CHARACTERISATION OF DIOECY IN NUTMEG (*Myristica fragrans* Houtt.)

By
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THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Science in Horticulture

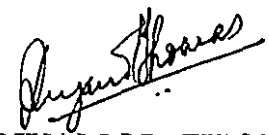
Faculty of Agriculture
Kerala Agricultural University

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

1997

DECLARATION

I hereby declare that this thesis entitled "**Characterisation of dioecy in nutmeg (*Myristica fragrans* Houtt.)**" is a *bona fide* record of research work done by me during the course of research and this thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



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Certified that this thesis, entitled "**Characterisation of dioecy in nutmeg (*Myristica fragrans* Houtt.)**" is a record of research work done independently by **Miss Priyamol Thomas,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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ACKNOWLEDGEMENT

I bow my head before Lord Jesus who was with me throughout my work and bestowed on me good health and caring friends to share my problems. His blessings alone helped me to complete this small endeavour.

I have great pleasure to place on record my sincere thanks to Dr. Rema Menon, Associate Professor, Banana Research Station, Kannara and Chairperson of my Advisory Committee for her keen interest, erudite guidance and constant encouragement. Her valuable advice and unstinting support rendered at all stages of the work contributed most to the completion of the study.

I am extremely grateful to Dr. E.V. Nybe, Professor and head i/c, Department of Plantation Crops and Spices for his propitious support and critical scrutiny of the manuscript.

No words can truly express my profound gratitude to Dr. A. Augustine, Associate Professor, AICRP on M&AP and member of my Advisory Committee for his everwilling help, scholarly suggestions, sustained interest and the facilities provided during the course of my research work.

I am very much grateful to Dr. V.S. Sujatha, Assistant Professor, Department of Plantation Crops and Spices and member of my Advisory

Committee for her valuable suggestions and advice during the various stages of the study. Her critical comments indeed helped me a lot in improving the text.

I duely acknowledge Dr. P.V. Prabhakaran for his valuable advice and help rendered in the statistical analysis of the data.

I am thankful to Dr. R. Kesavachandran for his support and help given to me for photographic works.

With immense pleasure I thank each and every member of Department of Plantation Crops and Spices for their support and encouragement.

I am extremely grateful to the scientists, farm manager, other staff members and labourers of CPCRI, Kannara for all the facilities provided to me during the course of my research work.

I wish to thank Estellita teacher for her constant encouragement and cheerful advice.

My heartfelt thanks are extended to Bindu Chechy, Reetha Chechy, Sathya Chechy, Seena Chechy and Jalaja Chechy for their help and affection.

A word of thanks to Ajith Mohan, Matheesh, Babu and Praveen.

My cordial thanks are due to my loving friends, Anu, Maveli, Manju, Chandru, Rosamma, Deuthi, Bavrah, Sreekala and Naija.

The award of Junior Fellowship by Kerala Agricultural University is gratefully acknowledged.

I wish to thank Sri. O.K. Ravindran, C/o Peagles, Mannuthy for the neat typing and prompt service.

I am forever beholden to my parents and brothers for their boundless affection, support and prayers.

I am deeply indebted to my uncle, aunty, Roychetan and Rennychetan for their affection, care, encouragement, support and prayers. My gratitude towards them is beyond words.

I also thank my grandfather for his blessings and constant prayers.

I affectionately thank my Mary Aunty, Lysa Aunty and Mini Aunty for their prayers and encouragement.

PRIYAMOL THOMAS

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Introduction

1. INTRODUCTION

Nutmeg, (*Myristica fragrans* Houtt.) is an important tree spice, yielding two products of commercial value, nutmeg and mace. It is a spreading evergreen tree belonging to the small, primitive family Myristicaceae. Nutmeg, which originated in the Moluccas islands in the East Indian Archipelago, was introduced to India and other tropical countries during the 18th century. Nutmeg is cultivated in many tropical countries like Malaysia, Indonesia, West Indies, India and Sri Lanka.

Both nutmeg and mace are used as spice and in medicine. In eastern countries, they are used more as a drug than as a condiment. Nutmeg is said to have stimulative, carminative, astringent and aphrodisiac properties. The volatile oil present in both spices contains small amounts of myristicin and elemicin,which are narcotic and poisonous when consumed in large quantities. The husk or pericarp is made into sweetmeats and jellies. Nutmeg butter, the fat extracted from the kernel is used in the manufacture of scented oils, perfumes, soaps and as flavouring agent in cookery and confectionery.

In nutmeg, apart from the long prebearing period, dioecy is recognized as one of the major problems of cultivation. The seedling progenies segregate into females and males in 1:1 proportion (Nichols and Pryde, 1958). The sex of the tree cannot be determined until flowering has commenced which normally takes 5 to 7 years. Male trees are required only in 1:10 ratio in a plantation for effective pollination. Expecting a 1:1 segregation into male and female, 50 per cent of the total trees in any nutmeg plantation raised through seedlings will be males. Cutting down of the excess male trees results in irregular spacing, loss of time and resources to the grower.

Presently, the only option to overcome dioecy in nutmeg is the use of vegetatively propagated material or top working of the excess male plants. Eventhough several vegetative methods have been reported with varying degrees of success, the large scale adoption of these methods is constrained due to the nonavailability of orthotrops in sufficient numbers which are required for the production of plants with normal growth habit. So seedlings continue to be the major propagating material. Hence, the identification of sex of the plants in the seedling stage remains a viable alternative for which a definite methodology has not been evolved so far.

Several attempts have been made in the past to identify the sex of nutmeg plants at the seedling stage on the basis of

morphological variations in mature male and female plants (Flach 1966; Nayar *et al.*, 1976). Chemical methods were also tried with limited success (Phadnis and Choudhari, 1971). The present programme envisages a comprehensive study of the dioecious nature in nutmeg through morphological observations and chemical analysis. The programme also aims to determine the factors contributing towards sex expression in nutmeg and to identify morphological or chemical markers for the early detection of sex in nutmeg plants.

Review of Literature

2. REVIEW OF LITERATURE

The nutmeg (*Myristica fragrans* Houtt) belongs to the small, primitive family Myristicaceae of the order Laurales with about 18 genera and 300 species. *Myristica* is the largest genus of which Sinclair (1968) lists 72 species. New Guinea is considered to be the centre of origin and distribution of the genus (Sinclair, 1958).

The seeds of several species are used locally for medicinal purposes. The seeds and arils of *Myristica argentea* Warb, the Papua nutmeg from New Guinea and those of *Myristica malabarica* Lam, the false or Bombay nutmeg from Malabar Coast of India are used as adultrants of the genuine products from *M. fragrans*. *M. argentea* possesses a peculiar odour and flavour while *M. malabarica* is practically odourless and tasteless.

The principal synonyms for *M. fragrans* are *M. officinalis* L.f., *M. moschata* Thunb, *M. aromatica* Swartz and *M. amboinensis* Gandoger (Purseglove, 1974).

2.1 Morphology

Nutmeg is a spreading dioecious evergreen tree, growing 5-13 m high, sometimes attaining 20 m height. All parts of the plant are aromatic.

2.1.1 Branches

In nutmeg, two types of branching, erect (orthotropic) and spreading (plagiotropic) are observed. But orthotropic branches are few in number. The twigs are glabrous, slender and greyish brown (Purseglove *et al.*, 1981)

2.1.2 Leaves

The leaves of nutmeg have been described as alternate, glabrous and exstipulate, with a petiole length of about 1 cm. The shape of the leaves may be elliptic or oblong-lanceolate with an acute base and the apex acute or slightly accuminate (Purseglove *et al.*1981).The leaves contain an essential oil. Meyer (1941) obtained a yield of 1.56 per cent oil by steam distillation of dried leaves. Khan and Krishnaswamy (1953) have reported yields of 0.41 to 0.62 per cent from fresh leaves in India. The leaf oil prepared by Meyer (1941) contained 80 per cent alphapinene and 10 per cent myristicin.

2.1.3 Growth pattern

Nutmeg trees are found to be slow growers when compared to other perennial trees. Shoot growth in nutmeg is found to be cyclic, a period of growth followed by a quiescence. Nazeem *et al.* (1981) observed six flushes during a period of one year.

2.1.4 Inflorescence and flowers

The male and female inflorescences are similar, glabrous and axillary with the flowers in umbellate cymes in which there are 1-10 flowers in the male inflorescence and 1-3 in the female (Talbot, 1976). The flowers which measure upto upto 1 cm in length are fragrant, waxy and fleshy. Leslia (1963) reported that the female flowers were larger than the male and that the calyx tube appeared more oval in shape.

2.1.5 Fruit

The fruit is a fleshy drupe, broadly pyriform, yellow, smooth, 6-9 cm long. Ridley (1912) described the fruits when ripe as one of the most beautiful fruits in nature. There is a circumferential longitudinal ridge with persistent remains of the stigma. When ripe the succulent, aromatic yellow pericarp splits into two halves along the suture to expose the seed and the red aril.

2.1.6 Seed

Purseglove *et al.*(1981) described the seeds as round or oval in shape with brown testa. The kernel is broadly ovoid, greyish brown and ruminate and consists of convoluted dark brown perisperm, lighter coloured endosperm and a small embryo.

2.2 Dioecy in nutmeg

Nutmeg is typically dioecious with male and female flowers on different trees, but hermaphrodite plants with male and female flowers on the same plant and hermaphrodite flowers are noticed occasionally. The sex of the tree cannot be identified until flowering has commenced which normally takes 5 to 7 years (Purseglove *et al.*, 1981)

Dienum (1931) reported that on an average 55 per cent of the seeds from a female plant will be female, 40 per cent male and 5 per cent bisexual. Nichols and Pryde (1958) observed that segregation of progenies into male and female was in 1:1 proportion. Flach (1966) recognised two different sexes, a female flowering sex, a male flowering sex, the latter being subdivided into four different groups viz. male, bisexual males, bisexuals and bisexual females. Hermaphrodite plants produce only smaller number of fruits and fairly high per cent of double nuts. Sastri (1962) reported that occasionally male trees after a number of years produced female flowers and eventually became females.

Morphological variability in relation to dioecy has been reported with regards to leaf size, venation and nature of branching (Janse, 1898 and Prestoe, 1948). Flach (1966) observed that female trees had a significantly higher stem

diameter during the prebearing stage while no significant difference in terms of plant height was observed.

Nazeem *et al.* (1981) studied the flowering pattern in nutmeg and reported that the male and female trees showed variation in flowering. In females, flowering was constrained to seven months whereas in males flowering was observed throughout the year. Maximum flowering in both cases occurred in July followed by October.

2.3 Determination of sex in nutmeg

Several workers devised different methods to identify the sex of nutmeg seedlings. Janse (1898) stated that male trees had smaller leaves and less horizontal branches. But this difference was not clear and prominent enough in young seedlings and hence it was not possible to determine the sex of the plant in the seedling stage.

Prestoe (1948) reported that the sex of nutmeg seedlings less than 30 cm high could be identified by observing the leaf form and venation. Leaves of female trees would be nearly elliptical with more or less straight veins, whereas male trees would posses nearly obovate leaves with their veins running to the more pronounced point of the leaf. Flach (1966) observed a slight difference in the tree size between female and male trees.

Methods used by Flach (1966) to determine sex linked chromosomes did not reveal a heteromorhic chromosome pair. No difference in the number, shape and size of the chromosome was noticed in mitosis of trees of different sexes by him.

Phadnis and Choudhari (1971) reported difference in the colour reaction of leaf extracts of male and female plants with ammonium molybdate reagent. They observed the development of `faint green' colour for male plant and `sea green' colour for females with ammonium molybdate reagent. They also noticed that colour differences were more marked in the fifth leaf collected from old flush than the second leaf from the new flush.

Nair *et al.* (1976) postulated a method for distinguishing the sex by the shape of calcium oxalate crystals in the lower epidermal cells of leaves of plants of at least two years age. Male plants showed a single larger rhomboidal or prismatic crystal with rectangular or square flat faces. Female plants had a cluster of small crystals.

Packiyasothy *et al.* (1991) analysed the essential oil content of leaves, its composition and phenolic profile of dried leaves and reported that the essential oil content was more in adult female trees. This difference was more clear in the leaves of five year old plants and in seedlings they could not quantify the oil content. They also reported that the

Thin layer chromatography profile of phenolics in leaves of adult male plants showed two additional phenolic compounds compared with that of female plants.

Krishnamoorthy *et al.* (1992) studied the reliability of some of the earlier reported methods to identify the sex at seedling stage. They observed that the crystal pattern of calcium oxalate was well differentiated in 70 per cent males and 78 per cent females. In seedlings the crystal pattern was not definite and so sex could not be differentiated in them based on crystal study. They conducted a detailed study of the characters like leaf shape, size of leaves, venation, colour of new sprouts, days for germination of seeds and concluded that none of these characters can be considered as a marker for sex in nutmeg seedlings. Among the various colour tests tried using leaf exudates and alcoholic leaf extracts of male and female plants ammonium molybdate test was found reliable. This test was reproducible in the case of all the mature plants tested but not in the seedlings.

2.4 Influence of sex on morphological characters

Differences in the morphological traits have been observed in the male and female plants of different dioecious species. The possibility of using morphological characters as sex markers have been investigated by many.

Lacombe (1980) observed significant differences in the internode length, growth rate and plastochron of male and females of dioecious hemp *Cannabis sativa*. He reported that sex of the plants could be determined at the age of 15 days based on early vegetative characters.

In jojoba, *Simmondsia chinensis* which is a dioecious desert shrub, the females were found to have larger leaves and more open canopies than males (Kohorn, 1994). Chen *et al.* (1985) reported 100 per cent correlation between sex and length/width (L/W) ratio of leaves of jojoba. He observed that L/W ratio of all the females was greater than average and that of all the males was smaller.

In odum (*Milicia excelsa*), a forest tree species, the females were found to possess more spreading crown and thicker stems than the males (Nyong *et al.*, 1994). A study of the male and female plants of *Morus alba* and *Ficus carica* by Kotaeva *et al.* (1982) revealed that females of both species were having larger leaves, denser and more spreading crown than males.

Machon *et al.* (1995) reported that in the dioecious perennial *Asparagus officinalis* the male plants produced more but thinner stems than the females. In the dioecious palm *Chamaedorea tepijelote*, the male plants showed spatial variation in growth rate but not the females. Both sexes had

different rates of production of leaves among years and males produced significantly more inflorescence than females (Oyama, 1990).

2.5 Phenolics and sex expression

The term phenolics embraces a wide range of plant substances which possess a common aromatic ring bearing one or more hydroxyl substituents. In the natural state phenolics play an important role in plants, the hormone balance, disease resistance and protection of injured tissue from infection (Crompton and Preice, 1986). Phenolics in high concentrations are toxic to plant cells themselves (Tepper and Anderson, 1984). Hence phenolics will be present in plants in small quantities.

The number of phenolic compounds present and the content were found to be differing in males and females of some dioecious species. Singh *et al.* (1974) subjected the leaf extracts of *Carica papaya* to ten colourimetric tests specific to phenolics and determined the sex of the plant with an accuracy of 86 per cent using Folinciocalteau reagent.

Billau *et al.* (1987) conducted high performance liquid chromatography (HPLC) of phenolic compounds in storage roots of asparagus during vegetative period and found that male

plants contained less caffeic acid and chelidonic acid and more coniferin than the female plants.

In the dioecious plant sorrell (*Rumex acetosella*) the content of hydroxy cinnamic acid (P-caumaric acid and ferulic acid) and hydroxy benzoic acids (vanillic acid) were found to be more in the leaves and reproductive organs of male plants than female plants (Dyurdevich *et al.*, 1992).

2.6 Protein and isozyme pattern

In recent years the analysis of protein or isozyme by polyacrylamide gel electrophoresis (PAGE) has been considered as a unique and powerful technique for ascertaining genetic relationship in plants. Further PAGE provides a tool for species and cultivar identification where morphological and cytological data are inadequate (Wilkinson and Beard, 1972).

Isozymes are multiple molecular forms of enzymes that catalyse the same reaction but differing in physicochemical properties (Market and Moller, 1959). Among organic molecules isozymes are very useful aids in deciphering the evolutionary relationship within different groups of plant and animals (Oliver and Zapater, 1985).

Isozyme analysis has been reported to be useful in varietal characterisation and classification in a variety of

crops. Different male cultivars of date palm were identified using esterase and peroxidase banding pattern (Al- Jibouri, 1988). Durham *et al.* (1989) used nine different isozyme systems for the clonal identification of peach. Bhat *et al.* (1992) demonstrated the usefulness of analyses of esterase, acid phosphatase and catalase isozymes to distinguish the different cultivars of banana.

Munoz *et al.* (1982) observed that sex of the adult and juvenile plants of papaya could be identified using peroxidase zymogram where the male plants had more bands than females. Suganuma and Iwasaki (1983) analysed peroxidase isozymes in the leaves of date palm electrophoretically and reported that there was difference in the zymogram of male and female plants. The female zymogram showed two additional bands and sex of the seedlings could be identified at two leaf stage using peroxidase isozyme pattern.

Peroxidase isozyme pattern was successfully used by Sriprasertek *et al.* (1988) to distinguish the sex and cultivar of tissue culture derived plants of papaya. In *Asparagus officinalis* association of malate dehydrogenase locus with sex determining genes was reported by Maestri *et al.* (1991). Shuang-Xi and Xue-Feng (1995) analysed peroxidase isozymes from different organs and tissues of male and female plants of asparagus using PAGE and reported that male plants had one

band less than females in the zymograms of callus. Stem apices from tissue culture also showed same result.

McDaniel *et al.* (1979) analysed the leaf proteins from individual plants of jojoba by PAGE and reported that male and female plants were readily distinguishable due to quantitative and qualitative differences in fast migrating proteins. Miege (1981) reported that tubers from male and female plants of *Dioscorea opposita* could be differentiated by electrophoresis of albumin present in it.

Kovaleva *et al.* (1980) analysed proteins in the stem apices of dioecious hemp by immunoelectrophoretic method and reported a protein unique to female plants. Talyshinskii (1982) reported greater electrophoretic mobility for proteins in the leaves of polyploid male forms of mulberry than in females.

Materials and Methods

3. MATERIALS AND METHODS

The studies were carried out at the College of Horticulture, Vellanikkara during 1995-97.

Experimental material

Fifteen each of well differentiated male and female trees of 20 years age were selected from the garden maintained at the Central Plantation Crops Research Institute (CPCRI) Research Centre, Kannara. Hundred each of one year and two year old seedlings were raised and a random sample of thirty plants each was selected from them for morphological observations and biochemical analyses.

3.1 Morphological characterisation

3.1.1 Mature plants

3.1.1.1 Plant characters

The following biometric observations of mature trees were recorded.

3.1.1.1.1 Plant height

The height of the plant from ground level to the tip of the longest branch was measured and expressed in metre.

3.1.1.1.2 Spread of the plant

The maximum horizontal extension of branches in the North-South and East-West direction was measured and their product gave a measure of the spread of the plant.

3.1.1.1.3 Collar girth

The collar girth of the plants at 5 cm above ground level was recorded in metre

3.1.1.1.4 Height at first branching

The height of the plant from the ground level to the first branching point was measured in metre.

3.1.1.1.5 Stem diameter at branching

The diameter of the stem at the point where branching started was measured in metre.

3.1.1.1.6 Number of main branches

The number of main branches in one metre length starting from the first branching point of the plant was observed and recorded.

3.1.1.1.7 Canopy shape and orientation of branches

The canopy shape and orientation of the branches were recorded by visual observation.

3.1.1.2 Leaf characters

From the selected male and female plants, 30 leaves each were collected and the following leaf characters were recorded.

3.1.1.2.1 Leaf length

Length of leaves was recorded in centimetre.

3.1.1.2.2 Leaf width

Width of the leaves at the widest portion was measured and expressed in centimetre.

3.1.1.2.3 Length/width ratio

The length by width ratio was worked out for each leaf.

3.1.1.2.4 Leaf area

The leaf area was worked out by multiplying the maximum length and width of the leaf with a correction factor of 0.7 reported by Menon *et al.* (1990).

3.1.1.2.5 Shape of leaves

The shape of leaves was noted visually and expressed as elliptic or oblanceolate.

3.1.1.2.6 Length of petiole

The petiole length of 30 leaves from each plant was measured and the mean was worked out.

3.1.1.2.7 Number of leaves per flush

In a plant ten flushes were observed and number of leaves produced in each flush was recorded and the average was worked out.

3.1.1.2.8 Stomatal index

In order to ascertain the stomatal index, peelings of the lower epidermis were taken from the base, middle and tip of leaf lamina and number of stomatal openings as well as number of other cells were counted per microscopic field. The stomatal index was worked out using the following formula:

$$\text{Stomatal index} = \frac{\text{Number of stomata}}{\text{Number of stomata} + \text{Number of other epidermal cells}} \times 100$$

Two mature leaves selected at random were observed per plant and mean stomatal index was worked out.

3.1.1.2.9 Calcium oxalate crystal pattern

To study the crystal pattern, the 10th leaf from the tip of a shoot was selected and the lower epidermis was peeled off with a sharp blade and observed directly under the microscope.

3.1.1.3 Flower characters

The following flower characters were observed.

3.1.1.3.1 Number of flowers per unit area of canopy

The number of flowers per unit area (0.25 m^2) of canopy was recorded using a quadrat of the same size.

3.1.1.3.2 Flower size

From each plant, ten flowers were collected and the length and diameter of the flowers were measured in centimetre..

3.1.1.4 Fruit characters

From the fifteen selected female trees, ten fruits each were collected in a random manner and the fruit shape, fruit weight, seed weight and fresh weight of the mace were recorded.

3.1.2 Seedling observations

From the selected female plants, seeds were collected and hundred seedlings were raised. Thirty seedlings were selected randomly for taking observations. The seedling observations include seedling height, collar girth, height at branching, stem diameter at branching, total number of branches, leaf length, leaf width, L/W ratio, leaf area and petiole length.

3.2 Biochemical analyses

Biochemical analyses were carried out at the biochemistry laboratory of the College of Horticulture, Vellanikkara.

3.2.1 Mature plants

The following biochemical analyses of the mature plants were done.

3.2.1.1 Essential oil content of leaf

Essential oil was estimated by clevenger apparatus. From each sexually well differentiated male and female plant, three mature leaf samples of 60 g each were collected for distillation. The oil was collected in the clevenger trap for 2½ hours and expressed as percentage.

3.2.1.2 Estimation of total phenol

Total phenol in leaf was estimated by Folin Ciocalteau method (Mahadevan and Sridhar, 1986).

One gram of freshly collected leaf sample was crushed in a mortar and pestle with 10 ml methanol and centrifuged for 20 minutes. The residue was again centrifuged twice for 15 minutes each adding 5 ml methanol each time to ensure complete extraction of phenol. From the methanol extract ten ml was evaporated to dryness and 10 ml distilled water was added to dissolve the phenols. Since the phenol content was found high, 1 ml water extract was made upto 100 ml with distilled water. From the made up solution, 1 ml was taken in a test tube and 2 ml distilled water was added. To the diluted solution, 0.5 ml Folin-Ciocalteau reagent was added followed by the addition of 2 ml of Na_2CO_3 20 per cent after 3 minutes. The contents were mixed thoroughly by shaking

and the absorbance was read at 650 nm in a UV-vis spectrophotometer.

Total phenol content was calculated from a standard curve of catechol (0.1 g in 100 ml) and was expressed in percentage.

3.2.1.3 Thin layer chromatography of phenolics

Preparation of TLC plates: A slurry of silica gel G was prepared by adding 100 ml distilled water to 50 g silica gel. The slurry was stirred for 5 minutes and air bubbles removed by vacuum system. Plates of 0.25 mm thickness were prepared by TLC plate preparation system. The plates were activated at 120°C for half an hour just before use. The plates were cooled at room temperature and used for sample analysis.

Five ml methanol extract of phenolics of male and female plants were taken separately and acid hydrolysed with 10 ml 2M HCl for half an hour by boiling in a water bath. After cooling, the extracts were treated with ethyl acetate in a separating funnel. The ethyl acetate extract was concentrated by boiling on a water bath till the volume is reduced to 0.5 ml. From the concentrated sample, 5 μl was spotted on the plate and eluted with 10 per cent acetic acid in chloroform.

The plates were first sprayed with FolinCioculteau reagent and then with 20 per cent Na_2CO_3 to develop the spots. The Rf valuesof the spots were calculated as

$$\frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent}}$$

3.2.1.4 Ammonium molybdate test

The ammonium molybdate test was carried out following the procedure described by (Phadnis and Choudhari, 1971). Ammonium molybdate reagent was prepared by dissolving 10 g molybdic acid in a mixture of 14 ml of ammonium hydroxide and 27 ml of distilled water. This solution was then slowly poured into a cool mixture of 50 ml of nitric acid and 114 ml of distilled water.

The fifth leaf from the tip of a twig was collected from sexually well differentiated plants and from seedlings. The samples collected were dried in the sun for three days and thereafter in an oven for 3 hrs. The midribs and petioles of the samples were discarded and the remaining parts were finely powdered in a grinder. From the powdered sample, 0.1 g was accurately weighed and transferred to a conical flask and added 25 ml of distilled water. The mixture was shaken in a shaker at 200 rpm for 20 minutes and filtered. From the filtrate, 5 ml was used for the test. Five drops of ammonium molybdate reagent was added to 5 ml filtrate. The mixture was heated to boiling, cooled and the colour developed was noted visually after 30 minutes. The absorbance was recorded at 490 nm using a UV-vis spectrophotometer.

3.2.1.5 Protein pattern by electrophoresis

Polyacrylamide gel electrophoresis (PAGE) using vertical slab gel was carried out for protein pattern. Acrylamide monomers were polymerised with N-N methylene bis acrylamide [CH_2 (NHCONH = $CH_3)_2$ bis] to obtain the gel. N,N,N,'N' tetramethyl ethylene diamine (TEMED) was added as polymerisation initiator and freshly prepared ammonium persulfate as catalyst.

Polyacrylamide gel was preferred because of its chemical inertness, high resolution, ease in handling, transparency of the gel and easiness in preparation.

Preparation of the gel

The following stock solutions were prepared

Solution A

Tris	-	38.3 g
TEMED	-	0.46 ml
IN HCl	-	48 ml
Distilled water	-	200 ml
pH	-	9

Solution B

	7.5% polymerisation	10% polymerisation
Acrylamide	30.0 g	40.0 g
Bisacrylamide	0.9 g	1.2 g
Made upto	100 ml	

Solution C

Ammonium persulfate	-	0.14 g
Distilled water	-	100 ml

Preparation of gel column

Gels of 7.5 per cent and 10 per cent acrylamide were tried. The size of the gel slab was 16 cm x 14 cm x 0.01 cm. Solution A and B were prepared and stored in amber coloured bottles at 0-4°C. Solution C was prepared fresh each time. Stock solution of A, B and C were taken in 1:1:2 proportion and mixed thoroughly. The solution was applied by a syringe in between glass plates and kept in the polymerisation stand. Combs were placed at the top to make the wells.

Extraction buffers

Extraction buffers of different composition were tried to extract active protein from leaf tissue. The composition of different extraction buffers tried are given below.

1. Tris buffer - 21.199 g
   Citric acid - 2.626 g
   Vitamin C - 0.52839 g
   L-Cystein HCl - 0.52689 g
   Made upto - 500 ml
   pH - 7

2. Tris buffer - 21.1995 g
   Citric acid - 2.626 g
   L-Cystein HCl - 0.52689 g
   ß-Mercaptoethanol - 0.039065 g
   pH - 7
   Made upto - 500 ml

3. Citrate buffer

   Reagent A

   Citric acid - 10.505 g
   Made upto - 500 ml

   Reagent B

   Sodium citrate - 14.705 g
   Made upto - 500 ml

Sixteen ml of reagent A and 34 ml of reagent B were mixed together and pH was adjusted to 5.3. Then the solution was

made upto 100 ml. From the made up solution 50 ml was made upto 100 ml to prepare 50 ml solution.

4.	Tris buffer	-	1 M
	NaCl	-	50 m*M*
	$CaCl_2$	-	1 m*M*
	EDTA	-	50 m*M*
	pH	-	8.5
5.	Tris HCl	-	28.8 g
	Citric acid	-	2.626 g
	Vitamin C	-	0.52839 g
	L-Cystein HCl	-	0.52689 g
	Insoluble PVP	-	0.5 g
	ß-Mercapto ethanol	-	0.39065 g
	Made upto	-	500 ml
	pH	-	7

One millilitre of this buffer was taken and made upto 100 ml after adding 17.115 g of sucrose at the time of extraction.

Insoluble PVP was used to chelate the polyphenols and thus prevent oxidation by polyphenol oxidase enzyme. Sucrose was added to increase density.

Preparation of sample

Leaf samples of both tender and mature leaves were collected in liquid nitrogen. One gram sample was ground in 5 ml extraction buffer in a prechilled mortar and pestle. The homogenized material was centrifuged at 1500 rpm for 15 minutes in a refrigerated centrifuge at 5°C. After centrifugation the clear supernatant was collected and stored in a refrigerator and loaded in the well at the time of electrophoresis.

Acetone powdering of sample

Since active protein groups could not be extracted from fresh leaf sample, acetone powdering of leaf samples was also tried.

Leaf samples were collected and acetone powder was prepared by blender homogenizing 25 g of tissue in two successive 100 ml aliquots of cold acetone. The homogenate was collected by filtering through Whatman number 40 filter paper. The homogenate was air dried until free of acetone odour, the resulting dry powder was weighed and freeze dried.

One gram of acetone powder was ground in 10 ml extraction buffer in a prechilled mortar and pestle in an ice bath. The extract collected was centrifuged in a refrigerated centrifuge

for 15 minutes. The supernatant collected after centrifuging was loaded in the well for electrophoresis.

Electrophoretic running

Electrode buffer solution

Tris	-	6 g
Glycine	-	28.8 g
Made upto	-	1000 ml
pH	-	8.3

After polymerisation the gels were transferred to electrophoresis unit. Upper and lower tanks were filled with precooled electrode buffer. Ten and twenty micro litre samples were applied to each well with transfer pipette. Upper tank was connected to the cathode and lower one to the anode. The analysis of protein was carried out in anionic system. Bromophenol blue (0.002%) was added to the upper tank as the tracer dye.

A constant current of 20 m A was applied for the first half an hour and increased to 40 m A and maintained till the end of running. A cooling system was attached to the electrophoresis unit for heat dissipation and electrophoresis was carried out at 4°C for 6 hrs.

After running, the gels were immersed in 0.01 per cent Coomassie brilliant blue R 250 in 15 per cent trichloroacetic acid overnight. Seven per cent acetic acid at 50-60°C was used for destaining.

3.2.1.6 Isozyme analysis

Polyacrylamide gel electrophoresis of peroxidase was carried out as in protein electrophoresis. Staining and developing of isozyme band was done as mentioned below.

Staining solution

Benzidine	-	0.208 g
Acetic acid	-	18 ml
H_2O_2 3%	-	100 ml
Water	-	80 ml

Fresh stain was prepared each time. Benzidine 0.208 g was dissolved in 18 ml and heated to boil for complete dissolution of benzidine. Hydrogen peroxide was added just before staining. Kept it as such overnight and destained with 7 per cent acetic acid.

3.2.2 Seedlings

The total phenol content estimation and ammonium molybdate test of one year old and two year old plants were carried out.

3.3 Statistical analysis

The data collected were statistically analysed and analysis of variance was done as described by Panse and Sukhatme (1978). The characters which were found significant after analysis of variance were subjected to discriminant function analysis to develop a discriminant function to differentiate between male and female plants.

Results and Discussion

4. RESULTS AND DISCUSSION

The results of the investigations on "Characterisation of diocey in nutmeg (*Myristica fragrans* Houtt.)" conducted during 1995-1997 in the Department of Plantation Crops and Spices and Biochemistry Laboratory, College of Horticulture, Vellanikkara are presented and discussed in this chapter.

4.1 Morphological characterisation

4.1.1 Mature plants

4.1.1.1 Plant characters

The data with regard to plant characters of male and female plants of nutmeg are presented in Table 1a and 1b. Analysis of variance table for plant characters is given in Appendix-I.

4.1.1.1.1 Plant height

The height of male plants was found to range from 7.0 m to 14.8 m whereas in female plants it varied from 7.2 to 13.6 m. The average height of male and female plants were 11.43 m and 10.22 m respectively (Table 1a). The analysis of variance indicated no significant difference in this

character between male and female trees and values are found to be broadly overlapping.

4.1.1.1.2 Spread of plant

The mean spread of male plants was observed to be 64.24 m^2 which in turn was lower than that in females which registered 74.15 m^2. Wide variation was noted in the spreading pattern of both male and female plants. The spread of male plants varied from 24.01 m^2 to 89.25 m^2 whereas in females it ranged from 30.8 m^2 to 111.3 m^2 (Table 1a). The difference in this character was not significant enough to discriminate between male and female plants.

4.1.1.1.3 Collar girth

Collar girth of female plants was observed to range from 0.64 to 1.43 m and that of male plants from 0.61 to 1.49 m (Table 1a) the mean collar girth of female plants (1.06 m) was found to be slightly larger than that of male (0.97 m). However, analysis of variance did not reveal any pronounced difference between male and female plants.

4.1.1.1.4 Height at branching

Eventhough wide variation was noted in the branching heights of male and female trees, no distinguishable difference existed between males and females in this

character. The branching height varied from 0.41-2 m in males and 0.62 to 2.01 m in females. The mean branching height of male and female plants were 1.17 m and 1.26 m respectively (Table 1b).

4.1.1.1.5 Stem diameter at branching

At branching, a maximum of 1.14 m stem diameter was observed for male plants and 1.28 m in female plants. Minimum stem diameter observed for male plants was 0.51 m and 0.59 m in females. Average stem diameter of female plants (0.953 m) was found to be slightly higher than that of males (0.815 m) (Table 1b). Stem diameter at branching of male and female plants were found to be broadly overlapping and hence it can not be taken as a discriminating character between the males and the females.

4.1.1.1.6 Number of main branches

The number of main branches observed along one metre length of the tree was almost same for male and female plants. The number of branches varied from 6 to 20 in males and 10 to 21 in females (Table 1b). The mean number of branches in males was 14.13 and in females it was 15.33.

Table 1a. Plant characters

	Height of plant (m)		Spread (m^2)		Collar girth (m)	
	M	F	M	F	M	F
Mean	11.433± (0.58)	10.22± (0.58)	64.24± (5.67)	74.15± (5.67)	0.977± (0.06)	1.063± (0.06)
Range	7.0- 14.8	7.2- 13.6	24.01- 89.25	30.8- 111.3	0.61- 1.49	0.64- 1.43

Table 1b. Plant characters

	Height at branching (m)		Stem diameter at branching (m)		No. of branches at 1 m ht	
	M	F	M	F	M	F
Mean	1.175± (0.12)	1.261± (0.12)	0.815± (0.06)	0.953± (0.06)	14.133± (0.92)	15.33± (0.92)
Range	0.41- 2.0	0.62- 2.01	0.51- 1.14	0.59- 1.28	6- 20	10- 21

The values in the brackets denote standard error

4.1.1.1.7 Canopy shape and branching pattern

In both males and females, the canopy shape was found to be conical and branching pattern was plagiotropic. Male and female plants did not show any difference in these characters.

In several studies on dioecious tree species, attempts have been made to relate the sex with plant characters such as height and stem diameter. Thomas and Lafrankie (1993) studied the sex, size and interyear variation in flowering among dioecious forest trees of the family Euphorbiaceae in Malayan rainforest. They reported that in small statured species *Aprorusa microstachya* and *Baccaurea parviflora* the males grow to a larger height than females. But they also reported overlapping of height distribution of males and females in all the tall statured species.

High stem diameter in female plants has been reported in *Milicia excelsa* by Nyong *et al.* (1994) and in *Asparagus officinalis* (Machon *et al.*, 1995). In nutmeg, Flach (1966) observed that the female trees had a significantly high stem diameter during the prebearing stage while no marked difference in terms of plant height existed.

In the present study, observation on plant characters of selected male and female trees showed no significant differences in the spread of the plant and stem diameter at

branching. In respect of plant height, collar girth and height at branching no definite pattern indicative of sex has emerged.

4.1.1.2 Leaf characters

The observations in respect of leaf characters are presented in Table 2a and 2b. Analysis of variance table for leaf characters of mature male and female plants is given in Appendix-II.

4.1.1.2.1 Leaf length

The length of leaves in male plants ranged from 7.19 cm to 13.85 cm whereas in females it was 9.05 cm to 13.67 cm. The average length of male and female plants were 11.07 cm and 11.66 cm respectively (Table 2a) and indicated no significant variation.

4.1.1.2.2 Leaf width

Leaf width was found to vary significantly between male and female plants. The average leaf width in male plants (3.74 cm) was found to be lower than that in female plants (4.61 cm). In general, the female plants were observed to have broader leaves than the male plants.

4.1.1.2.3 Length by width (L/W) ratio

Analysis of variance revealed significant variation in the L/W ratio between male and female plants. Male plants were found to possess higher L/W ratio than females since the females were found to have broader leaves. The mean L/W ratio of male plants (2.95 cm) was observed to be higher than that of females (2.56 cm) (Table 2a).

Chen *et al.* (1985) reported significant difference in the L/W ratio between male and females of jojoba. They observed that L/W ratio of all the males was smaller. So highly significant positive correlation was observed between sex and L/W ratio. In nutmeg also the L/W ratio registered by all the male plants was higher than the mean value.

4.1.1.2.4 Leaf area

The leaf area recorded in male plants ranged from 11.61 cm^2 to 49.14 cm^2 (Table 2b). The corresponding range in females was 21.08 cm^2 to 55.68 cm^2. The mean leaf area of female plants (37.59 cm^2) was found to be larger than that of the males (29.57 cm^2). But overlapping of leaf areas of male and female plants was observed.

Kotaeva *et al.* (1982) studied the male and female plants of *Morus alba* and *Ficus carica* and reported that the females of both the species had larger leaves than the males. In

nutmeg also most of the females studied were found to have larger leaves than the males though some overlapping was found in the distribution of leaf area. This observation is supported by an earlier finding of Janse (1898) who recorded smaller leaves in male trees of nutmeg.

4.1.1.2.5 Petiole length

No pronounced difference in the petiole length was observed between male (1.01 cm) and female (1.05 cm) plants (Table 2b).

4.1.1.2.6 Number of leaves/flush

Both male and female plants produced the same number of leaves/flush with a range of 2-4 leaves (Table 2b). No sex linked difference was evident in respect of this character.

4.1.1.2.7 Stomatal index

Stomatal indices were also not found to vary between male and female plants. Average stomatal index of male plants was found to be 6.86 and that of females 6.79 (Table 2b).

4.1.1.2.8 Leaf shape

The observation recorded in respect of leaf shape and nature of leaf tip and leaf base are furnished in Table 3 and Table 4. It can be seen that in both males and females the

Table 2a. Leaf characters

	Leaf length (cm)		Leaf width (cm)		l/w ratio	
	M	F	M	F	M	F
Mean	11.07± (0.44)	11.667± (0.44)	3.745± (0.18)	4.61± (0.18)	2.95± (0.08)	2.567± (0.08)
Range	7.19- 13.85	9.05- 13.67	2.28- 5.02	3.84- 5.78	2.73- 3.21	2.08- 3.27

Table 2b. Leaf characters

	Leaf area (cm^2)		Petiole length (cm)		No. of leaves/ flesh		Stomatal index	
	M	F	M	F	M	F	M	F
Mean	29.57± (2.56)	37.59± (2.56)	1.01± (0.03)	1.05± (0.03)	6.4± (0.13)	3.4± (0.13)	6.86± (0.24)	6.79± (0.24)
Range	11.61- 49.14	21.08- 55.68	0.8- 1.2	0.9- 1.2	2- 4	2- 4	5.67- 7.98	5.1- 7.79

The values in the brackets denote standard error

Table 3. Leaf shape in male plants

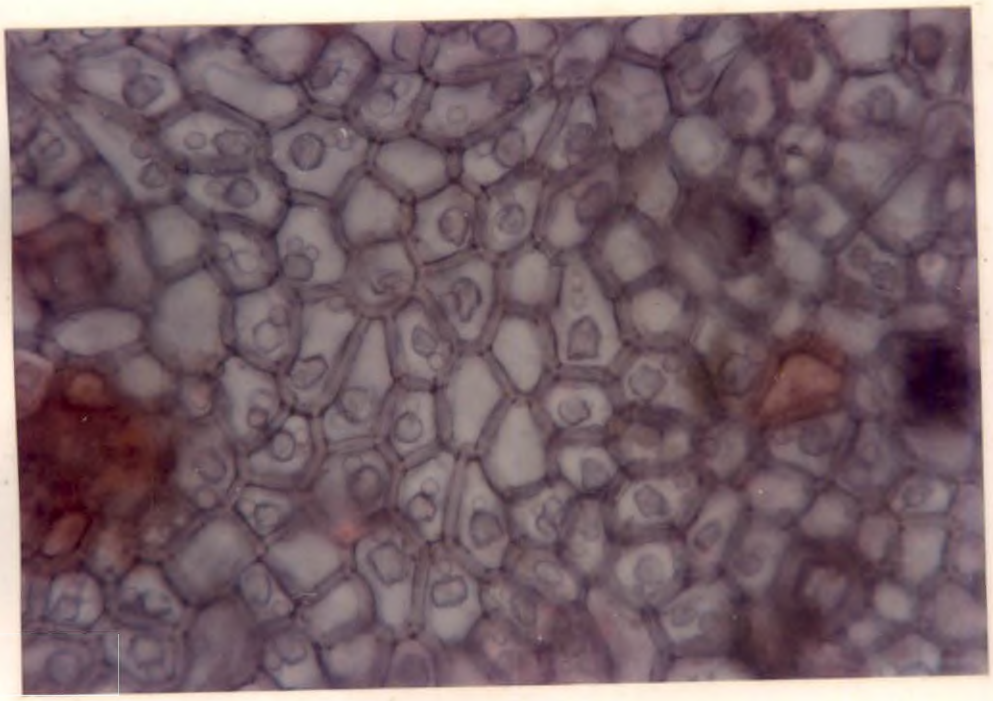
Sl. No.	Leaf shape	Leaf tip	Leaf base
1	Oblanceolate	Acuminate	Attenuate
2	Oblanceolate	Acuminate	Attenuate
3	Oblanceolate	Acuminate	Attenuate
4	Oblanceolate	Acuminate	Attenuate
5	Oblanceolate	Acuminate	Attenuate
6	Oblanceolate	Acuminate	Attenuate
7	Oblanceolate	Acuminate	Attenuate
8	Oblanceolate	Acuminate	Attenuate
9	Elliptic	Acute	Acute
10	Elliptic	Acuminate	Acute
11	Oblanceolate	Acuminate	Attenuate
12	Oblanceolate	Acuminate	Attenuate
13	Elliptic	Acute	Acute
14	Elliptic	Acuminate	Attenuate
15	Elliptic	Acuminate	Acute

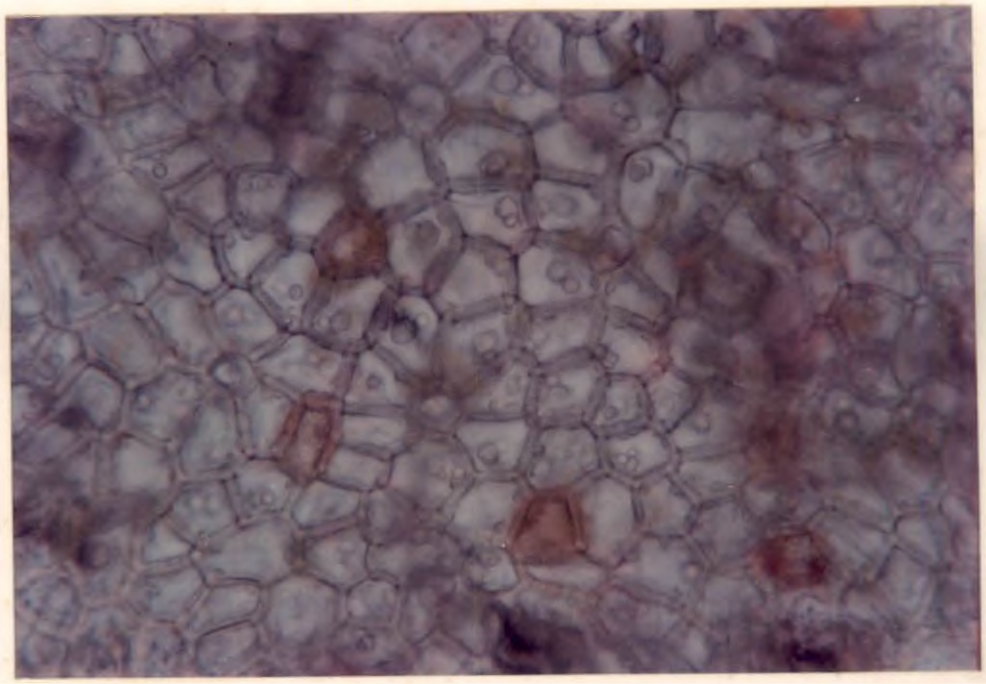
Table 4. Leaf shape in female plants

Sl. No.	Leaf shape	Leaf tip	Leaf base
1	Elliptic	Acuminate	Acute
2	Elliptic	Acuminate	Acute
3	Oblanceolate	Acute	Attenuate
4	Oblanceolate	Acuminate	Attenuate
5	Elliptic	Acute	Acute
6	Oblanceolate	Acuminate	Attenuate
7	Elliptic	Acuminate	Acute
8	Oblanceolate	Acuminate	Attenuate
9	Oblanceolate	Acuminate	Attenuate
10	Oblanceolate	Acuminate	Attenuate
11	Oblanceolate	Acute	Attenuate
12	Oblanceolate	Acuminate	Attenuate
13	Elliptic	Acute	Acute
14	Oblanceolate	Acuminate	Attenuate
15	Elliptic	Acute	Acute

Plate 1. Calcium oxalate crystal pattern in lower leaf epidermis of male nutmeg tree

Plate 2. Calcium oxalate crystal pattern in lower leaf epidermis of female nutmeg tree





leaf shape was predominantly oblanceolate. Elliptic leaf shape was also observed. The leaf tip was predominantly accuminate in both male and female plants. A small percentage of acute leaf tip was also accorded. The leaf base showed a marked tendency towards attenuate nature though acute bases occurred to a limited extent.

4.1.1.2.9 Calcium oxalate crystal pattern

The data relating to calcium oxalate crystal pattern in the lower epidermal cells are presented in Table 5. In the adult male plants 64.34 per cent of the cells observed were found to possess rhomboidal crystals and 35.65 per cent of cells showed a clustered pattern. In contrast, with regard to female plants, 65.9 per cent of the total cells observed had clustered calcium oxalate crystals and 34.01 per cent cells revealed rhomboidal cells (Plates 1 and 2).

Nair *et al.* (1976) reported that the sex of nutmeg plants could be distinguished based on the shape of calcium oxalate crystals in the lower epidermis of leaves of plants of at least two years age. The crystal pattern observed by them in male and in female is in conformity with the present studies. Krishnamoorthy *et al.* (1992) recorded rhomboidal (70%) and clustered (78%) crystal pattern in male and female plants of nutmeg respectively. In the present study, the observation recorded is supportive of the aforesaid result to a great

Table 5. Calcium oxalate crystal pattern in nutmeg leaves

Age of the plant	Total no. of cells observed (mean of 15 plants)	Cells with clustered crystals		Cells with rhomboidal crystals		Cells with crystals of no definite pattern	
		No.	%	No.	%	No.	%
Adult plant male	1136	405	35.65	731	64.34	-	-
Adult plant female	1288	850	65.99	438	34.01	-	-
1 year old seedling	962	461	47.92	269	27.96	232	24.11
2 year old seedling	1028	534	51.94	278	27.04	216	21.01

Plate 3. **Inflorescence of a male nutmeg tree**

Plate 4. **Inflorescence of a female nutmeg tree**

extent. However, the presence of compound crystals of female type in male plants and vice versa complicates the reliability of this method.

The crystal pattern in both one year and two year old seedlings did not reveal much variation. A higher percentage of clustered crystals were observed in both groups along with crystals of no definite pattern. Since both typesof crystal pattern are observed in one year and two year old seedlings no definite conclusion regarding sex could be derived based on this character

4.1.1.3 Flower characters

The data regarding flower characters are given in Table 6 and analysis of variance table for flower characters is given in Appendix-III.

4.1.1.3.1 Number of flowers per unit area

The number of flowers in 0.25 m^2 area of the selected male and female plants recorded are presented in Table 6. The number of flowers in male plants varied from 198-358 per 0.25 m^2 with a mean of 285.6. In female plants it varied from 55-102 per 0.25 m^2 with a mean of 75.93 per 0.25 m^2. In a male inflorescence clusters of 1-10 flowers were observed whereas the female flowers were seen mainly solitary or in clusters of 1-3 flowers (Plates 3 and 4). The male plants

were found to produce three times more flowers than female plants. This may be because, females of dioecious species often have higher reproductive effort than males because of their production of fruits. Gehring and Linhart (1993) reported that in the dioecious species *Silene latifolia* the females produced larger but fewer flowers than males. Carr (1991) reported that in american holly (*Ilex opaca*) on an average, individual males produced 7.4 times as many flowers as in female trees.

4.1.1.3.2 Flower size

The mean diameter of female flowers were found to be larger than that in males. But analysis of variance did not show any significant difference in the flower diameter of male and female plants (Table 6). Leslia (1963) reported that female flowers were larger than that of male flowers in nutmeg. Danilin (1973) studied sexual dimorphism in some dioecious tree species like *Populus tremula*, *P. balsamifera*, *Salix caprea* and *Hippophae rhamnoides* and reported that the only reliable indicator of sex was flower bud diameter which was larger in male than in female trees.

Length of male flowers varied from 0.71 cm to 0.95 cm with a mean of 0.85 cm and that of female flowers varied from 0.88 cm to 1.1 cm with a mean of 0.93 cm (Table 6). Nyong *et al.* (1994) reported that in *Milicia excelsa*, the male

Table 6. Flower characters of male and female plants

	No. of flowers/ unit area		Flower diameter (cm)		Flower length (cm)	
	M	F	M	F	M	F
Mean	285.6± (45.3)	75.93± (45.3)	1.91± (0.06)	2.13± (0.06)	0.85± (0.02)	0.93± (0.02)
Range	198-358	55-102	1.82-2.08	1.92-2.34	0.71-0.95	0.88-1.1

The values in the brackets denote standard error

flowers were longer and slender than females. But in nutmeg analysis of variance did not reveal any significant difference between male and female flowers in terms of flower length.

4.1.1.4 Fruit characters

The data relating to fruit characters recorded for the fifteen selected plants are furnished in Table 7. Analysis of variance table for fruit characters is given in Appendix-IV. It can be seen that the fruit characters are highly varying and significant difference was observed between plants in respect of all the characters studied. This may be because of the seedling origin of female plants.

4.1.2 Seedlings

4.1.2.1 Plant characters

The plant characters of one year and two year old seedlings were studied and are summarised in Tables 8a and 8b respectively.

4.1.2.1.1 Seedling height

The height of one year old plants ranged from 30 cm to 57 cm with a mean of 35.86 cm. In two year old plants the height varied from 80-112 cm with a mean of 94.46 cm.

Table 7. Variation in fruit characters of the selected female plants

Plant No.		Mean fruit length (cm)	Mean peri-meter (cm	Mean fruit weight (g)	Mean mace weight (g)	Mean seed weight (g)
1	round	5.95± (0.12)	15.40± (0.18)	54.51± (2.02)	0.86± (0.13)	7.60± (0.4)
2	round	6.97± (0.12)	17.40± (0.18)	80.08± (2.02)	2.11± (0.13)	11.70± (0.4)
3	round	6.64± (0.12)	15.99± (0.18)	65.01± (2.02)	1.87± (0.13)	9.76± (0.4)
4	pyriform	7.71± (0.12)	14.87± (0.18)	58.31± (2.02)	1.33± (0.13)	9.22± (0.4)
5	round	7.26± (0.12)	15.87± (0.18)	69.01± (2.02)	1.57± (0.13)	9.17± (0.4)
6	pyriform	7.39± (0.12)	15.28± (0.18)	62.42± (2.02)	1.61± (0.13)	8.66± (0.4)
7	pyriform	8.22± (0.12)	15.20± (0.18)	67.04± (2.02)	2.24± (0.13)	8.78± (0.4)
8	round	7.96± (0.12)	15.71± (0.18)	60.97± (2.02)	1.98± (0.13)	8.88± (0.4)
9	pyriform	8.11± (0.12)	15.24± (0.18)	64.10± (2.02)	2.04± (0.13)	8.82± (0.4)
10	round	7.14± (0.12)	13.97± (0.18)	47.76± (2.02)	1.21± (0.13)	7.95± (0.4)
11	round	8.41± (0.12)	17.60± (0.18)	87.26± (2.02)	3.43± (0.13)	12.29± (0.4)
12	round	7.09± (0.12)	15.23± (0.18)	48.82± (2.02)	1.74± (0.13)	11.12± (0.4)
13	pyriform	8.24± (0.12)	16.10± (0.18)	74.57± (2.02)	2.93± (0.13)	11.57± (0.4)
14	round	8.01± (0.12)	14.90± (0.18)	59.55± (2.02)	1.63± (0.13)	8.85± (0.4)
15	round	8.06± (0.12)	15.48± (0.18)	63.77± (2.02)	1.52± (0.13)	8.06± (0.4)

The values in the brackets denote standard error

4.1.2.1.2 Collar girth

The mean collar girth of one year old plants were found to be 1.85 cm and that of two year old plants 3.77 cm.

4.1.2.1.3 Height at branching

In one year old plants branching was not observed. But in two year old plants the mean branching height was found to be 36.76 cm which showed high variation between individual plants. In two year old plants the mean stem diameter at branching was found to be 2.61 cm.

4.1.2.1.4 Total number of branches

The number of branches in two year old plants also showed high variation between individual plants. The number of branches varied from 0 to 11 with a mean of 6.86.

4.1.2.2 Leaf characters

The leaf characters of one year old and two year old seedlings are presented in Table 8a and 8b respectively.

4.1.2.2.1 Leaf length

The leaf length of one year old seedlings were found to vary from 9.2 cm to 14 cm with a mean of 11.46 cm (Table 8a).

In two year old plants, the mean leaf length was found to be 11.63 cm which ranged from 9.46 cm to 14.13 cm (Table 8b).

4.1.2.2.2 Leaf width

The mean leaf width of one year old plants were found to be smaller (3.67 cm) than that of two year old plants (4.14 cm). The minimum leaf width of one year old plants were found to be 2.60 cm and that of 2 year old plants 3.39 cm (Table 8a and 8b). The maximum leaf width of one year old plants were 5.30cm and that of two year old plants 5.14 cm.

4.1.2.2.3 L/W ratio

The L/W ratio of one year old plants ranged from 2.59 to 4.35 with a mean of 3.16. In two year old plants, the ratio varied from 2.13 to 3.32 with a mean of 2.78 (Table 8a and 8b).

4.1.2.2.4 Leaf area

In one year old plants the mean leaf area was found to be 30.17 cm^2 which varied between 17.68 cm^2 to 51.52 cm^2. In two year old plants the leaf area ranged from 23.03 cm^2 to 50.41 cm^2 with a mean of 34.51 cm^2 (Table 8a and 8b).

Table 8a. Variation in characters of one year old plants

	Characters	Mean	Range	Coefficient of variation
1	Seedling height (cm)	35.36	30-57	15.46
2	Collar girth (cm)	1.85	1.4-2.5	15.5
3	Leaf length (cm)	11.46	9.2-14	10.2
4	Leaf width (cm)	3.67	2.6-5.3	16.89
5	L/W ratio of leaf	3.16	2.59-4.35	15.15
6	Leaf area (cm^2)	30.17	17.68-51.52	25.88
7	Petiole length (cm)	0.92	0.7-1.06	9.89
8	Phenol content (%)	0.649	0.39-1.57	38.98
9	OD value of ammonium molybdate test	0.442	0.18-0.71	39.26

Table 8b. Variation in characters of two year old plants

	Characters	Mean	Range	Coefficient of variation
1	Seedling height (cm)	94.46	80-112	9.68
2	Collar girth (cm)	3.77	2.5-5	16.4
3	Height at branching (cm)	36.76	13-66	38.13
4	Stem diameter at branching (cm)	2.61	1-4	23.3
5	Total number of branches	6.86	0-11	42.85
6	Leaf length (cm)	11.63	9.46-14.13	11.77
7	Leaf width (cm)	4.14	3.39-5.14	11.3
8	L/W ratio	2.78	2.13-3.32	10.79
9.	Leaf area (cm^2)	34.51	23.03-50.41	21.38
10	Petiole length (cm)	1.03	0.94-1.23	5.82
11	Phenol content (%)	1.403	0.20-1.88	25.00
12	OD value of ammonium molybdate test	0.451	0.162-0.72	38.13

4.1.2.2.5 Petiole length

The mean petiole length of one year old plants were found to be 0.92 cm and that of two year old plants 1.03 cm. In one year old plants the petiole length varied between 0.70 cm and 1.06 cm. In two year old plants the petiole length ranged from 0.94 cm to 1.23 cm (Table 8a and 8b).

The data presented in Table 8a and 8b in respect of seedling characters of one year and two year old seedlings reveal wide range of variation. The coefficient of variation ranged from 9.89 to 39.26 per cent in one year old seedlings. The least amount of variation was exhibited with regard to petiole length followed by leaf length (10.2 per cent) and L/W ratio of leaf (15.15 per cent). The variation was highest with regard to OD value of ammonium molybdate (39.26 per cent) followed by phenol content (38.98 per cent). In two year old seedlings, the variation observed with regard to different characters ranged from 5.82 (petiole length) to 42.85 (number of branches). The trend observed for all other characters were similar to those recorded for one year old seedlings.

The morphological parameters recorded in the case of one and two year old seedlings did not conform to a definite pattern and did not give any indication as far as sex is concerned. However, these plants are to be planted in the field and observed until flowering to verify any positive

correlation of morphological characters at seedling stage with sex of mature plants.

4.2 Biochemical characterisation

4.2.1 Mature plants

4.2.1.1 Essential oil content of leaf

The data on essential oil content of leaf are presented in Table 9. The leaf oil content in male plants ranged from 0.33 to 1.0 per cent whereas in females the range observed was 0.5 to 1.16 per cent. Mean oil content in female plants (0.73 per cent) was found to be slightly higher than that of male plant (0.68 per cent).

Packiyasothy *et al.* (1991) reported a higher oil content in the leaves of female trees than in male trees. The present study is in conformity with the above observation.

4.2.1.2 Total phenol content of leaf

The total phenol content in male plants ranged from 0.6 to 2.3 per cent with an average of 1.42 per cent. In female plants the total phenol content varied from 0.4 to 2.3 per cent with a mean of 1.22 per cent. Of the fifteen male plants analysed ten plants had phenol content of 1.4 per cent and above. On the other hand only five plants of the selected

Table 9. Essential oil and total phenol content of leaves and absorbance reading of ammonium molybdate test of mature plants

	Oil content (%)		Total phenol (%)		Absorbance 490nm	
	M	F	M	F	M	F
Mean	0.681± (0.05)	0.732± (0.05)	1.421± (0.14)	1.223± (0.14)	0.381± (0.04)	0.594± (0.04)
Range	0.33- 1.00	0.5- 1.16	0.6- 2.3	0.4- 2.3	0.19- 5.5	0.36- 0.82

The values in the brackets denote standard error

Plate 5. TLC profile of phenolics in male and female nutmeg plants


flavonoid
←Phenol→
M
F

fifteen female plants showed a total phenol content of 1.4 per cent and above. The rest registered lower phenol content than 1.4 per cent (Table 10).

In the present study male plants showed high phenol content and low essential oil content whereas female plants registered high essential oil content and low phenol content.

4.2.1.3 Thin layer chromatography of phenolics

The TLC profile of phenolic extract after acid hydrolysis showed a single phenolic spot with Rf value 0.32 and a flavonoid spot with Rf value 1.0. Both male and female plants displayed the same pattern (Plate 5). It may be an indication of the origin of secondary products from the same basic group for both male and female plants.

Packiyasothy *et al.* (1991) reported two additional phenolic spots in the TLC profile of male plants than that of female plants. Presence of more phenolic groups and high concentration of phenolics in the male plant indicate that synthesis and accumulation of phenolics have a direct relation to the sexuality and fruit setting of nutmeg plants. Sastri (1962) reported that occasionally male trees after a number of years produce female flowers and may eventually become females. This may be because of a change in the metabolic pathway or variation in the metabolite concentration.

Table 10. Essential oil and total phenol content of leaves of mature plants

Sl. No.	Male		Female	
	Total phenol %	Oil content %	Oil content %	Total phenol %
1	0.81	0.66	1.0	0.898
2	1.98	0.55	0.83	1.88
3	1.39	0.33	1.16	1.806
4	1.85	0.72	0.83	1.786
5	1.77	1.00	0.66	0.47
6	0.60	0.83	0.77	1.20
7	1.49	0.66	0.50	0.968
8	1.06	0.66	0.66	1.064
9	2.31	0.50	0.60	1.35
10	1.65	0.72	0.50	0.54
11	1.87	0.66	0.66	2.38
12	0.69	0.50	0.60	0.868
13	1.36	0.83	0.83	0.736
14	0.96	0.93	0.66	1.20
15	1.52	0.66	0.66	1.26

4.2.1.4 Ammonium molybdate test

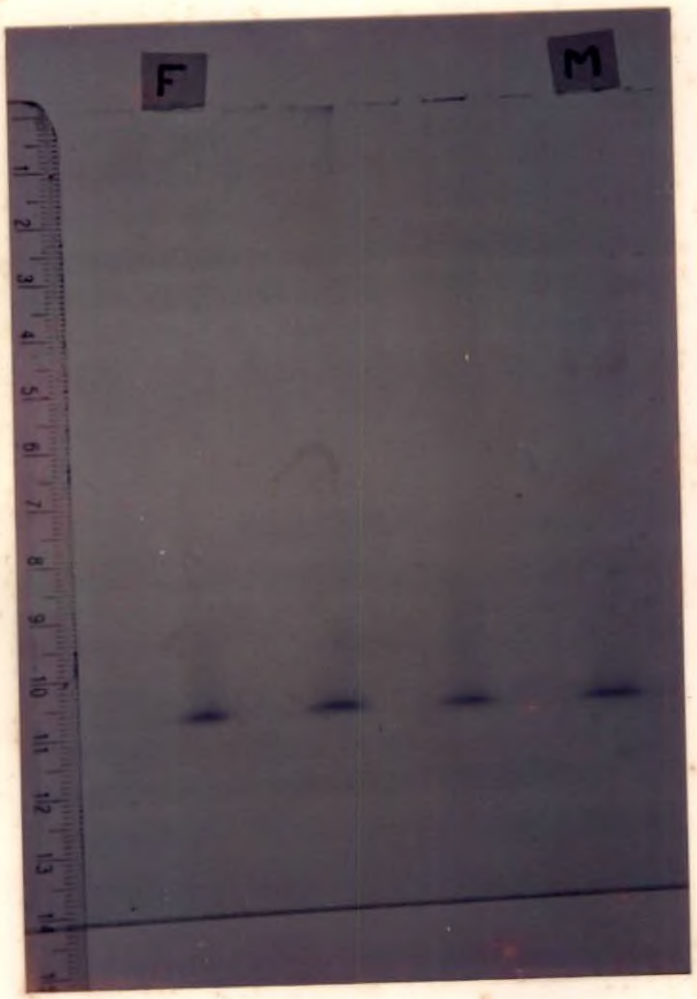
The colour reaction of ammonium molybdate reagent with leaf extract of male and female plants were observed (Table 11). Both male and female plants displayed dark green and light green colour on reaction with ammonium molybdate. So visual demarcation of male and female based on colour reaction method was in contrary to the report of Phadnis and Choudhari (1971) and Krishnamoorthy *et al.* (1991). The absorbance was read at 490 nm. The OD value of male plants ranged from 0.194 to 0.575 and that of female plants from 0.364 to 0.824. Though some male plants showed higher OD values, the general trend of OD value in female plants is higher than that of male plants. This result is in conformity with the observation of Phadnis and Choudhari (1971) who reported higher absorbance values for female plants than that of male plants.

The differential colour reaction for different sexes obtained with ammonium molybdate reagent indicate that different sexes have different components which appear to react actively with ammonium molybdate. Horovitz (1954) reported that genes controlling maleness and femaleness may be producing specific substances which react differently to different chemicals.

Table 11. Colour observed and absorbance reading of mature male and female plants

No.of plant	Male plant		Female plant	
	Colour developed	Absorbance at 490 nm	Colour developed	Absorbance at 490 nm
1	light green	0.194	dark green	0.693
2	light green	0.258	dark green	0.739
3	dark green	0.557	dark green	0.736
4	light green	0.294	dark green	0.824
5	dark green	0.472	light green	0.375
6	dark green	0.527	light green	0.364
7	light green	0.291	dark green	0.704
8	dark green	0.575	dark green	0.475
9	dark green	0.336	light green	0.384
10	dark green	0.320	dark green	0.526
11	dark green	0.460	dark green	0.626
12	dark green	0.340	dark green	0.685
13	dark green	0.354	dark green	0.495
14	dark green	0.465	dark green	0.783
15	dark green	0.385	dark green	0.498

Plate 6. **Protein pattern in male and female nutmeg plants**


F
M

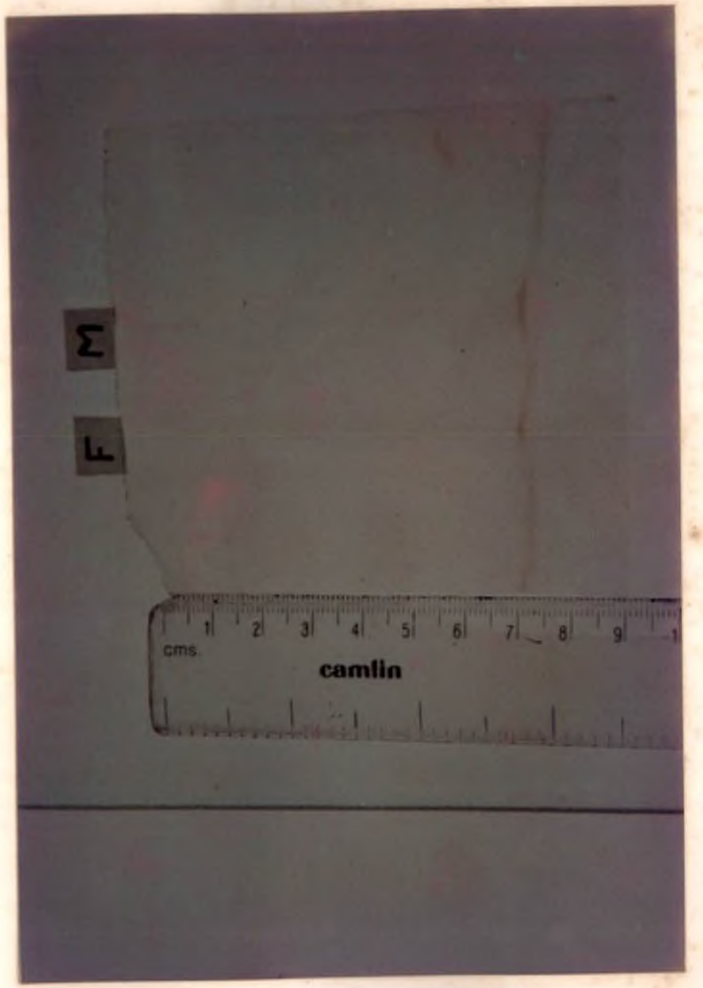
4.2.1.5 Protein pattern by electrophoresis

Different extraction buffer systems were tried for isolating active protein groups from leaf tissue. Tris buffer with different constitutents and citrate buffer were tried. Since active proteins could not be isolated from fresh tissue using the different buffer systems, acetone powdered samples were extracted with tris buffer. But this method also was found to be ineffective. Finally fresh leaf samples were collected in liquid nitrogen and extracted with tris buffer of lower ionic concentration which contained antioxidants ascorbic acid and L-cystein Hcl, insoluble polyvinyl pyrolidine (PVP) to remove phenolic interferences and ß-mercaptoethanol to break disulphide linkage. Even after such precautions, electrophoresis showed the presence of a single fast moving protein with Rm value 1.0 (Plate 6). This indicates that the leaves may have high content of protein degrading metabolites or protein inhibiting enzymes like protease. The fast moving band obtained was same for both male and female plants.

Peroxidase

A single band of protein showing peroxidase activity was obtained when stained with benzidine (Plate 7). The Rm value of peroxidase was same as that of protein band. This again is supporting the observation on electrophoresis of protein as

Plate 7. **Peroxidase enzyme pattern in male and female nutmeg plants.**


M
F
cms
camlin

given above. The isozymes may also be degraded due to the presence of protease enzyme or other inhibiting metabolites. Single isozyme banding was observed because of the same size and structure of peroxidase enzyme as that of the protein band obtained. Use of synthetic buffer or other extraction system after removing the inhibiting or degrading factors may give active protein groups which can be used for screening the plants successfully.

4.2.2 Seedlings

4.2.2.1 Total phenol content of leaf

Phenol content of one year old plants was found to be lower than that of two year old plants. The mean phenol content in one year old plants were found to be 0.64 per cent with a range of 0.39-1.57 per cent (Tabla 8a and 8b). In two year old plants, the phenol content varied between 0.20 and 1.88 per cent with a mean of 1.40 per cent which is comparable with that of mature plants.

4.2.2.2 Colour reaction with ammonium molybdate

The mean OD value in one year old plants were found to be 0.442 and in two year old plants it was 0.451 (Table 8a and 8b). In one year old plants the OD value ranged from 0.18-0.71 with a high degree of variation between individual plants. In two year old plants, the value varied between 0.16

and 0.72. Both in seedlings and mature male and female plants, OD value of ammonium molybdate showed high degree of variation between individual plants. So the method of differentiating sex of seedlings based on colour reaction with ammonium molybdate is not reliable.

4.3 Discriminant function analysis of the data

The data collected were subjected to discriminant function analysis after analysis of variance. Ten characters viz., spread of plant, maximum leaf width, L/W ratio of leaf, leaf length, leaf area, leaf oil content, phenol content, collar girth, stem diameter at branching and absorbance reading of ammonium molybdate were subjected to discriminant function analysis to find out the characters which show maximum variability between male and females.

Maximum leaf width (85.44 per cent), L/W ratio (32.10 per cent), absorbance of ammonium molybdate test (38.27 per cent) and stem diameter at branching (18.75 per cent) showed maximum per cent of variability which indicate that males and females differ maximum in these characters (Table 12). Of these four characters two combinations of three characters each were subjected to discrimination analysis to derive discriminant function coefficients. F-value and centroid discriminant score were found to be more for combination of L/W ratio, stem diameter at branching and absorbance reading of ammonium

Table 12. Discriminant function analysis to establish the difference between male and female plants

Sl. No.	Variables	Variable mean		t value	Discriminant function coefficient	% Variability
		M	F			
1.	Spread of plant	64.24	74.12	-1.23	-0.01	1.24
2.	Maximum width of leaf	3.74	4.62	-3.52	-7.75	85.44*
3.	L/W ratio of leaf	2.96	2.57	3.68	-6.48	-32.10*
4.	Maximum leaf length	11.07	11.66	-0.96	3.87	-29.02
5.	Leaf area	29.57	37.59	-2.22	0.10	11.05
6.	Leaf oil	0.68	0.74	-0.86	2.171	-1.49
7.	Phenol content	1.42	1.22	1.03	3.304	8.26
8.	Collar girth	0.98	1.06	-0.94	0.41	-0.44
9.	Absorbance of amm. molybdate test	0.39	0.59	-4.09	-14.79	38.27*
10.	Stem diameter	0.82	0.95	-1.74	10.81	18.36*
	Grand centroid	-17.22	-25.146			
	F value	4.031				

* High percentage variability

molybdate test (Table 13). The discriminant function developed is given below.

4.799 x L/W ratio of leaf - 5.53 x stem diameter at branching - 10.43 x absorbance reading of ammonium molybdate test.

The function was substituted by the corresponding observations of male and female plants to develop individual score for male and female plants (Table 14). The scores of male plants ranged from 2.27 to 10.12 and that of female plants -2.67 to 4.81. The standard deviation and standard error of the scores were worked out. The males are expected to be distributed between scores of 1.83 and 9.45 and females are expected to lie between 5.685 and -3.97. But overlapping of the scores of male and female plants were observed and so the discriminant function is found to have an accuracy of 60 per cent. The function is not applicable in seedlings because, the character of stem diameter at branching of mature plants used in the function is not comparable with that of seedlings. So the function requires further refinement.

From the above mentioned results it can be seen that the expression of characters varied considerably among the male and female plants of nutmeg. It was not possible to attribute the sex in nutmeg to a particular character or a group of characters. However morphological characters like stem diameter at branching, L/W ratio of leaf and leaf width were

Table 13. Discriminant function analysis of characters showing maximum variability

Sl. No.	Variables	Variable mean		t value	Discriminant function coefficient
		M	F		
1.	Maximum leaf width	3.74	4.62	-3.52	-0.995
2.	Stem diameter	0.82	0.95	-1.74	-3.244
3.	Absorbance reading	0.39	0.59	-4.09	-9.94

F value for testing T^2 = 7.793

Centroid Discriminant scores for male and female -10.239, -13.59

Sl. No.	Variables	Variable mean		t value	Discriminant function coefficient
		M	F		
1.	L/W ratio of leaf	2.96	2.56	3.68	4.799
2.	Stem diameter at branching	0.86	0.95	-1.74	-5.53
3.	Absorbance reading of amm molybdate:	0.39	0.59	-4.09	10.43

F value for testing T^2 = 11.105

Centroid discriminant scores for male and female 5.631, 0.847

Table 14. Discriminant scores of selected male and female plant

Sl. No.	Male	Female
1	10.12	-1.08
2	7.12	-2.67
3	5.19	-2.08
4	5.23	1.00
5	6.07	2.20
6	4.32	2.46
7	6.19	-1.70
8	3.13	4.81
9	4.92	4.76
10	5.33	3.69
11	2.27	-1.50
12	8.15	0.80
13	6.07	-0.04
14	5.74	0.61
15	4.58	1.59

observed to be contributing towards sex expression. The various biochemical tests carried out were not useful in discriminating the sex of nutmeg. Seedlings exhibited wide variation in the expression of characters and did not conform to a definite pattern.

The seedlings have to be established in the field and observed until flowering to verify any positive correlation of morphological characters with sex expression. Electrophoretic methods employed in the present study did not give any definite results due to the degradation of protein. So the technique has to be refined further to resolve the proteins without degradation. Molecular techniques like Restriction Fragment Length Polymorphisms (RFLPs) or Random Amplified Polymorphic DNAs (RAPDs) may be useful in resolving the sex problem in nutmeg.

Summary

5. SUMMARY

The present investigations on "Characterisation of dioecy in nutmeg (*Myristica fragrans* Houtt.)" was carried out during the period 1995 to 1997 in the Department of Plantation Crops and Spices, College of Horticulture,Vellanikkara. The salient results of the study are summerised hereunder.

The study consisted of the morphological observations and biochemical analysis of sexually well differentiated male and female plants and one year and two year old seedlings.

Among the different morphological characters studied such as plant height, spread of the plant, collar girth, height at branching and number of main branches in one metre height, no significant differences were noted between male and female plants.

In the case of leaf characters, elliptic and oblanceolate leaves were observed in both male and female plants. No significant difference was observed in the leaf lengths of male and female plants. In the selected fifteen male and female plants, average leaf width of male plants were found to be less than that of female plants. Female plants observed were found to have broader leaves.

The length by width ratio of leaves also found to be differing between males and females. The L/W ratio of males was observed to be larger than that of females. Though the mean leaf area of female plants was larger than that of male plants, the difference was not statistically significant. There was no difference in the number of leaves produced per flush and petiole length of male and female plants. The stomatalindicesof both male and female plants were worked out and no pronounced difference was noted between male and female plants.

In the study of calcium oxalate crystals in the lower leaf epidermis, 64.34 per cent cells of male plants showed rhomboidal crystals and 35.65 per cent cells showed clustered pattern. In the adult female plants, 65.90 per cent of total cells observed were found to have clustered and 34.01 per cent possessed rhomboidal crystals.. In one year and two year old seedlings both type of crystal pattern is observed along with crystals of no definite pattern. So no definite conclusion regarding sex could be derived based on crystal pattern in seedlings.

The number of flowers produced per unit area in males is found to be three times more than that of female plants. High variation was noted in the fruit characters of the female plants.

The essential oil content was found to be slightly more in the leaves of female plants than that of male plants. But in male plants, the phenol content was found to be slightly higher than that of females. The TLC profile of phenolic extract showed one phenolic spot with Rf value 0.32 and a flavonoid spot with Rf value 1.00 which were same for both male and female plants.

With respect to the colour reaction of ammonium molybdate with leaf extracts of male and female plants, both male and female plants showed dark green and light green colour. The OD values were found to be highly varying though a general trend of high OD value was observed for female plants.

A single fast moving protein band was obtained when leaf samples collected in liquid nitrogen were extracted with a buffer of lower ionic concentration containing insoluble PVP to remove phenol interference and ß-mercaptoethanol to break disulphide linkage. The single protein obtained showed peroxidase activity when stained with benzidine.

In seedlings, the morphological characters studied showed high variation between individual plants. The morphological characters of seedlings did not conform to a definite pattern and did not give any indication as far as sex is concerned.

Among the biochemical characters studied in seedlings, the phenol content was found to be lower in one year old plants than in two year old plants. In two year old plants, the phenol content was found to be comparable with that of mature plants. In seedlings, the OD values of colour test with ammonium molybdate showed high variation between individual plants.

A discriminant function was developed with L/W ratio of leaf, stem diameter at branching and OD value of ammonium molybdate test as variables which gave discriminant scores of 60 per cent accuracy to male and female plants. This function is not applicable in seedlings since the variable viz., stem diameter at branching of mature plants and seedlings are not comparable.

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* Originals not seen

Appendices

Appendix-I

Analysis of variance for plant characters of mature male and female plants

Source	d.f. .	Mean square	F value
Plant height	1	1.925	0.378
Spread of plant	1	737.45	1.527
Collar girth	1	0.055	0.884
Stem diameter at branching	1	0.141	3.026
Height at branching	1	0.056	0.260
Number of branches at meter height	1	10.80	0.842

Appendix-II

Analysis of variance for leaf characters of mature male and female plants

Source	d.f.	Mean square	F value
Leaf length	1	2.614	0.920
Leaf width	1	5.633	12.09**
Length/width ratio	1	1.152	13.55**
Leaf area	1	482.563	4.927
Petiole length	1	0.027	0.15
Number of leaves/ flush	1	0.000	0.000
Stomatal index		0.002	0.15

** Significant at 1% level

Appendix-III

Analysis of variance for flower characters

Source	d.f.	Mean square	F value
Number of flowers/ unit area	1	10741.3	4.858*
Flower length	1	0.007	0.7
Flower diameter	1	0.029	0.48

* Significant at 5% level

Appendix-IV

Analysis of variance for fruit characters

Source	d.f.	Mean square	F value
Fruit length	14	4.898	31.819 **
Fruit perimeter	14	8.682	25.868 **
Fruit weight	14	1138.702	27.927 **
Seed weight	14	22.021	13.567 **
Mace weight	14	4.213	26.357 **

** Significant at 1% level

CHARACTERISATION OF DIOECY IN NUTMEG (*Myristica fragrans* Houtt.)

By
PRIYAMOL THOMAS

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Science in Horticulture

Faculty of Agriculture
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1997

ABSTRACT

Investigation on "Characterisation of dioecy in nutmeg (*Myristica fragrans* Houtt.)" was carried out in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 1995-97 to study the dioecious nature in nutmeg plants.

Among the different morphological characters studied such as plant height, spread of the plant, collar girth, height at branching and number of main branches in one meter length no significant differences were noted between male and females.

Among the leaf characters, average leaf width was found to be larger in female plants compared to male plants. The length by width ratio of leaves was found to be larger in male plants than in female plants.

The calcium oxalate crystal pattern in the lower leaf epidermis was not found to be a reliable indicator of sex because both in male and female plants rhomboidal and clustered crystal pattern were observed.

Among the biochemical characters studied, the essential oil content was found to be slightly higher in the female plant leaves than in those of male plants. But in the male leaves the phenol content was found to be slightly higher.

The TLC profile of phenolic extract showed one phenolic spot with Rf value of 0.32 and a flavanoid spot with Rf value 1.0 which were same for both male and female plants.

With regard to the colour reaction of ammonium molybdate with leaf extracts of male and female plants, both male and female plants showed dark green and light green colour. The OD values were found to be highly varying though a general trend of high OD values was observed for female plants.

On electrophoresis a single fast moving protein band showing peroxidase activity was obtained which was found to be same for both male and female plants.

In seedlings the morphological characters studied showed high variation between individual plants. The observations did not confirm to a definite pattern and did not give any indication as far as sex is concerned.

Among the biochemical characters studied in seedlings, the phenol content was found to be lower in one year old plants than in two year old plants. The OD values of colour test with ammonium molybdate showed high variation between individual seedlings.

A discriminant function was developed with L/W ratio of leaf, stem diameter at branching and OD value of ammonium molybdate best as variables which gave discriminant scores of sixty per cent accuracy to male and female plants.