Methods

Morphological studies

The budgrafted plants of induced as well as spontaneous male sterile clones were raised in polybags (65 x 35 cm) and planted in nursery in three replications, each having five plants per type. The morphological characters like height, girth, number of flushes, number of leaves in a flush, specific leaf weight, stem index, petiolar index and foliar index were observed. Foliage characters were recorded from middle leaflets of 100 mature leaves selected at random. From each flush of growth three leaves were selected from top middle and bottom. Stem index, petiolar index and leaf index were determined following Mendes (1969) and specific leaf weight as suggested by Chatterton et al. (1972) as shown below:

Petiolar index = \( \frac{\text{Diameter of the petiole at its half length}}{\text{Length of petiole}} \)

Foliar index = \( \frac{\text{Diameter of the principal vein at its half length}}{\text{Length of vein}} \)

Stem index = \( \frac{\text{Thickness of stem at its half length}}{\text{Length of whorl}} \)
Budgrafted plants of these materials were induced to flower early, at the age of 30 months by ring barking (Saraswathy Amma, 1975). Flower size, i.e. length and breadth of both male and female flowers, just prior to anthesis were recorded. The data were subjected to statistical analysis. In the case of spontaneous male sterile clones flowers were collected from mature trees. Seeds were collected and the morphology and seed size were noted. Observations were taken from 100 flowers and seeds selected at random. Correlation of characters were also calculated in the case of growth attributes of polybag plants of male sterile clones.

Cytological studies

Mitosis:

For mitotic studies tender leaves were pretreated with saturated aqueous solution of Paradichlorobenzene (PDB) and kept at 10°C for 2.5 to 3.0 h. The pretreated leaves were thoroughly washed in water and preserved in alcohol-acetic acid (3:1). Leaf-tips were washed and hydrolysed in 1 N HCl for 20 to 25 min at 60°C. After thorough washing in water they were kept overnight in 2% acetocarmine. Squash preparations were made in 45% acetic acid and the slides were made permanent by acetic-butanol series.
Meiosis:

For meiotic studies, male flower buds, at the appropriate stage of development, from the male sterile clones as well as control were collected and fixed in modified Carnoy's fluid (3:1:1), alcohol, acetic acid, chloroform. After 24 h, the materials were transferred to 3:1 alcohol-acetic acid. Staminal columns were dissected out and stained overnight in 2% acetocarmine. Preparations were made in 45% acetic acid and observations were taken from 100 pollen mother cells, selecting at random 10 cells from 10 slides from temporary mounts.

Pollen and cytological studies were repeated for three consecutive flowering seasons.

Palynological studies

For morphological studies flowers were collected and preserved in 70% alcohol. Acetolysis was done by the standard procedure (Erdtman, 1952; Nair et al., 1977). Pollen grains were examined and measurements of equatorial diameter, polar diameter, exine thickness and pore diameter were taken at a magnification of 400 X by means of an
ocular micrometer using light microscope. A total of 100 pollen grains, 10 each from 10 slides, selected at random were used for measurements of pollen characteristics.

SEM studies were carried out at the National Botanical Research Institute, Lucknow. The acetolysed pollen grains were placed on adhesive tape attached to an aluminium stub. The samples were coated with gold (200 \( \text{Å} \)) JEOL ION sputter using a Coater (JFC 1000) and observed with a JEOL JSM 35 C Scanning Electron Microscope and photographed at 2000 X and 6000 X. Five samples were observed from each clone.

The methods and terminology used for the morphological studies of pollen grains were those followed by Nair (1961, 1970).

For the assessment of pollen stainability as an index of pollen sterility, mature male flowers just prior to anthesis were collected and treated with (1:1) acetocarmine glycerine mixture. For studies on pollen germination, male flowers were collected just prior to anthesis and pollen grains were dusted in 20% to 25% sucrose solution with 0.01% boric acid and germinated by the "hanging drop" technique. The concentration of sucrose solution was selected after preliminary studies. Germination percentage was assessed by scoring 100 pollen grains from ten microscopic
fields after three hours of incubation. For pollen production studies (Mathur and Mohan Ram, 1986) mature flower buds just prior to anthesis were collected at random and the anther columns from five flowers were dissected out. Anthers were homogenized in 2 ml of 10% aqueous glycerine, using a glass homogenizer. Pollen counts were made using a haemocytometer and compound research microscope. Seven replicates were taken for each homogenate and the number estimated twice from which average per anther per flower was taken.

Male flower buds of male sterile, triploid and tetraploid along with control were collected at different stages of development. The length of flower buds was measured. Microsporocytes or microspores contained in each bud were stained with 1% aceticarmine solution and their diameter were measured. In each bud, the mean value of diameter of fifty sporocytes or spores was calculated. The relationship between flower size and size of the pollen mother cells was ascertained.

For studying the microsporogenesis the materials were fixed vacuum applied in 3:1 alcohol:acetic and preserved in 70% alcohol. Paraffin blocks of the materials were prepared (Johansen, 1940) and sections were cut at 10-12 μm. Staining was done with Delafields hematoxylin, safranine and fastgreen. Sections were
made permanent and mounted in Euparel and observations and photomicrographs were taken from the permanent slides.

**Estimation of DNA**

Young shoot tips were collected from diploid, triploids and tetraploid and pretreated with saturated solution of Para-dichlorobenzene (PDB) for 2.5 to 3.0 h at 10°C. The leaf tips were washed thoroughly with water and fixed in (3:1) alcohol-acetic acid for 24 h and preserved in 70% alcohol. The samples were hydrolysed in 1 N HCl for 20 to 25 min and washed thoroughly in water and kept in leuco-basic fuchsin at pH 3.6 overnight at low temperature. The samples were washed with SO₂ water for 30 min with three changes of 10 min each. The root tip of *Allium cepa* was also treated in the same manner as control. Squash preparations were made in a drop of glycerine. Photometric measurements were taken on Vickers Scanning Cytophotometer M 85a at the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow.

**Genetical studies**

Mature dry fruits resulting from open pollination before dehiscence, were collected from trees of GT 1, Ch 2, RRII 35 and
Mil 3/2 and the seeds were taken by opening the fruits. They were germinated in germination beds in the normal manner (Jopseh et al., 1980) and the rate of germination was assessed from the seventh day onwards up to the 19th day. From among the seeds which sprouted from 7th to 13th day of sowing, a nursery was established, adopting RBD with five replicates of forty seedlings per plot. The planting was done at a spacing of 30 cm between plants in four rows of ten seedlings each, the distance between rows being 30 cm. Observations were recorded from the sixteen inner plants in each plot. Height and basal girth of the seedlings were recorded at 12 and 24 months growth. At the age of 30 months, test tapping was done adopting half spiral alternate daily (S/2 d/2) system. Tapping was commenced at 15 cm height from the ground level and girth was also recorded. First ten tappings were to initiate and regulate flow. After ten days tappings, yield of the next five consecutive tappings was collected as cup lumps and oven dried at 60°C. The dry weight of the lumps was recorded when the moisture was removed. Yield recordings were repeated during two subsequent quarters also. The seedlings were stimulated by applying 0.1% of Ethrel (2 Chloro ethyl phosphonic acid), on the bark just below the tapping cut with a brush. Yield after stimulation was also recorded for five days. Girth after tapping was also measured after the third quarter. From among the seedlings based on yield and secondary characters forty one progenies
were selected. The growth attributes and yield of these selections were also recorded.

The male sterile clone GT 1 was crossed with four male fertile clones (RRII 105, RRII 118, RRIC 100 and RRIM 600). Hand pollinations were also carried out incorporating the fertile clones RRII 105 and PR 107 as female and male parent. The details of hand pollinations attempted are given in Table 2. The seedlings were multiplied vegetatively and ten budgrafted plants from each combinations were planted in the nursery. At the age of 24 to 30 months early flowering was induced by ring barking (Saraswathy Amma, 1975). The nature of sterility in the F1 progenies was studied employing cytological and palynological techniques.

STATISTICAL ANALYSIS

Analysis of variance

Analysis of variance in the morphological characters and growth attributes was done as suggested by Panse and Sukhatme (1957). The manifestation of genotypic (G) and environmental (E) effects on the observed value of a character was partitioned by the method of analysis (Kempthorne, 1957):
\[ V(X) = V(G) + V(E) \quad \text{or} \]
\[ \sigma^2_P(X) = \sigma^2_g(X) + \sigma^2_e(X) \]

where \( \sigma^2_P(X) \) is the phenotypic variance of character \( X \), \( \sigma^2_g(X) \) is the genotypic variance of \( X \) and \( \sigma^2_e(X) \) is the variance due to environment.

The extent of covariance between \( x \) and \( y \), due to genetic and environmental factor, was partitioned using the formula:

\[ \text{Cov} (xy) = \text{Cov} G(xy) + \text{Co} E(xy) \quad \text{or} \]
\[ \sigma p(xy) = \sigma G(xy) + \sigma E(xy) \]

where \( G(xy) \) is the covariance between \( x \) and \( y \) attributable to genotypes and \( E(xy) \) that due to environment.

**Correlation**

The phenotypic correlation coefficients were estimated as:

\[ r^\wedge p(xy) = \frac{\sigma^\wedge p(xy)}{\sigma^\wedge p(x) \cdot \sigma^\wedge p(y)} \]
where \( p(x) \) and \( p(y) \) are the estimated phenotypic standard deviation of \( x \) and \( y \).

**Co-efficient of variation**

The co-efficient of variation for phenotypic and genotypic traits were estimated as below:

**Phenotypic coefficient of variation:**

\[
C.V.p(x) = \frac{\sigma p(x) \times 100}{\bar{x}}
\]

and **genotypic coefficient of variation:**

\[
C.V.g(x) = \frac{\sigma g(x) \times 100}{\bar{x}}
\]

Heritability \((h^2)\), general combining ability (GCA), genetic advance (GA) were calculated following the methods suggested by Singh and Chowdhery (1979). Simple and multiple correlations were also worked out with regard to yield and secondary characters.
Photomicrographs of cytological and palynological preparations were taken from suitable slides using Orthoplan large field Microscope (Leitz-Wetzlar, Germany) with WILD MPS 12 (Heerbrugg, Switzerland) attachment.
Table 1. Details of male sterile materials.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Origin</th>
<th>Cytotype</th>
<th>Nature of sterility</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Spontaneous sterility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRII 15</td>
<td>Indian</td>
<td>$2n = 3x = 54$</td>
<td>Spontaneous triploid</td>
</tr>
<tr>
<td>RRII 17</td>
<td>India</td>
<td>$2n = 2x = 36$</td>
<td>Male and female sterile</td>
</tr>
<tr>
<td>RRII 35</td>
<td>India</td>
<td>$2n = 2x = 36$</td>
<td>Complete male sterility</td>
</tr>
<tr>
<td>Ch 2</td>
<td>Malaysia</td>
<td>$2n = 2x = 36$</td>
<td>Complete male sterility</td>
</tr>
<tr>
<td>GT 1</td>
<td>Indonesia</td>
<td>$2n = 2x = 36$</td>
<td>Complete male sterility</td>
</tr>
<tr>
<td>(b) Induced sterility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRII 105</td>
<td>Colchiplody</td>
<td>$2n = 4x = 72$</td>
<td>Tetraploid partial sterility</td>
</tr>
<tr>
<td>Polyploid</td>
<td>辜chiploid种</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induced triploid</td>
<td>Diploid x tetraploid</td>
<td>$2n = 3x = 54$</td>
<td>Partial sterility</td>
</tr>
<tr>
<td>Mutagen induced</td>
<td>EMS induced</td>
<td>$2n = 2x = 36$</td>
<td>Complete male sterility</td>
</tr>
<tr>
<td>Radiation induced mutant</td>
<td>Gamma rays 3000 r seed treatment</td>
<td>$2n = 2x = 36$</td>
<td>Complete male sterility</td>
</tr>
</tbody>
</table>
Table 2. Details of hand pollinations.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Combinations</th>
<th>Hand pollinations done</th>
<th>Final success %</th>
<th>Seeds obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GT 1 x RRII 105</td>
<td>200</td>
<td>2.5</td>
<td>15</td>
</tr>
<tr>
<td>2.</td>
<td>GT 1 x RRIC 100</td>
<td>200</td>
<td>2.7</td>
<td>18</td>
</tr>
<tr>
<td>3.</td>
<td>GT 1 x RRII 118</td>
<td>200</td>
<td>3.0</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>GT 1 x RRIM 600</td>
<td>215</td>
<td>2.3</td>
<td>15</td>
</tr>
<tr>
<td>5.</td>
<td>RRII 105 x PR 107</td>
<td>200</td>
<td>2.5</td>
<td>18</td>
</tr>
</tbody>
</table>