Chapter – II

MATERIALS AND METHODS
2.1 Materials

Azadirachta indica A. Juss (Synonyms, Melia indica (A. Juss.) Brandis, Melia azadirachta Linn. and Melia parviflora Moon) belongs to the family Meliaceae, commonly known as Neem, is the plant for the present study. Its phenology, flower, fruit, seed and seedling characters were studied using samples collected from eleven locations in Kerala and Tamil Nadu.

2.2 Methods

2.2.1 Bibliographic investigations

Literature pertaining to the taxonomy, distribution, phenology, flower, seed and seedlings were collected from various florae (Hooker 1875 - 1897; Gamble 1915-1936; Mathew, 1983). The literature available in the libraries of Mahatma Gandhi University, Kottayam; Botanical Survey of India, Southern Circle, Coimbatore; Tropical Botanical Garden and Research Institute and University of Kerala, Thiruvananthapuram were also collected periodically and updated.

2.2.2 Locations

India lying entirely in the northern hemisphere, and extends between 8° 4' to 37° 6' North latitude and 68° 7' to 97° 25' East longitude is measuring about 3214 km from north to south between extreme latitudes and about 2933 km from east to west between the extreme longitudes. Geographically, India is a Peninsular elevation of the great Eurasian land mass and the major crust movement during early periods made a sharp demarcation of major structural and physiographic divisions with its own characteristic features, namely the Peninsula, Extra-peninsula and Indo-Gangetic
Alluvial plain. The Peninsular region is structurally, stratigraphically and physiographically quiet in contrast to the other divisions. It is flattened on one side by the Eastern Ghats and on the other by the Western Ghats. Between the Western Ghats and Arabian Sea, lies the narrow coastal strip, while between Eastern Ghats and Bay of Bengal have broader coastal area. The Peninsular plateau with hill ranges in the middle and coastal plains in the west and east have profound effect on the climatic and edaphic condition of this region. The geological regions, climate, physiography and other complex environmental features of the Peninsular regions determine the wet tropical and semi arid conditions in the region as evidenced in Kerala and Tamil Nadu, the two administrative divisions in the Peninsular plateau. These natural or edaphic factors have contributed a great deal in determining the vegetation and biodiversity of these states (Fig. 2.1).

2.2.2.1 Kerala

Kerala occupies the southwest portion of the Peninsula, and is situated between $8^018'$ to $12^048'$ North latitude and $74^052'$ to $77^022'$ East longitude. It extends over a distance of about 560km along the west coast with width varying from 11 km to 124 km within a limited area of 38863 Sq.km. Kerala represents a wide variation in physical features including mountain plains and coastal areas. Geologically, Kerala consists of crystalline rocks of Archaean group, residual laterite, Warkalli formation and recent formations in a more or less north-south alignment. The soil is mostly dominated by lateritic and forest loam along with other minor groups. The soils like coastal alluvium, riverine alluvium, red loam, lateritic, greyish onattukara, acid saline, brown hydromorphic, hydromorphic saline, black soil and forest loam are available. In general, the climate is tropical and the coastal
location of the state and high variations of relief from the coast to Western Ghat areas influences the weather pattern in the state. While most of the areas are under tropical dry and wet climate, the highland region experiences cold temperate climate. Along the coast the temperature is moderate, whereas in the east it is very low. This type of temperature gradient has endowed the state with a unique agroclimatic condition, favourable for rich diversity in life forms. The rainfall throughout the state is quite high and mostly received from two monsoons - the southwest and the northeast. In general, the rainfall increases from the coast to the foothills and decreases on the hilltops. This general trend is particularly disrupted in the Palghat gap region.

The climate and the soil conditions are so conducive for the growth of luxuriant vegetation with all classical types. Generally the vegetation is tropical however, five major types wet-evergreen, semi-evergreen, moist-deciduous, dry-deciduous and montane with temperate plantations were noted. Grasslands and mangrove forests are also available in few isolated patches in the highlands and coastal areas, respectively. Apart from the multiple cropping and home gardens, large monoculture plantations of teak, eucalyptus, rubber, coconut, tea, coffee and spice crops are very common.

Physiographically, the state is broadly divided into three parallel topographical divisions, running south to north, the highlands (above 75m); midlands (10-75m); and lowlands (below 10m). The ICAR under NARP Programme divided the state into six agroclimatic zones, namely northern zone, southern zone, coastal zone, problem area zone (Onattukara, Kuttanadu, Pokkali and Kole), central zone and high altitude zone.
2.2.2.2 Tamil Nadu

It is situated in the southeastern extremity of the Peninsula (8° 05' to 13° 34' North latitude and 76° 14' to 80° 21' East longitude) and is the eleventh largest state in land area. It forms part of the Peninsular shield and about 3/4th of the total area is underlined by unclassified crystalline rocks of Archaean age. The sedimentary belt forms the major area all along the coast and it mainly consists of upper Gondwana, Cretaceous, Tertiary and Quaternary deposits. A major portion of the plain belongs to the Peninsular genesis and in the hills, the formation consists of charnockite genesis. The reddish brown and sandy soil occupies a large part of the area particularly in the interior districts. However, laterite soil, coastal alluvial and black cotton soil are also occur in some districts. The climate is basically tropical and is very warm and dry except in hilltops. Tamil Nadu gets less rain from both monsoons, but northeast monsoon is more active and most of the rainfall is occurring during the period. The vegetation of Tamil Nadu is varied depending upon climate, altitude, relief and slope of land and is broadly divided into coastal vegetation, island vegetation, vegetation of the interior plains and vegetation of the hills and mountains. The wide range of climatic conditions ranging from tropical to temperate and from semi-arid to humid contribute to the diversity in natural vegetation. Physiographically the state is broadly divided into two natural regions, the eastern coastal plain and the western hill region. The eastern coastal plain comprises the Coromondal plain, inner plain, dry southern plains, cauvery delta and a narrow stretch of coastal plain. The hilly western part covers the hill regions of the Nilgiris, Sheoroy, Kollimalies, Pachamali, Anamalai, Pulneys, Kalakkadu and Ashambu hills. The ICAR under NARP programme classified the state into seven agro-
Figure – 2.1: Map showing India with major physiographic divisions and study area
Figure - 2.1
Figure 2.2: Map showing the eleven study locations and the respective agroecosystems in the states of Kerala and Tamil Nadu.
LEGEND

- Study / Sampling Locations
- District Boundary
- State Boundary
- Waterbody
- Hilly upland
- Southern midland
- Northern midland
- Palakkad
- Coastal lowland
- Onattukara, Kuttanad and Kole
- High rainfall
- Thirunelveli plain
- Dry southern
- Cauvery delta
- Western plain
- North-Western plain
- Coromondal plain

Sampling Locations

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thrithelloor</td>
</tr>
<tr>
<td>2</td>
<td>Chittoor</td>
</tr>
<tr>
<td>3</td>
<td>Trivandrum</td>
</tr>
<tr>
<td>4</td>
<td>Chennai</td>
</tr>
<tr>
<td>5</td>
<td>Namakkal</td>
</tr>
<tr>
<td>6</td>
<td>Bannari</td>
</tr>
<tr>
<td>7</td>
<td>Thiruvaroor</td>
</tr>
<tr>
<td>8</td>
<td>Periyakulam</td>
</tr>
<tr>
<td>9</td>
<td>Tirunelveli</td>
</tr>
<tr>
<td>10</td>
<td>Nagercoil</td>
</tr>
<tr>
<td>11</td>
<td>Sengottai</td>
</tr>
</tbody>
</table>

Figure - 2.2
climatic zones, whereas NBSS & LUP (1990) brought the state into 3 regions (Eastern coastal plains, Eastern and Western Ghats regions).

2.2.2.3 Study locations

Kerala and Tamil Nadu, the two states in India representing the two major biogeographic zones have been taken for the study. After a general field survey, eleven study locations were identified covering both the states, three in Kerala and eight in Tamil Nadu (Fig. 2.2). The criteria used for the selection of study locations are; population of neem trees, physiography, topography, geology, climate, edaphic conditions, ecology and general vegetation. The study locations are representing different ecosystem types having distinct variations in climate, topography, soil and vegetation (Table - 2.1). The basic details about study locations were collected from Soil toposheets, various maps and literature.

2.2.3 Field study

The present study was concentrated on the understanding of the variability in the different characters of neem trees present in various ecosystems of Kerala and Tamil Nadu. Thus the data collection involved exploration, field observations, collection of samples and its laboratory analysis. The search of literature showed that the flowering and fruiting of neem trees will spread only three to six months and hence the field works and laboratory studies were planned accordingly. Field study consisted of the exploration and selection of sampling trees, phenological studies, flower and fruit bearing capacity studies. The methods adopted for the field study is given below.
Table - 2.1: Details of the study locations in Kerala and Tamil Nadu.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Sampling location</th>
<th>Ecosystem type</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Soil Type</th>
<th>Topography</th>
<th>Average annual Rainfall (mm)</th>
<th>Average annual Temp (°C)</th>
<th>District/State</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thrithelloor</td>
<td>Northern Midland</td>
<td>8°21'</td>
<td>74°09'</td>
<td>Laterite</td>
<td>Plain</td>
<td>3500</td>
<td>28±2°C</td>
<td>Trichur, Kerala</td>
</tr>
<tr>
<td>2</td>
<td>Chittoor</td>
<td>Palakkadu plain</td>
<td>10°25'</td>
<td>76°45'</td>
<td>Laterite, black</td>
<td>Plain</td>
<td>2500</td>
<td>32±2°C</td>
<td>Palakkad, Kerala</td>
</tr>
<tr>
<td>3</td>
<td>Trivandrum</td>
<td>Coastal plain</td>
<td>8°12'</td>
<td>76°21'</td>
<td>Coastal Alluvial</td>
<td>Plain</td>
<td>3000</td>
<td>28±2°C</td>
<td>Trivandrum, Kerala</td>
</tr>
<tr>
<td>4</td>
<td>Chennai</td>
<td>Coromandalal Plain / North Eastern Zone</td>
<td>13°09'</td>
<td>80°12'</td>
<td>Crystalline Rock</td>
<td>Coastal plain</td>
<td>640</td>
<td>38±2°C</td>
<td>Chennai, Tamil Nadu</td>
</tr>
<tr>
<td>5</td>
<td>Namakkal</td>
<td>Northern Western Zone</td>
<td>11°15'</td>
<td>78°15'</td>
<td>Crystalline Rock</td>
<td>Slopping</td>
<td>700</td>
<td>36±2°C</td>
<td>Namakkal, Tamil Nadu</td>
</tr>
<tr>
<td>6</td>
<td>Bannari</td>
<td>Western Zone</td>
<td>10°30'</td>
<td>77°05'</td>
<td>Latterite and Forest loam</td>
<td>Slopping</td>
<td>1000</td>
<td>35±2°C</td>
<td>Erode, Tamil Nadu</td>
</tr>
<tr>
<td>7</td>
<td>Thiruvaroor</td>
<td>Cauvery Delta Zone</td>
<td>10°45'</td>
<td>79°18'</td>
<td>Coastal Alluvial</td>
<td>Plain</td>
<td>1000</td>
<td>39±2°C</td>
<td>Thiruvaroor, Tamil Nadu</td>
</tr>
<tr>
<td>8</td>
<td>Periyakulam</td>
<td>Dry Southern Zone</td>
<td>8°52'</td>
<td>77°28'</td>
<td>Red/coastal sandy Rock</td>
<td>Slopping</td>
<td>1000</td>
<td>35±2°C</td>
<td>Teni, Tamil Nadu</td>
</tr>
<tr>
<td>9</td>
<td>Tirunelveli</td>
<td>Tirunelveli Plain/ Southern Dryland Zone</td>
<td>8°42'</td>
<td>77°45'</td>
<td>Sandy Black/ Red soil</td>
<td>Plain</td>
<td>660</td>
<td>38±2°C</td>
<td>Tirunelveli, Tamil Nadu</td>
</tr>
<tr>
<td>10</td>
<td>Nagereoil</td>
<td>High Rainfall Zone</td>
<td>8°12'</td>
<td>77°18'</td>
<td>Sandy loam</td>
<td>Plain</td>
<td>1500</td>
<td>38±2°C</td>
<td>Kanyakumari, Tamil Nadu</td>
</tr>
<tr>
<td>11</td>
<td>Sengottai</td>
<td>Hilly Upland</td>
<td>8°52'</td>
<td>77°28'</td>
<td>Chamockite</td>
<td>Hilly</td>
<td>1000</td>
<td>28±2°C</td>
<td>Tirunelveli, Tamil Nadu</td>
</tr>
</tbody>
</table>
### Studies on the Genetic Variability of Neem

**Candidate Tree Selection Format**

*(Use ✓ mark in the appropriate boxes)*

<table>
<thead>
<tr>
<th>No: ..............</th>
<th>Date: ..............</th>
</tr>
</thead>
</table>

1. Approximate age of the tree .......... DBH .......... Height: ..............

2. The Candidate tree meet the following minimum requirements.
   
   a. Possess a greater DBH than 30 cms
      
   Yes ❑ No ❑
   
   b. Possess a round or oval crown
      
   Yes ❑ No ❑
   
   c. Bole posses less persistent branch stubs, burrs bumping or knots
      
   Yes ❑ No ❑
   
   d. The trees are healthy and not much affected by disease or insect pests
      
   Yes ❑ No ❑
   
   e. The tree possess an height of more than 8 meters
      
   Yes ❑ No ❑

3. The most outstanding features of the tree

   .................................................................

4. Details of the locations (Town, roadside, field, households, protected compounds or near water bodies)

   .................................................................

5. Owner's Name and Address: .................................................................

   .................................................................

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**Studies on the Genetic Variability of Neem**  
Candidate Tree Assessment Data Sheet

1. No: ......................  
2. Location: ......................  
3. Site Quality: ...........................................  
4. Characters of Importance:

<table>
<thead>
<tr>
<th>Characters</th>
<th>Comparison Trees</th>
<th>Average</th>
<th>Superiority (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1    2    3    4    5    6    7    8    9    10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crown</td>
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<td>Height (Metres)</td>
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<td>Length (Metres)</td>
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<tr>
<td>Width (Metres)</td>
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<tr>
<td>Bole</td>
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<tr>
<td>DBH (cms)</td>
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<tr>
<td>Height (Metres)</td>
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<tr>
<td>Straightness</td>
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<tr>
<td>Bark thickness at DBH level (mm)</td>
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<tr>
<td>Disease / Pest incidence</td>
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</tbody>
</table>

Remarks if any: ..............................................................................................................

Collected by:                                                                                   

Box - 2.2
Figure - 2.3 : (a - d) : Diagrammatic representation of the characteristics and the quantitative measurements taken for crown and bole of neem trees

a) Illustration of the crown
b - d) Illustration of bole
b1 & b2) Straightness
c) Bole with bumping nodes / branch stubs
d) Waving boles
Figure 2.3

(a) Tree with labels:
- Crown Length
- Crown Height
- Crown Radius (mts)

(b 1) Tree with DBH (cm) label
(b 2) Tree showing different trunk shape
(c) Tree with Boles Height (mts) label
(d) Tree showing different trunk shape
2.2.3.1 Population and sampling tree selection

The selection of the sampling trees from the population of neem trees present in the study locations were done following the total score method described by Sidhu (1996). The population of neem trees within an area of 10 sq.km of the sampling location was thoroughly screened by conducting field survey and ten superior trees (candidate trees) with respect to growth, crown, bole (stem) and the resistance to diseases and pests, were selected and their details were entered in a Candidate Tree Selection format (Box – 2.1). The quantitative measurements of crown size, shape and size of bole, DBH and incidence of disease and pests were noted as given in Fig. 2.3 (a-d) in a Candidate Tree Assessment Data Sheet (Box – 2.2). Each trait of the candidate tree was analysed by giving a score ranging from 0-10 with 10 as maximum. The total scores of all the traits were computed together and those trees having a total score above the average in the population with a superiority percentage of 20 and above were taken as the superior most tree in the population of the respective sampling location.

2.2.3.2 Field observation

Systematic field visits were undertaken during different seasons of the year 1998-99 and 1999-2000 in all the study locations. Phenological data pertaining to the identified superior most tree (sampling tree) in the location was collected along with flower and fruit samples for laboratory studies. During the field visits, flower and fruit bearing capacity of the selected trees were also recorded.
2.2.4 Phenological studies

2.2.4.1 Phenology

The phenological behaviour of selected trees in each location was studied. The data pertaining to the periodic behaviour of the trees like leafing, flowering and fruiting at three stages of development were recorded as given below in a phenological data sheet (Box -2.3) for further analysis.

a) Foliage phenology
   a) Leaf emergence
      b) Maturation of leaf
      c) Abscission of leaf

b) Flowering phenology
   a) Initiation of flowering
      b) Peak flowering
      c) End of flowering
      d) Intermittent flowering

c) Fruiting phenology
   a) Initiation of fruiting
      b) Peak fruiting
      c) End of fruiting
      d) Intermittent fruiting

2.2.4.2 Flower bearing capacity

When flowering was at peak, twenty five twigs of selected trees were identified, tagged and number of panicle in a twig, average size of the largest and smallest panicle, average number of flowers in the smallest and largest panicles and
average distance between two adjacent panicles were recorded in the data sheet prepared (Box – 2.4) for collecting information in the field and brought for further analysis.

2.2.4.3 Fruit bearing capacity

The fruit bearing capacity of the selected trees were studied by noting the developed and mature fruits. One inflorescence of average size was tagged in twenty five twigs of the selected tree in each location and the number of flowers were recorded. During the peak period of fruiting the number of mature fruits developed in each tagged inflorescence was noted. The data were recorded in the data sheet prepared (Box – 2.5).

2.2.5 Sample collection

During the field visits sample collections were made separately for herbarium preparation, floral study, fruit and seed morphological studies. Herbarium was prepared following by the standard methods of Lawrence (1951) and deposited in the Herbarium of Department of Botany, NSS College, Changanassery and indexed. The flower samples were collected and fixed in formalin – acetic – alcohol (FAA) and stored in 70% ethyl alcohol in plastic containers. Anthers were collected and preserved in 70% ethyl alcohol for pollen studies. The mature fruits and seeds were collected from plants, put in labelled paper bags and used for cultivation, germination and seedling studies.
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Studies on the Genetic Variability of Neem

Phenological Data Sheet

1. No. .................. Date ....................... 

2. Location (State, District, Village)  

3. Address  

Botanical Name ................................... Vernacular Name: ....................................

4. Phenological Characters

<table>
<thead>
<tr>
<th>Months</th>
<th>JAN</th>
<th>FEB</th>
<th>MAR</th>
<th>APR</th>
<th>MAY</th>
<th>JUN</th>
<th>JUL</th>
<th>AUG</th>
<th>SEP</th>
<th>OCT</th>
<th>NOV</th>
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<tbody>
<tr>
<td>Leaf Emergence</td>
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<td>Maturation of Leaf</td>
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<td>Abscission of Leaf</td>
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<td>Peak Flowering</td>
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<td>End of Flowering</td>
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<tr>
<td>Beginning of Fruiting</td>
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</tbody>
</table>

Remarks if any  

.......................... .......................... ..........................

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Studies on the Genetic Variability of Neem

Data Sheet for Flower Bearing Capacity Studies

<table>
<thead>
<tr>
<th>Characters</th>
<th>Twig Numbers</th>
<th>Avg.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td>ii</td>
</tr>
<tr>
<td>Number of Inflorescence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size of the Panicle (length in cm)</td>
<td>largest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smallest</td>
<td></td>
</tr>
<tr>
<td>Number of Flowers in Panicle</td>
<td>Longest</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shortest</td>
<td></td>
</tr>
<tr>
<td>Length between two adjacent panicle in the twig (cm)</td>
<td>Maximum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td></td>
</tr>
</tbody>
</table>

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Studies on the Genetic Variability of Neem

Data Sheet for Fruit Bearing Capacity Studies

<table>
<thead>
<tr>
<th>Characters</th>
<th>Twigs Numbers</th>
<th>i</th>
<th>ii</th>
<th>iii</th>
<th>iv</th>
<th>v</th>
<th>vi</th>
<th>vii</th>
<th>viii</th>
<th>IX</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflorescence</td>
<td>Size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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Recorded by:
2.2.6 Morphological studies

The morphology of the selected trees of *Azadirachta indica* A. Juss at eleven locations in Kerala and Tamil Nadu was analysed for understanding the natural variation existing among the characters. The flowers, fruits, seeds and seedlings were selected for the study of morphological variation as they are the basic units of the reproductive system. The methods followed are described below:

2.2.6.1 Flower

Flowers were collected randomly from different twigs of the selected trees at each location for the study of morphological characters. Fifty flowers were observed in the laboratory for each location. The flowers were dissected and dehydrated following customary methods described by Johansen (1940) and staining was done using saffranin. The stained floral parts were observed under stereomicroscope, observations and quantitative measurements were carried out for the following parameters as given in Fig. - 2.4 (a-l). The pollen morphology was studied after acetolysing pollen following Erdtman (1952) and observed under light microscope.

a) Bracts - Present or Absent.

Caudacious or non-caudacious

b) Calyx (Fig. - 2.4.b) - Number

Shape

Size : Length and Width

Length/ Width ratio

Arrangement
c) Corolla (Fig. 2.4.c)

<table>
<thead>
<tr>
<th>Number</th>
<th>Shape</th>
<th>Size: Length and Width</th>
<th>Length / Width ratio</th>
<th>Arrangement</th>
</tr>
</thead>
</table>

d) Androecium (Fig. 2.4 d to g)

1) Stamens and Anther

<table>
<thead>
<tr>
<th>Number</th>
<th>Length</th>
<th>Width</th>
<th>Number of lobes</th>
</tr>
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2) Pollen grains

<table>
<thead>
<tr>
<th>Size</th>
<th>Shape</th>
<th>Exine character</th>
<th>Aperture type</th>
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</thead>
</table>

3) Staminal tube

<table>
<thead>
<tr>
<th>Length</th>
<th>Width at base, middle and tip</th>
<th>Hair - status and density</th>
</tr>
</thead>
</table>

e) Gynoecium (Fig. 2.4.h – j)

1) Space between style and staminal tube

2) Style

<table>
<thead>
<tr>
<th>Length</th>
<th>Type</th>
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3) Stigma

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<th>Width</th>
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</table>
4) Ovary

- Number of lobes and Type
- Structure,
- Number of locules
- Number of ovules in locule
- Placentation

2.2.6.2 Fruits and seeds

The mature and ripened fruits were collected from all selected trees. 100 fruits of uniform size and shape were used for the calculation of fresh weight. The fruits were further observed for shape and quantitative measurement of size length and breadth (Fig. – 2.4 k) for 100 fruits at each location.

Seeds were taken out from fully ripened mature fruits by squeezing and washed thoroughly in warm water. Removed the epicarp and mesocarp of the fruit by washing in running tap water. After depulping, the excess water was removed using blotting paper and fresh weight of 100 uniform shaped and sized seeds were recorded. The seeds were further dried in an oven at 60°C and weights were recorded at every 24 hrs until the weight become steady and constant. The final weight was recorded as the dry weight. The length and breadth of the seeds were also recorded for analysis (Fig. – 2.4.1).

2.2.7 Germination studies

Germination studies were carried out with seeds collected from eleven locations and the methods followed are described below:
Figure – 2.4 (a - l) : Diagrammatic representation showing the quantitative measurements taken for floral fruit and characters of a neem trees.

a) Flower
b) Calyx
c) Corolla
d) Staminal tube
e) Anther
f) Pollengrains
g) Pollengrains
h) L.S. of flower showing the space between style and staminal tube
i) Style
j) Cross section of ovary
k) Fruit
l) Seed
Figure - 2.4
2.2.7.1 Sterilization of seeds

Seeds collected and stored were surface sterilized with 0.1% HgCl₂ (Mercuric Chloride) for five minutes and thoroughly washed with running water and then in distilled water to remove the excess HgCl₂.

2.2.7.2 Test for viability

Viability of seeds collected were tested using tetrazolium test of Agarwal (1980). The living cells were made visible by the reduction of an indicator dye. The indicator dye used is the colourless solution of Tetrazolium salt (2,3,5 Triphenyl tetrazolium chloride ~ TTC). Within the seeds the dye interferes with the reduction process of living cells and accept hydrogenases. By hydrogenation of 2,3,5 Triphenyl tetrazolium chorinde, a red, stable and non-diffusible compound, Triphenyl formazone is produced in living cells which makes it possible to distinguish the red coloured tissue of seeds from the colourless dead ones.

The Solution-I was prepared by dissolving 9.078 gm of KH₂PO₄ in one litre of distilled water. Solution-II was prepared by dissolving 11.876 gm of Na₂HPO₄ in one litre of distilled water. 400ml of Solution-I was mixed with 600 ml of Solution – II. To this mixture, 10 gm of Tetrazolium salt was added and dissolved and the pH was adjusted to 7.0. It was stored in an amber coloured bottle.

A sample of 400 seeds were considered for the test. The sterilized and presoaked seeds were dipped in Tetrazolium salt solution and placed in dark and warm place at 40°C for 8 to 10 hrs, till the red colour appeared. Soon after the appearance of red colour, Tetrazolium solution was removed and seeds were kept
submerged in water. The seeds were examined under a dissection microscope and viable seeds were counted and recorded.

2.2.7.3 Pre-conditioning of seeds

The pre-conditioning of seeds were done by soaking the seeds in water for three hours to modify hard seed coats, remove inhibitors, soften seed and reduce time of germination. This treatment will overcome seed coat dormancy if any, and stimulate germination.

2.2.7.4 Chemical stimulation

Chemical stimulation were also studied tried to increase the germination percentage of seeds and seedling growth. Different concentrations (50, 100 and 500 ppm) of Thiourea (CSCNH₂)₂ and Gibberellic acid were prepared using distilled water. Healthy and viable seeds were immersed in the solutions for 24 hrs (Hartman and Kester, 1972). The treated seeds were sowed and cultured.

2.2.7.5 Germination

The treated seeds and control seeds were sown in pots (30x30cm) containing pot culture mixture. Soil samples were analysed for physical and chemical characteristics in the Soil Testing Laboratory at Kozha, Kottayam. The pot mixture was prepared by using tested soil, sand and cow-dung manure in 1:1:1 ratio. For germination and raising seedling, three seeds were sown in each pot. Ten replicates were maintained for each treatment of every location. Watering was done on every day depending on the water retaining capacity of the soil. Radicle emergence was
considered as the criterion for germination. The following parameters were recorded on every day up to 30th day (Fig. - 2.5.a-c).

1. Number of seeds sown.
2. Number of seeds germinated
3. Germination percentage
4. Seedling height (cm)
5. Number and characteristics of cotyledons
7. Percentage of seedling survival.
8. Fresh and dry weight (biomass) of seedling.

2.2.7.6 Germination percentage

Every tenth day after sowing the number of germinated seeds were counted and the germination percentage was calculated as:

\[
\text{Germination Percentage} = \frac{\text{Number of seeds germinated}}{\text{Total No. of seeds sown}} \times 100
\]

2.2.7.7 Speed of germination

The speed of germination was calculated as described by Maguire (1962),

\[
\text{Speed of Germination (x)} = \frac{\text{Number of normal seedling}}{\text{Days to first count}} + \frac{\text{Number of normal seedling}}{\text{Days to final count}}
\]
2.2.7.8 Mean Germination Time

The germination time was calculated as described by Ellis and Roberts (1981) based on the formula given below:

\[ MGT = \frac{\sum nd}{\sum ni} \]

Where \( nd = \) Number of seeds germinated \( \times \) Number of days taken for germination.

\( ni = \) Total number of germinated seeds.

2.2.7.9 Vigour index

It was calculated as given below:

Vigour index = Standard Germination \( \times \) (Shoot length + Root length)

2.2.7.10 Survival of seedlings

On the 60\textsuperscript{th} day of sowing seeds, the survival of seedlings was recorded and expressed as percentage of total seeds germinated.

2.2.7.11 Characteristics of cotyledons

The following characteristics of cotyledons was noted both qualitatively and quantitatively as given in Fig. – 2.5 a.

1. Number
2. Shape
3. Colour
2.2.7.12 Characteristics of eophylls

Eophylls characteristics noted are as shown in Fig. 2.5.b.

1. Phyllotaxy
2. Shape

2.2.7.13 Seedling growth

Seedling growth was measured on 30th day. The shoot and root length was measured in cm from the transition zone to the shoot tip for shoot length and from transition zone to the root tip for root length (Fig. 2.5.c). The effect of chemical stimulants - Thiourea and Gibberellic acid are also noted as earlier.

2.2.7.14 Fresh and dry weight of seedling

Five seedlings were taken for the measurement of fresh and dry weight of seedling after 30 days of sowing. The seedlings were washed and removed all soil particles and fresh weight of both shoot and roots were noted separately. The seedlings were further dried in an oven at 30°C and weight was measured on every 24 hrs until the weight become steady and constant. The final weight was recorded as the dry weight and expressed in mg.

2.2.8 Statistical analysis

The data were analysed statistically to test whether there is any significant difference between the superior trees at various locations with respect to the characters studied. Analysis of variance, phenotypic and genotypic coefficients of
variation, heritability and genetic divergence were estimated using various statistical methods as described below:

2.2.8.1 Genetic variability

a) Analysis of variance (ANOVA)

Analysis of variance was carried out for all the characters studied as per the procedures prescribed (Panse and Sukhatme, 1954). The test of significance was carried out with reference to the standard 'F' Table (Fisher and Yates, 1963).

2.2.8.2 Genetic parameters

2.2.8.2.1 Phenotypic and genotypic coefficients of variation.

The Phenotypic and Genotypic Coefficients of Variations (PCV and GCV) were estimated following Burton and Devane (1953) for the characters studied:

a) Phenotypic coefficient of variation (PCV)

where \( \sigma_p \) = Phenotypic SD.

\[
PCV = \frac{\sigma_p \times 100}{\bar{X}}
\]

\( \bar{X} \) = grand means for the particular character

b) Genotypic coefficient of variation (GCV)

\[
GCV = \frac{\sigma_g \times 100}{\bar{X}}
\]

\( \sigma_g \) = genotypic standard deviation for a particular character

\( \bar{X} \) = grand means for the particular character
Figure – 2.5 (a - c) : Diagrammatic representation showing the quantitative measurements taken for seedling characters

a) Cotyledon
b) Eophylls
c) Seedlings
Figure - 2.5
2.2.8.2.2 Heritability ($H^2$)

Heritability ($H^2$) in the broad sense is the fraction of the total variance, which is heritable, was estimated as percentage, following Jain (1982) as:

$$H^2 = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

where $\sigma^2_g$ = genotypic variance

$\sigma^2_p$ = phenotypic variance

2.2.8.2.3 Genetic divergence (GD)

Genetic Divergence of the superior trees in different location was estimated by $D^2$ statistics as per Mahalanobis (1928, 1936) and is computed as:

$$D^2_x = \sum_{i=1}^{p} \sum_{j=1}^{p} (\lambda^n)_{ij}$$

where, $x =$ Number of metric traits in point, $p =$ Number of Superior trees $di$ and $dj =$ The differences between the mean values of two genotypes for $i^{th}$ and $j^{th}$ characters respectively.

$\lambda^n =$ Dispersion matrix reciprocal to the common dispersion matrix.

After computing the relative genetic distance between the sampling trees in every location, they were clustered into genetically divergent clusters as per iterative relocation algorithm suggested by Friedman and Rubin (1967) and modified by Suresh and Unnithan (1996). The mean intra cluster distances were computed using the formula:  

51
\[ \sum (d_{ij})^2 / n \]

Where \( d_{ij}^2 \) = The distance between \( i^{th} \) and \( j^{th} \) trees in each location in the same cluster.

\( n \) = Number of values

The mean inter cluster distances were worked out using the distances between all possible combinations of the clusters obtained. The sum of distances between all possible combinations of the trees in each location in a pair of clusters were taken. The sum of \( D^2 \) values divided by the product of the number of trees in each cluster gave the inter-cluster distance between the particular pair of clusters. The mean inter and intra cluster distances were then tabulated.