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# IDENTIFICATION OF SEX OF NUTMEG SEEDLINGS BASED ON MORPHOLOGICAL AND CHEMICAL CHARACTERS

B. KRISHNAMOORTHY, T. JOHN ZACHARIAH, P.N. RAVINDRAN, and A. GOPALAM[1]

*National Research Centre for Spices ,Calicut 673 012, Kerala*

## ABSTRACT

**The main problem facing nutmeg cultivation is the segregation of the seedling progenies into males and females in the ratio of 1:1. Several methods have earlier been reported for the possible identification of the sex at the seedling stage. The present paper deals with the suitability of some of these methods at the nursery stage. The study of crystal patterns in the epidermal cells of the leaves revealed that the crystal differentiation was very clear in the mature leaves in almost 70 per cent of male and 78 per cent of female plants, whereas inthe case of seedlings, the crystal pattern was not definite and so the sex could not be differentiated in seedlings based on crystal study. The study of the seedling morphological traits like sprout colour, days for germination, leaf shape, size, venation, etc. led to the conclusion that none of these characters can be taken as a guide to sex the nutmeg plant. Out of a number of colour tests carried out on the leaf exudates or alcoholic leaf extracts of male and female trees, the ammonium molybdate alone gave satisfactory differentiation between male and female nutmeg trees. This test gave a 'faint green' colour with the male and a 'sea green' colour with the female plant. This is reproducible in the case of all the trees tested, but not in the seedlings.**

## INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is an important tree spice, yielding two products of commercial value, nutmeg and mace. The main problem facing nutmeg cultivation is the segregation of seedling progenies into females and unproductive males in the ratio of 1:1. A definite methodology to identify the sex at seedling stage itself is lacking. Determination of sex of the seedlings based on characters such as leaf form and venation (Prestoe,1984), colour of sprouts (Purple female; Green-male), seedling vigour (more vigorous seedlings-male) and chromosome morphology (Flach,1966) had been reported. Phadnis and Choudhary (1971), reported a colourimetric test, using a reaction with ammonium molybdate that can help to identify the sex of the plant at the seedling stage. Nayar *et al.* (1976) reported that the shape and size of the calcium oxalate crystals in the epidermis of the mature leaves vary between male and female plants. But none of these characters had been tested so far on a field scale. The present paper examines the suitability of some of the reported methods in the identification of male and female seedlings at the nursery stage. Results of a study conducted to evolve an easy test to differentiate female and male seedlings is also presented.

## MATERIALS AND METHODS

Calcium oxalate crystal pattern was studied in 1st to 10th leaf of ten each of one and two year old seedlings. Besides, the crystal patterns in 10 male and 10 female plants of about eight years of age were also studied, where 10 leaves starting from the apex of a shoot were analysed in each tree. The lower epidermis was peeled off and observed directly under the microscope.

For studying the morphological characters, seedlings of 16 selected trees planted at the National Research Centre for Spices Farm at Peruvannamuzhi during 1980 were utilized. Time required for germination, colour of the sprout (green or purple), shape of the leaf (obovate, spindle or elongate), leaf size (medium, broad or narrow) and venation of leaf were recorded for all the seedlings. The seedling characters recorded were correlated with the sex of the plants.

A number of colour tests were carried out. The leaf exudates of 20 each of male and female trees were collected in 15 ml ethanol. Leaf extracts were also used, for which 10 leaves from different positions of 10 each of male and female trees were collected and dried at 50°C, powdered and 5g of the powder was extracted with chloroform or methanol and the tests were carried out using the extract.

[1] Present address : National Academy of Agricultural Research and Management, Hyderabad



## RESULTS AND DISCUSSION

### (a) Crystal study

The epidermal cells are polygonal with straight lateral walls. The 9th and the 10th leaves bear uniseriate deciduous hairs, scattered along the lower epidermis of the leaves of the seedlings. The cells contain pale oil globules. In the epidermal cells of mature leaves of male plants a single large rhmoboidal or prismatic crystal with rectangular or squarish flat faces occurs. In the epidermal cells of the female plants, the crystals are clustered to form large compound spherical massess (crystoliths). Some epidermal cells of the male plants also have compound crystals of the female type and simple prismatic crystals of the male types are found to be very clear in mature leaves in almost 70 per cent of male and 78 per cent of female plants.

But in the case of one year old and two year old seedlings, the crystal pattern is not definite and variations were noted among the various leaves in the same seedling(Table I). In one year old seedlings, clustered crystals occurred in 55 per cent seedlings, while in 30 per cent, rhomboidal crystals were noticed; and in 15 per cent of the plants no such definite pattern existed. In the case of two year old seedlings the corresponding figures were 49, 36 and 15 per cent. Thus, the sex could not be differentiated in one or two year old seedlings, based on crystal study.

The percentage of cells with clustered crystals and that with prism-like crystals, vary in the different leaves of the same seedling of both one and two year old (Table II).

In the case of adult plants, upto the 4th leaf from the tip, there is no definite trend in crystal differntiation. From the 5th leaf onwards, upto the 10th leaf, the crystal pattern became quite definite as seen from Table III. Thus, in mature seedlings (4-5 years old) the differentiation of sex could be made out by an analysis of the crystal pattern in the leaf epidermal cells. How this crystal pattern difference is brought about in male and female trees is not known. Because of the specificity of the crystal pattern, the calcium oxalate crystals are shown to have taxonomic value(Kuestor, 1956).

### (b) Morphological study

Out of the seedlings planted, 18 numbers turned out to be males and 19 females. Out of the 18 males, 10 had purple sprouts and 8 green sprouts. Out of the 19 females, 10 had purple sprouts and 9 green sprouts. Seeds resulting in male plants took on an average of 48.93 days for germination (range: 24 to 69 days) and those seeds from which female plants were obtained took an average of 47.8 days for germination (range: 37 to 79 days). Foliar characters like size, shape and venation also did not give any indication as far as the sex is concerned. All seedlings showed curved venation. On an average, the male plants have flowered earlier(about 72.2 per cent flowered in 5 years time), whereas 70 per cent females flowered

**Table I. Crystal pattern in nutmeg (leaves, lower epidermis)**

| Age of the plant | Total No. of cells observed (mean of 10 plants) | Cells with clustered crystals | | Cells with prism-like crystals | | Cells with crystals having no definite pattern | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| 1 year old seedling | 1149 | 633 | 55 | 347 | 30 | 169 | 15 |
| 2 year old seedling | 1197 | 583 | 49 | 429 | 36 | 185 | 15 |
| Adult plant (male) | 1063 | 321 | 30 | 742 | 70 | — | — |
| Adult plant (female) | 1287 | 1007 | 78 | 280 | 22 | — | — |

**Table II. Variations of crystal patterns in the leaves of the same seedling**

| Leaf No. | 1- Year Old seedling (Mean of 10 plants) | | | | | | | 2- Year old seedling (Mean of 10 plants) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of cells observed | Cells with clustered crystals | | Cells with prism-like crystals | | Cells having no definite pattern | | No. of cells observed | Cells with clustered crystals | | Cells with prism-like crystals | | Cells having no definite pattern | |
| | | No. | % | No. | % | No. | % | | No. | % | No. | % | No. | % |
| 1st leaf (from below) | 102 | 48 | 47 | 25 | 25 | 29 | 28 | 115 | 54 | 47 | 40 | 35 | 21 | 18 |
| 2nd leaf | 124 | 47 | 38 | 39 | 31 | 38 | 31 | 109 | 57 | 52 | 38 | 35 | 14 | 13 |
| 3rd leaf | 101 | 42 | 41 | 31 | 31 | 28 | 28 | 102 | 60 | 59 | 35 | 34 | 7 | 7 |
| 4th leaf | 110 | 75 | 63 | 83 | 28 | 11 | 9 | 138 | 63 | 46 | 51 | 37 | 24 | 17 |
| 5th leaf | 113 | 73 | 65 | 26 | 23 | 14 | 12 | 108 | 50 | 46 | 42 | 39 | 16 | 15 |
| 6th leaf | 115 | 72 | 63 | 37 | 32 | 6 | 5 | 100 | 49 | 49 | 40 | 40 | 11 | 11 |
| 7th leaf | 137 | 73 | 53 | 45 | 33 | 19 | 14 | 119 | 51 | 43 | 46 | 39 | 22 | 18 |
| 8th leaf | 128 | 85 | 66 | 37 | 29 | 6 | 5 | 148 | 83 | 59 | 41 | 28 | 19 | 13 |
| 9th leaf | 97 | 59 | 61 | 39 | 36 | 3 | 3 | 146 | 63 | 43 | 57 | 39 | 26 | 18 |
| 10th leaf | 113 | 59 | 53 | 39 | 34 | 15 | 13 | 112 | 48 | 43 | 39 | 35 | 25 | 22 |

**Table III. Variations in the different leaves of the mature plant**

| No. of leaf from the apex of the branch | Female Plant | | | | | Male Plant | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of cells observed | Cells with clustered crystals | | Cells with prism like crystals | | No. of cells observed | Cells with clustered crystals | | Cells with prism-like crystals | |
| | | No. | % | No. | % | | No. | % | No. | % |
| 1st leaf | 125 | 74 | 59 | 51 | 41 | 117 | 70 | 60 | 47 | 40 |
| 2nd leaf | 116 | 67 | 58 | 49 | 42 | 125 | 79 | 63 | 46 | 37 |
| 3rd leaf | 121 | 76 | 63 | 45 | 37 | 142 | 83 | 58 | 59 | 42 |
| 4th leaf | 132 | 84 | 64 | 48 | 36 | 112 | 40 | 36 | 72 | 64 |
| 5th leaf | 140 | 121 | 86 | 19 | 14 | 109 | 11 | 10 | 98 | 90 |
| 6th leaf | 135 | 120 | 89 | 15 | 11 | 87 | 11 | 13 | 76 | 87 |
| 7th leaf | 137 | 122 | 89 | 15 | 11 | 86 | 6 | 7 | 80 | 93 |
| 8th leaf | 142 | 128 | 90 | 14 | 10 | 94 | 7 | 8 | 87 | 92 |
| 9th leaf | 139 | 118 | 85 | 21 | 15 | 95 | 5 | 5 | 90 | 95 |
| 10th leaf | 100 | 97 | 97 | 3 | 3 | 96 | 9 | 9 | 87 | 91 |

only after 6 years. The data presented in Table IV indicate clearly that none of the morphological characters can be used for identification of sex of the nutmeg plant.

(c) **Colour tests**

Tables V and VI give the result of the chemical tests carried out. Only in the case of the Laba test and Ammonium molybdate test, differences between male and female plants were observed. With the leaf exudate, Laba test gave light violet colour in the case of male plants, while in the case of female plants, 80 per cent of the cases gave a light violet colour, while in 20 per cent of the cases, the colour was deeper. The colour indicates the presence of methylene



**Table IV. Seedling characters of adult nutmeg trees**

| Sl. No. | Seedling characters | | Male | Female |
|---|---|---|---|---|
| 1. | Sprout-colour-Green | | 44.4% | 47.3% |
| 2. | Sprout colour-Purple | | 55.6% | 52.7% |
| 3. | Days for germination - | Range | 24 to 69 | 37 to 79 |
| | | Mean | 48.93 | 47.8 |
| 4. | Time required for flowering | Range | 3-6 years | 3-6 years |
| | (after planting) | Mean | 4.3 years | 5.5 years |
| 5. | Leaf shape | Obovate | 41.2% | 60.0% |
| | | Spindle | 52.9% | 33.3% |
| | | Elongate | 5.38% | 6.7% |
| 6. | Leaf size | Medium | 80.0% | 64.3% |
| | | Broad | 6.7% | 35.7% |
| | | Narrow | 13.3% | Nil |
| 7. | Venation | Curved | All | All |

**Table V. Colour tests in nutmeg using leaf extracts**

| Sl.No. of leaf | Salkowski test | | Laba test | | Ellagic acid test | | Lieber-mann Burchard test | | Al.-chloride test | | Iodine & Al.Chl. test | | Ammonium molybdate test | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | F | M | F | M | F | M | F |
| 1. | Dark purple | Purple | Light green | Green | Dirty green residue | Light green residue | Green | | Light yellow | | Pink colour | | Faint green Col. | Sea green Col. |
| 2. | " | Dark purple | Light green | Purple | Light Green | Light greedn | " | | " | | " | | " | |
| 3. | " | " | " | Light green | " | Dirty Green | " | | " | | " | | " | |
| 4. | " | " | " | Dark green | " | " | " | | " | | " | | " | |
| 5. | " | Light purple | Dark Green | Light Green | " | " | " | | " | | " | | " | |
| 6. | " | Dark purple | Green | Green | Dirty Green | " | " | | " | | " | | " | |
| 7. | Light purple | " | Purple | " | " | " | " | | " | | " | | " | |
| 8. | Dark Purple | Dark purple | Green | Light Greem | " | " | " | | " | | " | | " | |
| 9. | " | " | " | " | " | " | " | | " | | " | | " | |
| 10. | " | " | " | " | " | " | " | | " | | " | | " | |

dioxy compounds. The ammonium molybdate test gave a faint green colour with the male plant and a sea green colour with the female plant. This colour difference is reproducible in the case of all the plants tested. The ellagic acid test gave a fairly positive indication of the presence of the compound in both

**Table VI. Chemical tests using leaf exudate**

| Sl. No. | Colour test | Details of the test | Reaction (male leaves) | Reaction (female leaves) | Inference |
|---|---|---|---|---|---|
| 1. | Salkowski test | To ethanolic plant extract, chloroform and sulphuric acid added | Development of wine red colour | | Presence of steroid compounds |
| 2. | Laba test | Gallic acid was added to ethanolic plant extract acidified with sulphuric acid | Light violet colour develops in all | Light violet colour in 80% samples; Very thick violet colour in 20% cases. | Presence of methylene dioxy compound |
| 3. | Aurone test | A few ml of dilute aqueous ammonia was placed in a glass stoppered test tube; a loose plug of cotton wool was wedged just above the liquid, but not touching it. A flower was dropped on the plug and the tube was stoppered. | No colour developed | | Absence of aurones |
| 4. | Ellagic test | A small quantity of leaf tissue was macerated with methanol. The alcoholic extract was separated and treated with a few drops of 5% acetic acid and a few crops of 5% sodium nitrite solution. | The solution turned mild yellow. | | Low amount of ellagic acid present. |
| 5. | Syringin test | Freshly hand cut sections of young stem were mounted in a drop of acsueous sulphuric acid (1:1 $H_2S_4$ : $H_2O$) and examined under the microscope. | Development of green colour was observd in the tissues. | | Positive reaction for the phenolics. |
| 6. | Molybdate test | 10 g molybdic acid dissolved in a mixture of 14 cc of ammonium hybroxide and 27 cc of distilled water. This solution was then slowly poured into a cool mixture of 50 cc of nitric acid and 114 cc distilled water. 10 drops of this reagent were added to 5 cc of leaf extract; | Faint green | Sea green | Presence of phenolic glucosides |



| Sl. No. | Colour test | Details of the test | Reaction (male leaves) | Reaction (female leaves) | Inference |
|---|---|---|---|---|---|
| | | mixture heated to boiling on a hol plate, cooled and the reading recorded after 30 mts. | | | |
| 7. | Aluminimum chloride test | A few drops of aluminium chloride were added to the methonolic extract. | No development colour | | Absence of 5' hydroxy flavanoids. |
| 8. | 2, 4-D N pH test | A few drops of 2, 4-D N pH were added to the methonolic extract. | -do- | | Absence of CHO group in the extract. |

the sexes. Both Salkowski and Liebermann-Burchard tests gave positive indication of the presence of steroids, but again there was no difference between the two sexes. These tests were repeated with methonolic extract of the dried leaves. Molybdate test showed a consistent difference between the two sexes. These tests were repeated with methonolic extract of the dried leaves. Molybdate test showed a consistent difference between the two sexes. Laba test gave green colour instead of violet and no difference could be seen between male and female plants, except for the difference between them in the rate of synthesis and accumulation of the methylene dioxy compounds. This difference is clearly seen in the older leaves. Such differences in the intensity were also noted in the case of Salkowski test, but was not consistent. Thus, ammonium molybdate test alone is found to give satisfactory differences between male and female nutmeg plants. Here the colour difference (faint green in males vs. sea green in females) could be attributed to the relative content of certain phenolic glucosides.

The authors have carried out a detailed chromatographic investigation of the leaf extracts, of male and female nutmeg plants, and found that in the male, a spot at rf0.8 consistently appears in TLC plates. Further investigation of IR and MS have shown the presence of 1-dimethyl 2-ethyl butyl methyl ketone degradation product of B-sitosterol. The details of this study are reported elsewhere (Zachariah *et al.* 1986).

In summary, it may be stated that among the methods available at present, the ammonium molybdate test is the most reliable one in the identification of the sex in the nutmeg.

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