Chapter 2

MATERIALS AND METHODS

The oleiferous crucifer *Eruca sativa* Mill. [*syn. E. vesicaria* ssp. *sativa* (Mill.) Thell.], a member of Brassicaceae (Cruciferae), has been investigated from five sources for genetics of self-incompatibility, male sterility, chromosome behaviour and SEM studies, for exomorphological features of pollen and anthers of male fertile and male steriles. The sources of various materials utilized and the methods employed for these studies are given below.

2.1 THE MATERIAL

Seeds of *E. sativa* from five different sources representing both cultivated and wild forms were procured (Table 2). It may be noted that the study includes two

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Variety</th>
<th>Code for text</th>
<th>Source</th>
<th>No. of plants raised</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Indian cultivated (ITSA)</td>
<td>IC</td>
<td>IARI, New Delhi, India</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Indian wild (ES8*)</td>
<td>ES</td>
<td>Courtesy of Prof. K. Hinata, University of Tohoku, Sindai, Japan (origin near Solan, India)</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Egypt</td>
<td>EG</td>
<td>NBPGGR</td>
<td>10</td>
</tr>
<tr>
<td>4.</td>
<td>Greece</td>
<td>GR</td>
<td>NBPGGR</td>
<td>5</td>
</tr>
<tr>
<td>5.</td>
<td>Sweden</td>
<td>SW</td>
<td>NBPGGR</td>
<td>5</td>
</tr>
</tbody>
</table>

*Escape from cultivation, collected by Prof. K. Hinata (Sendai, Japan) in India from about Solan (Shimla hills) numbered as ES8.*

# *Eruca sativa* Mill. is written as *Eruca sativa* L., on the title page of the thesis. The oversight could not be corrected as the title had already been submitted and approved. The error is regretted.
forms indigenous to India, namely a commercial variety called ITSA, obtained from Indian Agricultural Research Institute, New Delhi, and another wild form, growing as an escape from cultivation, collected by Prof. K. Hinata (Sendai, Japan) from about Solan-Shimla Hills, India, and designated by him as ES8. Each of the five kinds including those from Egypt, Greece and Sweden have been given a code, for brevity, and these have been followed consistently in the text. The number of plants raised from the original seed samples, called the parental generation are also given in Table 2.

First and subsequent Inbred-generation families were raised through selfings in the bud and the inbred families used for studies on self-incompatibility are summarised in Table 3 wherein the source of the various families is given together with the number of plants in each, and each family of the inbred-generation has been coded. In all 23 inbred bud-selfed families have been studied: 12 of $I_1$-generation, 8 of $I_2$-generation, and 3 of $I_3$-generation (Table 3). In addition, two families were obtained by crossing male-sterile plants that appeared in the $I_1$-generation family GR1.2 (Table 3) with their male fertile sibs. These two families GR2.3$^{SC}$ and GR2.4$^{SC}$ were also studied together with the $I_2$-generation families.

2.2 RAISING PLANTS

$E. \text{sativa}$ is an annual and is raised as a winter crop. The seed materials of the parental and the various inbred generations were sown in plots in the University Botanical Garden, in the month of October every year. For inbred generations a minimum of 13 sibs were planned to be utilized for analyses on SI, but some families where only fewer plants were obtained, such lesser-membered families were also investigated for the sake of interest. Flowering occurred in about 10 weeks from sowing, and it continued generally up to the end of March.
<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Parental material</th>
<th>Symbols for inbred families</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;-generation</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;-generation</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;-generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IC [Indian Cultivated]</td>
<td></td>
<td>IC1.1 (19)</td>
<td>IC1.2 (9)</td>
<td>IC1.3 (20)</td>
</tr>
<tr>
<td>2.</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; ES8 [North Indian Wild]</td>
<td>ES1.1 (22)</td>
<td>ES1.2 (13)</td>
<td>ES1.3 (15)</td>
<td>ES1.4 (14)</td>
</tr>
<tr>
<td>3.</td>
<td>EG [Egypt]</td>
<td>EG1.1 (20)</td>
<td></td>
<td>EG2.1 (16)</td>
<td>EG2.2 (13)</td>
</tr>
<tr>
<td>4.</td>
<td>GR [Greece]</td>
<td>GR1.1 (14)</td>
<td></td>
<td>GR2.1 (10)</td>
<td>GR2.2 (13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GR1.2 (17)</td>
<td></td>
<td></td>
<td>GR3.1 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GR3.2 (19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GR3.3 (19)</td>
</tr>
<tr>
<td>5.</td>
<td>SW [Sweden]</td>
<td>SW1.1 (9)</td>
<td></td>
<td>SW2.1 (10)</td>
<td>SW2.2 (18)</td>
</tr>
</tbody>
</table>

± As numbered by K Hinata

The numeral 1, 2 or 3 succeeding the abbreviation of parental family code denotes 1<sup>st</sup>-generation, 1<sup>st</sup>-generation and 1<sup>st</sup>-generation, respectively. The numerals following the inbred generation tells the serial number of the family. Figures within parentheses denote the number of plants within the family. Families inbred through sib crossing → arrows indicate the source of the 1<sub>2</sub> and 1<sub>1</sub>-generation.
2.3 METHOD OF BUD-SELFING FOR INBRED-GENERATIONS

It has been known in literature that *E. sativa* is self-incompatible and the block to self-pollen occurs on the surface of the stigma papillae. The incompatibility response is the strongest on the day of flower opening (Alam, 1936; Verma *et al.*, 1977). Thus it is possible to make effective self-pollinations some days prior to anthesis. In the materials utilized the desired plants were selfed in the bud 2-3 days before anthesis, and all these bud-selfings were successful. The buds were opened carefully, emasculated, artificially pollinated with self-pollen from mature flowers, and bagged for at least 5 days. In all cases where inbred generations are raised through bud-selfing only strongly self-incompatible plants were utilized. Strongly SI plants are those where self pollen do not germinate on stigma on the day of flower-opening. The only exception was family ES2.2 which was raised from a self-compatible plant (plant 13 of *I*₁-generation family ES1.3) to inquire into the nature of self-compatibility in the plant. The seeds obtained were stored in a dessicator and sown the following season.

2.4 DETERMINING COMPATIBILITY OR INCOMPATIBILITY OF A MATING

It is now well established that in the self-incompatible system of *Brassicaceae*, the recognition and rejection of incompatible self pollen occur at the stigma surface (Ockendon, 1971; Heslop-Harrison *et al.*, 1974; Heslop-Harrison, 1978; Shivanna, 1979; Roberts and Dickinson, 1981; Zuberi *et al.*, 1981; Nasrallah and Nasrallah, 1993; Nasrallah *et al.*, 1994b; Dickinson, 1995, 1999). Accordingly, the penetration of stigma papillae by pollen tubes of the germinated pollen provides a reliable marker to ascertain compatibility, and conversely the lack/denial of such penetration or lack of pollen germination signifies incompatibility.

The seed-set is dependent upon post-fertilization factors and hence would not be reliable in analysis of SI which phenomenon is essentially a pre-fertilization event. The cytological method is to be preferred over seed set. Besides, this method allows also the estimation of the relative scores of the compatible/incompatible pollen among different pollinations (Wallace, 1979a,b; Zuberi *et al.*, 1981). In this study, the inferences on the nature of pollinations are based primarily on the cytological examination of pollinated stigmas, after 18-24 hours, as a standard procedure advocated by Zuberi *et al.*(1981), Lewis *et al.* (1988) and Zuberi and Lewis (1988).

### 2.4.1 THE CYTOLOGICAL METHOD

It is also known as the direct method to determine compatibility or incompatibility of a mating. There are 2 ways to follow the cytological method i) Fluorescence microscopy of pollinated stigmas for callose detection, the deposition of which constitutes the so-called rejection response (Kho and Baer, 1968; Nettancourt, 1977; Verma, 1983; Knox, 1984a) and ii) Light microscopy of pollinated stigmas stained suitably to differentiate between compatibly-reacting and the incompatibly reacting pollen.

Both of these methods have been utilized wherever necessary, although routinely the light microscopy method has been followed for sake of convenience of handling large numbers of crosses simultaneously.
i) Fluorescence microscopy

Pollinated stigmas, after 24 hours, are treated with IN NaOH at 60°C for 1 hour, stained with water soluble 0.1% aniline blue in neutral 8N Na₂HPO₄, mounted in glycerine and examined under UV-fluorescence microscope (Kho and Baer, 1968).

This schedule detects callose in stigma papillae, as also in the pollen/pollen tubes, which signifies the rejection response to an incompatible pollination. The stigma papillae do not elaborate any callose when the pollination is compatible (Figs. 1 and 2). The callose rejection response of stigma papillae specifically allows the detection of incompatible pollinations, even when the pollen are able to hydrate and germinate but are denied entry into the papillae (Figs.3 and 4). In latter cases their pollen tubes also get occluded with callose (Fig. 4).

ii) Light microscopy

The second method does not require the use of a fluorescence microscope. Instead an ordinary light microscope is used. After 18-24 hours of pollination the pollinated stigmas along with styles were fixed in 1:3 acetic alcohol for about 1 hour. Stigmas alongwith the upper part of style were excised, placed on a glass slide, gently pressed and mounted in 2-3 drops of acid fuschin-light green stain in dilute lactic acid (Lewis and Crowe, 1958; Lewis, 1979a; Table 4). The slides are left to stain for about an hour and examined under a light microscope.

Table 4. Composition of pollen stain (from Lewis, 1979a)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acid fuchsin 1% aq.</td>
<td>2 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Light-green 1% aq.</td>
<td>2 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Lactic acid</td>
<td>40 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Water (dist.)</td>
<td>46 ml</td>
</tr>
</tbody>
</table>
Acceptance of compatible pollen of *Eruca sativa*. Decolorized aniline blue, UV fluorescence microscopy procedure, fixed 18-24 hours after pollination.

Fig. 1: No callose can be seen in the pollen or papillae through which the pollen tubes have penetrated, callose plugs however have been formed in penetrating pollen tubes. x 1200.

Fig. 2: Whole stigma view. x 350
Fig. 3, 4: Rejection reaction as evidenced by fluorescence microscopy. Decolorized aniline blue procedure, fixation 18-24 hours after pollination.

Fig. 3: Whole papillae aglow, evidence of very strong rejection, no pollen adhering. x 1200

Fig. 4: Hydrated and germinating pollen visible, strong callose deposition in papillae, pollen and whole pollen tube. x 1200.
Materials and Methods

In an incompatible pollination, either the pollen do not germinate, or when germinated their pollen tubes are unable to penetrate the stigma papillae. Such pollen are deeply stained as dark purple/pink as the cytoplasm of these pollen remains within the grains (Fig. 5). In case of compatible pollinations, during the 18-24 hour interval, the pollen grains had stuck firmly to the papillae, germinated and their pollen tubes succeeded in penetration of stigma papillae. The pollen tubes had grown down the style carrying the pollen cytoplasm at their tips. Such pollen grains are now emptied of their cytoplasmic contents. They appear as unstained and their pollen tubes penetrating the papillae can be easily detected (Fig. 6). This staining method allows further an estimate of the numbers of darkly stained incompatible pollen and the emptied unstained compatible grains on the carefully pollinated stigma, particularly in cases where two types of pollen behaviour is observed (Figs. 7 and 8). This method is very important in the understanding of the incompatibility system.

2.5 METHOD OF POLLINATION AND CODING

In order to be able to handle a large number of pollinations at a time and to ensure perfect labelling of the various matings, an extensive use of the pollination-box method was the most suited (Verma et al., 1977). A pollination-box of suitable size (10x10") was prepared by solidifying in it 1% agar-agar; and its surface was checkered into rows and columns with white paint. The rows were numbered A-J, and columns 1-10. As a rule the rows on the agar layer represented the plants tested as female, and the columns represented the plants tested as males against the several females. At one time a box accommodated a maximum of 100 pollinations 10 x d, and during the peak of the flowering season, more boxes were used simultaneously. For each box, numbers like A₁, A₂, A₃(i.e. Ax1,Ax2,Ax3) provided a code for the pollinations which were recorded scrupulously. The just-about-to-open flower-buds were picked up, carefully emasculated and were tucked in the squares of rows in the pollination box. The freshly-opened flowers with partially-dehisced anthers from plants to be used as the male parent were placed at the top of each column. Each
Plate 3.

Fig. 5: Acid fuchsin light green stain, light microscopy procedure, fixation 18-24 hours after pollination. x 1800

Fig. 5: Stain filled, dark, rejected germinated pollen grain. Pollen tube unable to penetrate papilla, forms a pad at papillar surface.

Fig. 6: Unstained, emptied, compatible pollen grains with their pollen tubes penetrating the stigma papillae.
Fig. 7, 8: Two types of pollen reaction from pollen of a single source/anther. Both compatible (emptied) and incompatible (full) pollen grains. x 1800.
Materials and Methods

Each of the pollinates, following staining of pollinated stigmas and light microscopy, was scored for three types of data i) the total number of pollen grains sticking firmly to the stigmatic papillae including the germinated ones; ii) the total number of pollen grains which had germinated, and it included also those where the pollen tubes had penetrated; and iii) the total number of grains whose pollen tubes actually penetrated the stigmatic papillae. The pollen grains which do not adhere firmly to stigma papillae float-away in the process of staining, and are eventually lost.

2.6 ANALYSIS OF POLLINATIONS

The pollination-box was kept overnight in the laboratory with the lid removed under insect-free conditions. After 18-24 hours of pollination the stigmas alongwith styles were processed for cytological examination as detailed under method of light-microscopy.
2.7 POLLEN FERTILITY

Freshly dehisced anthers were tested in 1 per cent acetocarmine for estimation of pollen fertility. The stainable pollen are taken to be fertile whereas the unstained ones are infertile (Kaul, 1988). Average estimates of per cent pollen fertility are based on the mean of 10 different preparations for each plant.

2.8 MEIOTIC STUDIES

Buds at suitable stages of development were fixed in 1:3 acetic acid-ethyl alcohol (ethanol) and squashes of pollen mother cells were made in 1 per cent acetocarmine and examined for various stages of meiosis. Mean chiasma frequency estimates in each case were made by examining 30 cells at diakinesis/early metaphase-1.

2.9 SEM OF ANTHER AND POLLEN

Inflorescences were fixed in 3 per cent glutaraldehyde in 0.1 M phosphate buffer. For SEM of pollen, anthers of the desired matured-flower were washed 4-5 times in the buffer solution, dehydrated in ethanol series, and air-dried (Sharma, 1992).

For SEM studies of intact anthers, buds at different stages were washed 4-5 times in phosphate buffer, followed by 4-5 washings in distilled water, and then dehydrated in graded series of ethanol-amyl acetate-CO₂. The specimens were then critical point dried (Sharma, 1992).

After drying each specimen was mounted on a copper stub with the help of double adhesive tape, gold plated for 3-5 minutes and scanned in a JOEL Scanning Electron Microscope at 10 KV at the Regional Scientific Instruments Centre Laboratory at Panjab University, Chandigarh.
Materials and Methods

2.10 MICROSPOROGENESIS

Inflorescences at various stages of development were fixed in formalin-acetic acid-50 per cent ethanol, FAA, in the ratio of 1:1:18. The buds fixed in FAA were passed through graded series of tertiary butyl alcohol and embedded in paraffin wax (60-62°C) (Johanson, 1940). Serial sections were cut on a Spencer 820 rotary microtome (American Optical Company, USA) at 6-8 μm. The sections were processed through a series of xylene ethanol grades, stained with Safranine and Fast Green combinations, and finally mounted in Canada Balsam. The slides were examined for the different stages of microsporogenesis, particularly to find out the differences between the male fertiles and the male steriles.