.228 | 0.103 | 0.158 | 0.159 | 0.149 | 0.078 | 0.062 | 0.121 | 0.06 | 0.104 | 0.063 | 0.047 | NA |
| 150 | 0.11 | 0.074 | 0.199 | 0.082 | 0.348 | 0.313 | 0.096 | 0.14 | 0.165 | 0.215 | 0.235 | 0.236 | 0.108 | 0.165 | 0.165 | 0.16 | 0.081 | 0.067 | 0.137 | 0.069 | 0.11 | 0.069 | 0.05 | NA |
| 180 | 0.114 | 0.076 | 0.208 | 0.086 | 0.358 | 0.33 | 0.099 | 0.16 | 0.19 | 0.222 | 0.241 | 0.241 | 0.115 | 0.17 | 0.166 | 0.167 | 0.086 | 0.071 | 0.147 | 0.073 | 0.114 | 0.074 | 0.052 | NA |
| 210 | 0.116 | 0.082 | 0.215 | 0.091 | 0.367 | 0.335 | 0.102 | 0.181 | 0.21 | 0.239 | 0.244 | 0.246 | 0.12 | 0.172 | 0.168 | 0.173 | 0.087 | 0.074 | 0.152 | 0.077 | 0.116 | 0.08 | 0.054 | NA |
| 240 | 0.121 | 0.086 | 0.219 | 0.094 | 0.373 | 0.34 | 0.105 | 0.201 | 0.212 | 0.251 | 0.249 | 0.25 | 0.123 | 0.175 | 0.169 | 0.178 | 0.092 | 0.076 | 0.156 | 0.08 | 0.121 | 0.086 | 0.057 | NA |
| 270 | 0.123 | 0.088 | 0.221 | 0.098 | 0.383 | 0.345 | 0.109 | 0.22 | 0.234 | 0.259 | 0.253 | 0.253 | 0.13 | 0.177 | 0.171 | 0.182 | 0.095 | 0.079 | 0.16 | 0.085 | 0.123 | 0.091 | 0.061 | NA |
| 300 | 0.127 | 0.089 | 0.226 | 0.1 | 0.388 | 0.353 | 0.111 | 0.23 | 0.244 | 0.264 | 0.255 | 0.256 | 0.133 | 0.181 | 0.172 | 0.187 | 0.097 | 0.08 | 0.164 | 0.09 | 0.127 | 0.095 | 0.063 | NA |

V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11. Peechi Local. TC12. Hirehalli Dwarf. EC1. Saigon 2. EC2. British Solomon Islands 1. EC3. Indonesia 2. S1. *Areca catechu* S2. *Areca triandra* S3. *Areca lutescens* S4. *Areca normanbyii*

**Table 11. Groups based on polyphenol oxidase activity studies**

| Sl.No. | Name of the accessions | A* Initial phase (activity upto 30secs.). | B* Avg. activity after 180-240 secs. | C* Activity at 180 secs. |
|---|---|---|---|---|
| 1 | Mangala | Low | Low | Low |
| 2 | Sumangala | Low | Low | Low |
| 3 | Sreemangala | High | Low | Low |
| 4 | Mohitnagar | Low | Low | Low |
| 5 | Samruthi | High. | High | Low |
| 6 | South kanara | High | High | High |
| 7 | Thirthahalli | Low | Low | Low |
| 8 | Mettupalayam Local | Low | High | High |
| 9 | Coimbatore Local | Low | High | High |
| 10 | Attur Local | High | High | Low |
| 11 | Kecheri Local | Low | Low | Low |
| 12 | Kunnamkulam Local | Low | Low | Low |
| 13 | Kannara Local | Low | Low | Low |
| 14 | Kasaragod Local | High | Low | Low |
| 15 | Thrissur Local | Low | Low | Low |
| 16 | Peechi Local | Low | Low | Low |
| 17 | Hirehalli Dwarf | Low | Low | Low |
| 18 | Saigon 2 | Low | Low | Low |
| 19 | Bri.sol.islands 1 | Low | Low | Low |
| 20 | Indonesia 2 | Low | Low | Low |
| 21 | *A. catechu* | Low | Low | Low |
| 21 | *A. triandra* | Low | Low | Low |
| 22 | *A. lutescens* | Low | Low | Low |
| 23 | *A. normanbyii* | NA | NA | Low |

**A.** 1. Low upto 10
2. High 10<

**C.** 1. Low upto 7
2. High 7<

**B.** 1. Low upto 10
2. High 10<

*(Values are expressed in units/ml)

# *Discussion*

# 5. DISCUSSION

During the early periods of research, classical strategies including comparative anatomy, physiology and morphology were employed in genetic analysis to determine inter and intra specific variability. In the recent years, the molecular markers have greatly complemented these classical approaches due to their efficiency and authenticity in detecting minor variations at molecular level.

One of the useful molecular markers to detect the genetic variations in the genotypes is isozyme. Isozymes are variant forms of an enzyme but similar in function and usually detectable through electrophoresis on account of the differences in their net electric charges. A large amount of variation can be detected through isozyme studies. The advantage of using isozymes to detect the variations lies in their co-dominant nature whereby the heterozygote could be distinguished from homozygote.

Isozyme characterization was done successfully in pepper, cassava, curcuma and in many other horticultural crops in order to identify the cultivars and to characterize the genotypes biochemically. However, perusal of literature revealed the absence of any serious studies on isozyme analysis in arecanut and hence it is imperative to take up such studies.

The present investigations primarily aim at the biochemical characterization of cultivars of *Areca catechu* and allied species through isoyme analysis using three isozymes namely peroxidase, esterase and polyphenol oxidase. Further, the study also attempts to assess the relationship between the yellow leaf disease (YLD) reaction and the banding patterns of the enzymes. Since less polymorphism was observed in the case of peroxidase and polyphenol oxidase, activity of these enzymes was also studied. Finally, the protein content of the accessions was also estimated in all the accessions for confirming the findings of other studies.

## 5.1 ISOZYME VARIATIONS

### 5.1.1 Standardization of Leaf Sampling

The plant sample for isozyme analysis has not been hitherto standardized in arecanut palm. According to Hengky and Hartana (1994), matured leaves constitute the ideal leaf sample for isozyme analysis in coconut. The relevance of leaf protein as a marker in genetic improvement of coconut has been highlighted by Cardena *et al.* (1998). With a view to identify the most ideal leaf sample in arecanut, the first, third, fourth and sixth fully opened leaves from the crown were analyzed for peroxidase, esterase and polyphenol oxidase. The leaves at different positions gave a similar banding pattern for all the three isozymes. In case of peroxidase and polyphenol oxidase, all the samples had given one band while in esterase three bands were observed. However, the fourth leaf from the crown was taken as the ideal leaf sample for isozyme analysis in arecanut as this leaf is reported as the D leaf for nutrient analysis in this crop by Mohapatra and Bhat (1985).

### 5.1.2 IsozymeVariation in *Areca* Species

All the three isozymes had shown significant variation among the species. For all the isozymes, *A. catechu* was taken as the standard accession and deviation from it was considered as variation. The Rm value of the peroxidase enzyme of *A. catechu* was 0.212 and the same value was recorded by the species *A. triandra* for the same isozyme. This enzyme did not distinguish between the species *A. catechu* and *A. triandra*. However, it could be used to distinguish another wild species *A. lutescens* from the cultivated one. The species *A. lutescens* lacked the common band (Rm 0.212) and had its only band at a different position (Rm 0.282). As the species *A. normanbyii* did not express any banding pattern in this method for peroxidase that accession was grouped separately.

*A. triandra* showed more similar banding pattern with the *A. catechu* and confirms the reports of Bavappa (1974), who have opined that the species *A. triandra*

is primitive when compared to *A. catechu*. In another report, Bavappa and Raman (1965) had suggested that the species *A. catechu* was more advanced than *A. triandra*.

With respect to esterase all the species except *A. normanbyii* could be distinguished based on the polymorphic banding pattern. While three bands were present in *A. catechu* (Rm 0.240, 0.266 and 0.466), the species *A. triandra* lacked the third common band (Rm 0.466). The species *A. lutescens* had its three bands at entirely different positions (Rm 0.173, 0.200 and 0.693). As *A. normanbyii* did not express banding pattern for esterase in this method, here also the species was separated into a different group.

It is to be noted that polyphenol oxidase could differentiate all the four species based on its banding pattern and hence could be effectively used as a biochemical marker. While *A. catechu* had its only band at Rm 0.250, *A. triandra* had its band at Rm 0.243. While *A. lutescens* had its two bands at different positions (Rm 0.200 and 0.341), *A. normanbyii* had only one band (Rm 0.341).

By taking cognizance of the variation in the banding pattern of all the three enzymes in the different species, it can be deduced that isozyme polymorphism could reliably be used to characterize the species in the genus *Areca*.

### 5.1.3 Isozyme Variation in *Areca catechu* L.

To analyze the intra specific variation in cultivars of *A. catechu*, five released varieties, twelve traditional cultivars and three exotic cultivars were used in the study. All the cultivars have shown minimum differences with respect to the banding pattern in case of all the three isozymes. Single band was observed for peroxidase and polyphenol oxidase whereas three bands were observed for esterase.

Released varieties of arecanut, which had not shown any variation in banding pattern for all the three isozymes analyzed were categorized into a single group.

Variation though little, existed in the traditional cultivars for the banding patterns of peroxidase and polyphenol oxidase. The differences were seen in TC3 (Mettupalayam Local) and TC10 (Thrissur Local) by having one extra band for both enzymes. However, they had similarity indices of 0.66 and 0.83 respectively with the other accessions of *A. catechu*.

There was no difference in the banding pattern of the cultivar Hirehalli Dwarf for all the three enzymes analyzed while comparing with majority of the *A. catechu* accessions. It came under the same group in which all the cultivars of *A. catechu* are present. This confirmed the report of Naidu (1963) who found no difference in inflorescence and floral characters of Hirehalli Dwarf when compared to other *A. catechu* accessions.

The presence of the exotic cultivar EC1 (Saigon 2) in the group of traditional and released varieties of *A. catechu* corroborated the findings of Bavappa (1974), who reported that the geographical diversity need not always be related to the genetic diversity. The similar report holds good for the traditional cultivars collected from various states of the country showing a nil variation in banding pattern for all the three isozymes analyzed. However, TC3 and TC10 showed variation with respect to banding patterns.

Two of the three exotic cultivars, EC2 and EC3 were different in their banding patterns for all the three isozymes. This was true as EC2 and EC3 had some extra bands in peroxidase and polyphenol oxidase and similar number of bands with different Rm values in esterase. However, the banding positions of the cultivar EC1 (Saigon 2) was quite similar with those of other accessions of *A. catechu* in all the three isozymes. This is in consonance with the results of Bavappa (1974), who grouped the cultivars from Saigon with the local collections based on cluster analysis using morphological characters.

As regards the genetic difference based on isozymes banding pattern between YLD tolerant cultivars and the susceptible cultivars, exotic cultivar EC2, which is reportedly tolerant to YLD had shown a striking difference for peroxidase and polyphenol oxidase. This accession had two extra bands for peroxidase (Rm 0.162 and 0.187) in addition to the common band (Rm 0.212). In case of polyphenol oxidase the cultivar EC2 had two bands at different positions (Rm 0.150 and 0.170). The common band to all the accessions (Rm 0.250) was conspicuous by its absence in EC2. However, this accession did not exhibit difference for esterase banding pattern.

The accession EC3, a cultivar from *A. triandra* reportedly to be tolerant to YLD had a very distant similarity with the wild form of *A. triandra* in banding pattern as evident from the zymograms. The esterase banding pattern for this accession showed striking difference with the other accessions of *A. catechu*. It had three bands at Rm values 0.186, 0.213, and 0.533 as against the Rm values of 0.240, 0.266 and 0.466 for *A. catechu* accessions.

Differences in the banding patterns in the form of presence of an additional band or absence of a common band could be correlated with disease resistance / tolerance as reported by Farkas and Stahmann (1942) in bean, Deyu (1985) in barley, Fei *et al.* (1997) in soybean and Gupta *et al.* (1995) in *Brassica.*

Even though the YLD tolerant cultivars EC2 and EC3 displayed explicit differences with respect to the isozyme banding patterns when compared to the susceptible cultivars, there intriguingly existed no similarity between the banding patterns of these tolerant cultivars. Moreover, Hirehalli Dwarf which is reported as a probable donor for YLD tolerence did not exhibit any difference in isozyme banding pattern when compared with the susceptible accessions.

Number of bands in peroxidase and polyphenol oxidase might not be completely adequate in order to classify the accessions analyzed. Thickness of the bands cannot be considered, because it may be due to a cluster of closely associated proteins

showing isozymic properties. However, the accessions were grouped based on the data generated from the study. Polymorphism could not be detected in the genotypes of *Areca* except in the case of two disease tolerant cultivars (EC2 and EC3) and two traditional cultivars (TC3 and TC10).

## 5.2 GENETIC SIMILARITY INDEX

The maximum similarity index of unity observed among the accessions of *A. catechu* except the traditional cultivars TC3 and TC10 indicated that they are similar with respect to all the three isozymes under the study. For the accessions TC3 and TC10, the similarity index with other accessions of *A. catechu* though not unity, registered high values. This indicated the close affinity of those accessions with the other accessions of *A. catechu*. Nonetheless, there existed considerable degree of dissimilarity between TC3 and TC10. As the present investigations were mainly focused on variation at the biochemical level, a better insight will be available if the morphological and economic characters of these lines are also taken into account.

Among the exotic cultivars, EC2 followed by EC3 exhibited dissimilarity with other accessions of *A. catechu* and other allied species. It was interesting to mention that there existed maximum dissimilarity between the cultivated form of *A. triandra* with its wild form (S2). Accessions EC2 and EC3 deserved special mention because it was reported to be tolerant to the YLD of arecanut.

The other wild species of *A. catechu* namely, *A. lutescens* and *A. normanbyii* did not show similarity with the accessions of *A. catechu*. The genetic similarity values indicated that the species *A. triandra* was intermediate between the species *A. catechu, A. lutescens* and *A. normanbyii.*

## 5.3 CLUSTER ANALYSIS

Dendrogram plotted from the pooled data from the three isozymes showed six clusters. Accession M10 along with "*tie*" accessions such as V1, V2, V3, V4, V5, M1,

M2, M6, M4, M5, M7, M8, M9, M11, M12, ECI and S1 comprise cluster I. All these accessions belong to the species *Areca catechu.* Cluster II, III, IV and V were solitary clusters consisting of only one accession under each. Species *A. lutescens* (S3) and *A. normanbyii* (S4) although grouped under one cluster i.e. cluster VI, due to the very low genetic similarity persisted between them (0.16) and no similarity with other accessions in the clusters, this cluster could be partitioned into two. *A. triandra* showed 50 per cent similarity with its closely related species, *A. catechu.* Clusters containing EC2 and EC3 were distinct and had very high dissimilarity over their counterparts. This points towards the possible relation of isozyme banding pattern with the tolerance of those cultivars to YLD.

To sum up, the isozyme banding pattern revealed the presence of interspecific variability in the genus *Areca.* The most dissimilar wild species were *A. normanbyii* and *A. lutescens. A. triandra* showed considerable similarity with the cultivated species *A. catechu.* The cross compatibility and the ease of production of interspecific hybrids between *A. catechu* and *A. triandra* (Bavappa 1974) corroborate this finding. However, isozyme studies within the cultivars of *A. catechu* could not reveal the existence of any polymorphism.

## 5.4 PROTEIN ESTIMATION

Quantitative estimation of protein was carried out by Lowry's method using the tris-buffer of pH 7.6. The quantity of the protein varied from 3.4 to 8.80 mg/ml extract. Comparison with the banding pattern of isozymes of the same samples revealed that protein is independent of the enzymes studied. It is supported with the activity studies of the above enzymes, which is reflected in the zymograms. Therefore, the protein content is independent of the factor / enzyme studied, which requires further screening and analytical approaches to exploit for this type of work.

Protein content of the accessions did not follow a uniform pattern among the cultivars of *A. catechu.* However, the estimation revealed the high quantity of proteins

in the disease tolerant types. The cultivars EC2 (British Solomon Islands 1), EC3 (Indonesia 2) and TC12 (Hirehalli Dwarf) had 7.6, 7.6 and 8.2 mg/ml extract of protein respectively. Interestingly the species *A. triandra*, which is reported as tolerant to YLD (Rawther *et al.*, 1982) recorded the highest protein content of 8.8 mg/ml extract. The presence of higher amounts of total protein invariably in all the four YLD tolerant accessions irrespective of the speeies might not be a mere coincidence but could have a possible influence on YLD tolerance.

As the *A. catechu* x *A. triandra* hybrids came into existence already, the further observations on economic and disease reactions would be beneficial to the scientific community to evolve more hybrids using this species. As the nuts of *A. triandra* could be used as masticatory, this would bring fruitful results.

The results of protein estimation are in line with the reports of Chowdappa *et al.* (2000), who recorded variations in the protein concentrations of the apparently healthy and YLD infected leaf samples. Bavappa and Nair (1974) reported that the hybrids produced using the dwarf mutant Hirehalli Dwarf had recorded only low YLD incidence and this dwarf mutant could be used as a donor for YLD tolerance and the higher protein content of this cultivar (8.20 mg/ml extract) suggested the presence of high concentrations of protein as one of the possible reasons for the tolerant reaction. Differences in the protein concentration and electrophoretic pattern have been reported in the disease tolerant and susceptible cultivars of several crops (Mc Coy, 1979).

## 5.5 ENZYME ACTIVITY STUDIES

### 5.5.1 Total Activity

Large variations were observed in the activity of the enzymes, peroxidase and polyphenol oxidase. The accessions could be divided into two groups as low and high activity groups based on the enzyme activity studies. There seems an immense scope for identifying the genotypes based on the enzyme activites if the classification followed a standard pattern for the low and high activity values. Graphical expression

of the activity of both the enzymes revealed that the activity levels varied among the species, varieties and traditional cultivars at constant time, which might be contributed by the feed back inhibition/end product inhibition (Figs. 6 and 7).

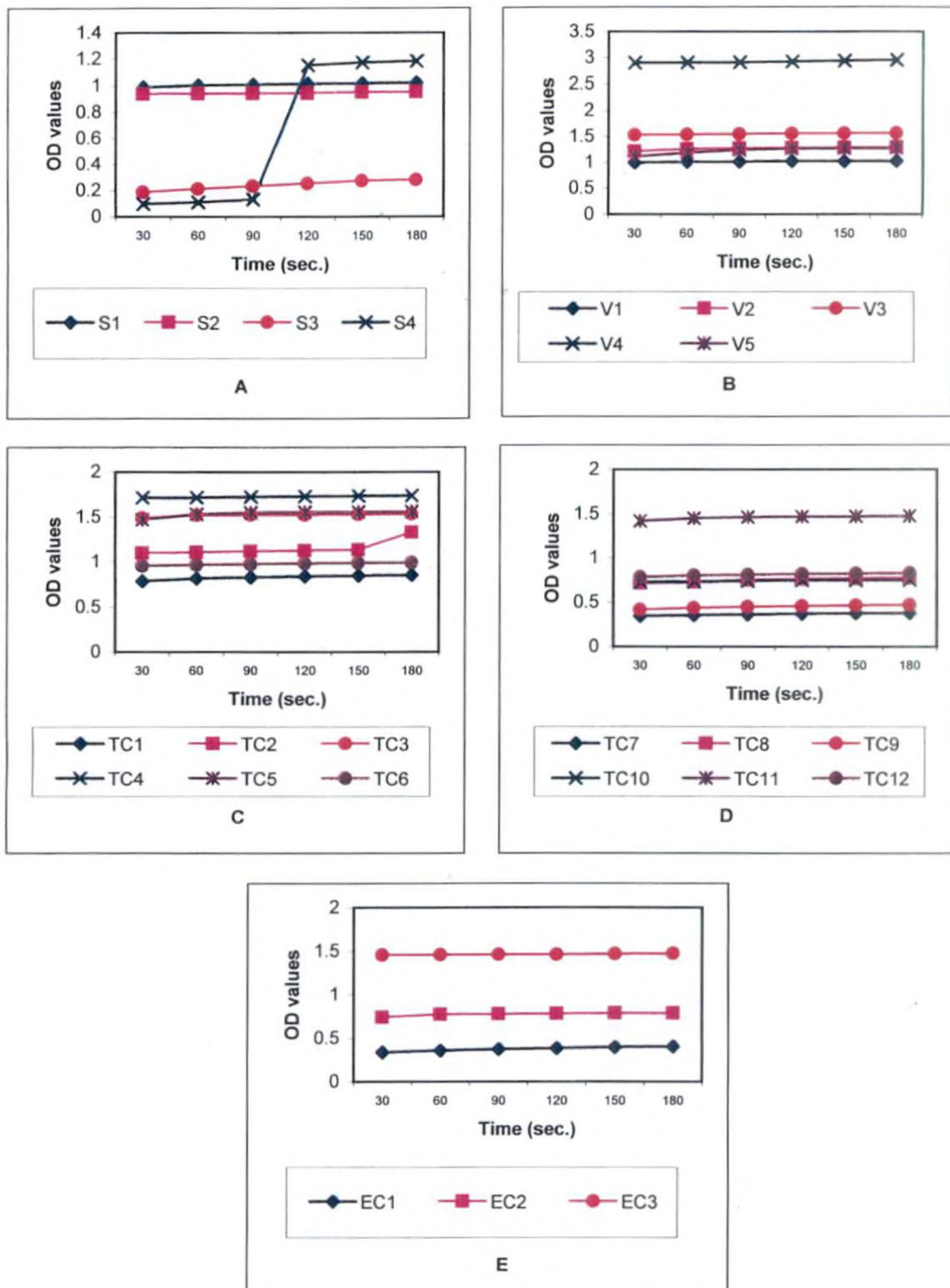
It was observed that the tolerance is related to the lower values of activity or decrease in the rate of reaction. The exotic cultivars EC2 (British Solomon Islands1), EC3 (Indonesia 2) and the traditional cutivar TC12 (Hirehalli Dwarf) in addition to the species *A. triandra* recorded very low activity values for both the enzymes. The possibility of using the enzyme activity rate as a criterion for screening of the genotypes for tolerance to YLD is worth exploring. This follows the reports of Pereira (2000), Reuveni (1991) and Ye *et al.* (1990), who all reported the association of low peroxidase activity with disease tolerance in various crops.

The enzyme polyphenol oxidase recorded very low activity values despite the earlier reports to the effect that genus *Areca* contains more phenols. This could be attributed to the non-accessibility of the enzyme polyphenol oxidase to the phenol substrate, the insufficiency of the substrate or the fact that the available phenol may not be a substrate for the enzyme. The specificity of the enzyme polyphenol oxidase present in this species could be explained through this specific nature of polyphenol oxidase.

### 5.5.2 Specific Activity

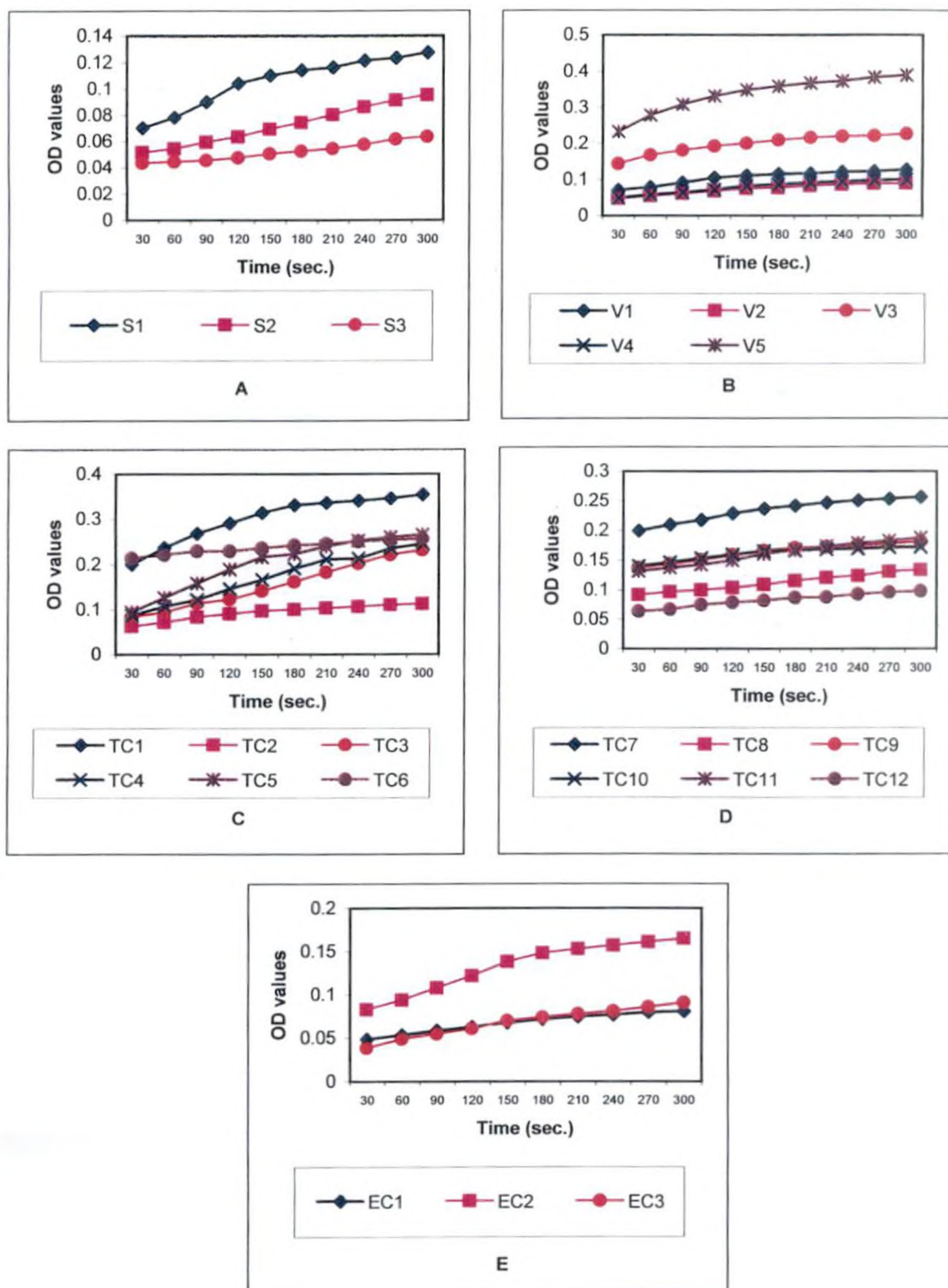
The specific activity of both the enzymes peroxidase and polyphenol oxidase was found to be constant and one and the same (1.16 units/mg protein/min. at $30^0$ C). This explains the genetic stability of the genus *Areca* irrespective of the variations in the morphological, anatomical and biochemical characteristics, which might be resulted because of acquired characters carried over through generations.

The present investigation has thus given an insight into the biochemical characterization of the varieties, traditional cultivars and species under the genus




V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. EC1.Saigon 2. EC2.British Solomon Islands 1. EC3.Indonesia2
S1. *Areca catechu* S2. *Areca triandra* S3. *Areca lutescens* S4. *Areca normanbyii*

**Fig. 6. Activity levels of peroxidase: (A) *Areca* species (B) Released varieties (C&D) Traditional cultivars (E) Exotic cultivars**




V1. Mangala. V2. Sumangala. V3. Sreemangala. V4. Mohitnagar. V5. Samruthi TC1. South Kanara. TC2. Thirthahalli. TC3. Mettupalayam Local TC4. Coimbatore Local. TC5. Attur Local. TC6. Kecheri Local. TC7. Kunnamkulam Local. TC8. Kannara Local. TC9. Kasaragod Local. TC10. Thrissur Local. TC11.Peechi Local. TC12.Hirehalli Dwarf. EC1.Saigon 2. EC2.British Solomon Islands 1. EC3.Indonesia2
S1. *Areca catechu* S2. *Areca triandra* S3. *Areca lutescens* S4. *Areca normanbyii*

**Fig. 7. Activity levels of polyphenol oxidase: (A) *Areca* species (B) Released varieties (C&D) Traditional cultivars (E) Exotic cultivars**

*Areca* and highlighted the possibility of using isozyme as a molecular marker. This information not only supplements and complements the classical approaches of inter and intra specific characterization of the genus through morphology, anatomy and physiology, but to a certain extent validates them also. Further investigation on molecular markers using the state of art technologies viz., Random amplified polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR) may possibly throw some additional light on the complex interrelationships among the cultivars varieties and species of the genus *Areca*.

# *Summary*

# 6. SUMMARY

The present investigations on "Isozyme variation in *Areca catechu* L. and allied species" were undertaken in the department of Plantation Crops and Spices and the Biochemistry laboratory of the College of Horticulture, Kerala Agricultural University, Vellanikkara during the period from 2000 to 2002. The study was carried out with the major objectives of working out the species relationship in the genus areca and biochemically characterizing the varieties/cultivars/types of arecanut palm by isozyme analysis. The study also aimed at bringing out possible association between the isozyme pattern and yellow leaf disease tolerance. The findings of the investigations are summarized hereunder:

Twenty-four accessions of arecanut collected from different centres viz., College of Horticulture, Vellanikkara; Central Plantation Crops Research Institute -Regional Station, Vittal; Agricultural Research Station, Arallam; State Seed Farm, Mannuthy; Tamilnadu Agricultural University, Coimbatore and farmers' fields were utilized for biochemical characterization studies. These included four species of the genus *Areca*, five released varieties, twelve traditional cultivars and three exotic cultivars of arecanut. Three of the accessions were tolerant to the yellow leaf disease of arecanut. The isozymes studied were peroxidase, esterase and polyphenol oxidase.

The isozyme variation in the species of the genus *Areca* was very noticeable, each species having a unique banding pattern. In peroxidase, both the species viz., *A. catechu* and *A. triandra* had a similar band (Rm of 0.212). The species *A. lutescens* had only band (Rm 0.282) at a different position. In esterase, the three bands in *A. catechu* were with Rm values of 0.240, 0.266 and 0.466. However, the species *A. triandra* lacked the band at the Rm 0.466. The species *A. lutescens* had its three bands at different positions (Rm 0.173, 0.200 and 0.693).

All the four species exhibited different banding patterns in case of polyphenol oxidase. While the species *A. catechu* exhibited only band at Rm 0.250 and *A.*

*triandra* at Rm 0.243. While *A. lutescens* had two bands at the different positions (Rm 0.200 and 0.341), the species *A. normanbyii* had only one band at Rm 0.341.

Five released varieties of arecanut viz., Mangala (V1), Sumangala (V2), Sreemangala (V3), Mohitnagar (V4) and Samruthi (V5) did not show any variation with respect to the banding patterns in any of the three isozymes studied and were hence categorized into one group. All these varieties had the maximum similarity index of 1 among them.

The group, traditional cultivars had shown very little variation for the three isozymes except in cultivars TC3 (Mettupalayam local) and TC10 (Thrissur local). They both had one additional band each in the case of peroxidase and polyphenol oxidase (Rm 0.237 and 0.350 respectively). The cultivar TC3 lacked one band in esterase (Rm 0.466), which all the other accessions had in common. However, they also had similarity indices of 66% and 83% with the other cultivars of *A. catechu.*

The exotic cultivars had shown a striking variation with respect to banding pattern in case of all the three isozymes. In peroxidase, the cultivar EC2 (British Solomon Islands1) had two extra bands (Rm 0.162 and 0.187) in addition to the common band (Rm 0.212). In esterase, cultivar EC3 had three bands at different positions (RM 0.186, 0.0.213, and 0.533), as against the positions of the common bands in the other accessions (Rm 0.240, 0.266 and 0.466). In case of polyphenol oxidase, the cultivar British Solomon Islands 1 had two bands (Rm 0.150 and 0.180), while the other accessions had only one band (Rm 0.150).

Interestingly, despite being a cultivar of *A. triandra* the cultivar Indonesia 2 matched neither with *A. triandra* nor with other accessions of *A. catechu* in the isozyme banding patterns. Only in the case of peroxidase, it had shown a somewhat similar pattern of banding as in the case of *A. triandra.*

Even though the YLD tolerant cultivars displayed explicit difference in the banding pattern when compared to susceptible cultivars, there was no similarity in the banding pattern of the tolerant accessions. Hence the possibility of relating the isozyme banding pattern with YLD tolerance is quite remote.

Based on the banding pattern of all the three isozymes, the accessions were divided into eight groups. All the released varieties, Saigon 2, all the traditional cultivars except TC3 and TC10 were categorized into one group. The traditional cultivars TC3 and TC10 were classified into individual groups. The exotic cultivars EC2 and EC3 were also categorized into individual groups. The remaining three species had one single group each.

This was supported by the dendrogram plotted using cluster analysis. The dendogram showing the genetic relationships among the accessions had seven clusters and in addition, it had one '*tie*' group.

The protein content of the accessions recorded a wide range (3.4 – 8.8 mg/ml extract). The protein contents of the cultivars EC2 (British Solomon Islands1), EC3 (Indonesia 2) and TC12 (Hirehalli Dwarf) were very high when compared to the other accessions. In addition to these cultivars, the species *A. triandra* also recorded high protein content (8.80 mg/ml extract).

The enzyme activity studies of the enzymes peroxidase and polyphenol oxidase revealed a high amount of variation in the accessions. The specific activity of the accessions was constant and one and the same for both the enzymes (1.16 units/mg protein/min at $30^0$ C). Based on the activity studies, the accessions were divided into two groups as low and high activity groups. The activity of the enzyme peroxidase recorded a range of 120 – 1455 units/ml. Likewise, the activity of the enzyme polyphenol oxidase had recorded a range of 22 – 208 units/ml.

The activity studies of peroxidase and polyphenol oxidase revealed very low values for the cultivars British Solomon Islands1, Indonesia 2 and Hirehalli Dwarf and species *A. triandra* when compared to other accessions. High protein content alongwith low enzyme activity levels in all the YLD tolerant accessions point towards a possible influence of these on YLD tolerant reaction.

The present investigations have thus given an insight into the biochemical characterization of varieties, traditional cultivars and species under the genus *Areca* and highlighted the possibility of using isozyme as a molecular marker. This information not only supplements and complements the classical approaches of inter and intra specific characterization of the genus through morphology, anatomy and physiology, but to a certain extent validates them.

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# ISOZYME VARIATION IN *Areca catechu* L. AND ALLIED SPECIES

By

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## ABSTRACT OF THE THESIS

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## 8. ABSTRACT

Investigations on "Isozyme variation in *Areca catechu* L. and allied species" were undertaken in the Department of Plantation Crops and Spices and the Biochemistry laboratory of the College of Horticulture, Kerala Agricultural University, Vellanikkara during the period from 2000 to 2002 with the major objectives of working out the species relationship in the genus *Areca* and biochemically characterizing the varieties/cultivars/types of arecanut by isozyme analysis.

Twenty four arecanut accessions comprising of four species, five released varieties, 12 traditional cultivars and three exotic cultivars collected from various states were included in the investigation. The isozymes studied were peroxidase, esterase and polyphenol oxidase.

The species viz., *Areca catechu, A. triandra, A. lutescens* and *A. normanbyii* were displayed variations with respect to the isozyme banding patterns, while no variation was observed among the five released varieties. Traditional cultivars numbering 12 had shown very little variation except in the case of TC3 and TC10. The exotic cultivars, which included two YLD tolerant accessions, had shown a striking variation when compared to other accessions of *A. catechu*. Based on the banding pattern of all the isozymes, the accessions were classified into eight groups. The dendrogram plotted using cluster analysis also vindicated this classification.

The protein content of the accessions ranged from 3.4 to 8.8 mg/ml extract. The protein content was very high in the YLD tolerant accessions when compared to the susceptible genotypes. The activity of the enzyme peroxidase recorded a wide range of 120 - 1455 units/ml while the range of polyphenol oxidase was 22 - 208 units /ml. The activity values of peroxidase and polyphenol oxidase were very low in all the YLD tolerant lines in comparison with other accessions. High protein content alongwith low enzyme activity levels recorded invariably by all the tolerant accessions point towards a possible influence of these biochemical parameters on YLD tolerant reaction.