CHAPTER–3
MATERIALS AND METHODS
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MATERIAL AND METHODS

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MATERIALS AND METHODS

A comprehensive detail of material used and methodology adopted during the course of the present investigation are presented in this chapter.

3.1 Experimental Material

The term rapeseed is used to cover sarson, toria and taramira, whereas the term mustard refers only to ‘rai’. Seeds of mustard (*Brassica juncea* L. Czem & Coss.) of two cultivars, namely Alankar and PBM16 were used as experimental material.

Seeds of the above cultivars were obtained from the Indian Agricultural Research Institute, New Delhi, India, and are recommended equally well for cultivation under drier and wet conditions.

3.1.1 Nomenclature

Indian *Oleiferous Brassicae* are divided into four groups.

3. Toria (lahi or maghi lahi) *Brassica campestris* L. var. Toria Duth
4. Taramira or tara (*Eruca sativa* Mill.)

In addition, there are two other species, namely *Brassica nigra* Koch (Banarasi rai) and *Brassica juncea* var. Rugosa (pahadi rai) which do not fall under any of the four groups, and are grown to a limited extent.

3.1.2 Botanical description

Rape and mustard are annual herbs. Roots, in general, are long and tapering. Toria is more or less a surface feeder but brown mustard has long roots, with limited lateral spreads, enabling its successful cultivation under drier conditions. Yellow sarson has both extensive and lateral spreads. The
height of the stem varies from 0.45m (in some cultivars of toria) to 1.90m (in yellow sarson). In toria and brown sarson, the branches arise at an angle of 30° to 40°. In yellow sarson, the branches arise laterally at an angle of about 10° to 20° and give the plant a narrow and pyramidal shape. The inflorescence is a corymbose raceme. In the case of yellow sarson, the four petals are spread apart, whereas in brown sarson and toria, the petals overlap or may be placed apart, depending upon the cultivar. The flowers bear a hypogynous syncarpous ovary. In brown sarson and toria, the ovary is bicarpellary, whereas in case of yellow sarson, it may be tri or tetra-carpellary.

The fruit is a siliqua. The pods are two, three or four valved, depending on number of carpels in the ovary. The flowers begin to open from 8 a.m. and continue upto 12 noons.

3.2 Experimental Site

Five field experiments were conducted at the Experimental field of the Aligarh Muslim University, Aligarh, India.

3.3 Agro-Climatic Conditions

3.3.1 Topography

Aligarh has an area of 5,024 sq. km and is situated at 27°52'N latitude, 78°51'E longitude and 187.45m altitude above sea level.

3.3.2 Climate

It has a semi arid and subtropical weather with severest hot dry summers and intense cold winters.

3.3.3 Temperature

The winter stretches from middle of the October till the end of March. A gradual decrease in the temperature in December and January is observed, reaching as low as 15°C and 13°C, and lowest recorded for any single day is 2°C and 0.5°C respectively. The summer season extends from April to the end of June. In this season, a gradual increase in temperature is recorded, which attains its maximum, sometimes in the month of June upto 46°C (Fig. 1).
3.3.4 Rainfall

The mean annual rainfall is about 847.3mm. More than 85% of the total downpour is derived during a short span of four months from June to September. The remaining rain drops are received during winter. These showers are very useful for winter crops. However, they are sometimes accompanied with high wind velocity and hailstorm (Figs. 2-3).

3.4 Meteorological Inputs

Meteorological data for the present study were documented at the Meteorological Observatory, Department of Physics, Aligarh Muslim University, Aligarh, India.

3.5 Soil Characteristics

Random soil samples were collected from various chosen spots, spread over the entire experimental crops upto depth of 15 cm, and analysed for physico-chemical characteristics of the soil. Data obtained on chemical characteristics and physical constant for soil are presented in Table 1. The soil was also analysed for moisture content at different growth stages of the crop (Table 1a).

3.6 Cultural Operations

The field experiments were laid out in randomized complete block design with three replicates for each treatment. The individual plot size was 10 sq m (2m X 5m).

3.6.1 Preparatory tillage

Before each trial, diligent ploughing of field was done to turn the soil for maximum aeration and weed eradication. The plots were made with proper boundaries along with necessary irrigation channels and were irrigated lightly before sowing to maintain proper moisture in the sub-surface of the soil.
Fig. 2-3. Variation in relative humidity and rainfall during experimental period at Aligarh
Table 1 Physico-chemical characteristics of soil of the field used for experiments

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (1 2)</td>
<td>8.0</td>
<td>8.4</td>
<td>7.8</td>
<td>7.9</td>
<td>7.6</td>
</tr>
<tr>
<td>EC (1 2) (m mhos/cm)</td>
<td>0.46</td>
<td>0.41</td>
<td>0.45</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>Available nitrogen (N) (kg/ha)</td>
<td>205</td>
<td>190</td>
<td>210</td>
<td>215</td>
<td>215</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Sowing (0d)</td>
<td>12.6</td>
<td>12.5</td>
<td>12.3</td>
<td>12.7</td>
<td>12.1</td>
</tr>
<tr>
<td>Flowering (60d)</td>
<td>11.5</td>
<td>8.6</td>
<td>11.4</td>
<td>8.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Pod fill (80d)</td>
<td>9.6</td>
<td>6.6</td>
<td>9.4</td>
<td>6.3</td>
<td>9.6</td>
</tr>
<tr>
<td>Pod maturity (100d)</td>
<td>8.2</td>
<td>4.2</td>
<td>8.3</td>
<td>3.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Harvest (120d)</td>
<td>6.0</td>
<td></td>
<td>6.2</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>
3.6.2 Sowing

The seeds were sown by the usual behind the plough method at a rate of 10 kg/ha. A distance of 30 cm between rows and 15 cm between plants in each row was maintained.

3.6.3 Thinning

After establishment of the crop i.e. after 12 days of sowing, seedlings were thinned to maintain the uniformity (12 plants/m²) of the plant population.

3.6.4 Crop protection

In order to check the aphid contagion, if any, insecticidal spray of Dimecron-100 was done. Hand weeding was done twice during the entire crop season to keep the experimental field free of weeds.

3.6.5 Irrigation

Before sowing 20 litre/m² irrigation was given to all plots maintained for irrigated and non-irrigated experiments. Thereafter this amount of irrigation was done only to the plots used for experiments done under irrigated conditions at 50d after sowing.

3.6.6 Plant sampling

Plants were sampled to record observations on various growth (Section 3.10), physiological (Section 3.11) and biochemical (Section 3.12) characteristics at different stages of plant growth. At maturity, plants were harvested to record yield characteristics (Section 3.13) by cutting at the ground level and were allowed for sun drying. After drying, threshing was done to clear the seeds for seed yield. Seeds were analysed for quality characteristics (Section 3.14).

3.7 Application of N Fertilizer

In Experiments 1 and 2, a recommended uniform soil application of 80 kg/ha was done to all plots, and in other experiments nitrogen (as urea) was applied according to the treatment and design of the experiments.
3.8 Application of Ethrel (2-Chloroethyl phosphonic acid)

Concentrations of ethrel used in experiments were sprayed as a.i. \( \mu L/L \) on leaves at 60d after sowing (flowering stage) at 600 litre/ha (600 mL/pot) using a hand sprayer (Hindustan Sprayers, New Delhi).

In mustard, flowering stage has been found as appropriate stage for spray (Khan, 1998; Khan et al. 2000). Early application of PGRs is not recommended because treatment effects are compensated for by prolonged duration of the crop photosynthesis and equal or increased assimilate partitioning to the seeds. This may result in non-significant difference in control and treated plants. The other reason for spray at flowering is that there exists internal hormonal imbalance during sink development and due to this, only 68% flowers develop into pods. Therefore, spray at flowering stage brings hormonal status to suitable concentration and restricts flowers and pods abortion. Moreover, plants may be benefited additionally by spray at this stage by increasing the capacity of photoassimilate assimilation and sink strength since plants inherent capacity of these started declining.

3.9 Application of Silver Thiosulphate

Silver thiosulphate (STS) at 1mM concentration was sprayed at 60d after sowing (flowering stage) at 600 litre/ha (600 mL/plot). Application of ethrel was used to promote ethylene evolution, while the action of this gaseous plant growth regulator was blocked using silver ions applied as silver thiosulphate (STS). The objective was to test the hypothesis that ethylene has a central role in mediating plant responses. Silver thiosulphate solution was used to block the action of ethylene, as silver ions have been proposed to reduce the capacity of ethylene to interact with its receptors (Beyer, 1976). Silver thiosulphate is readily absorbed and transported by plants (Morgan et al., 1993).
3.10 Experimentation

3.10.1 Experiment 1

This experiment was a factorial performed according to randomized complete block design during the winter season of 1998-99. The experiment was performed to assess the effect of leaf-applied 0, 100, 200, 400 and 600 µL/L of ethrel (2-chloroethyl phosphonic acid, 99.9 % a.i. CDH Bombay). The ethrel was applied on Alankar and PBM 16 cultivars of mustard (*Brassica juncea* L.) at 60 d after sowing (flowering stage). Alankar is a well adapted cultivar grown in the region, whereas PBM16 is a newly released cultivar. Ethrel was sprayed at a rate of 600 litre/ha (600 mL/plot) together with 0.5% teepol (a surfactant). In control group of plants, equal amount of de-ionized water with 0.5% teepol was sprayed.

The size of each plot was 10m² (2m X 5m). The seeds were sown by the usual behind the plough method at a rate of 10 kg/ha. Each treatment was replicated thrice. Irrigation was done once during the entire season of the crop. Spray of an insecticide (Dimecron-100) was done to check aphid contagion, if any.

The scheme of the treatments is summarized in the Table 2 and ANOVA is given in the Table 2a.

At 20 days interval i.e. 80 (pod fill), 100 (pod maturity) and 120d (harvest) after sowing, five plants from each plot were taken out with the help of hand hoe and various growth characteristics (Section 3.10) and biochemical characteristics (Section 3.12) were determined.

Physiological characteristics (Section 3.11) were studied at 80 and 100 d after sowing. At harvest (120 d after sowing), yield characteristics (Section 3.13) and quality characteristics (Section 3.14) were recorded.

3.10.2 Experiment 2

This experiment was also a factorial conducted simultaneously with Experiment 1 according to randomized complete block design, but the
Table 2. Scheme of treatments for Experiment 1 (1998-99)

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Ethrel (μL/L)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alankar</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBM16</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
| Crop      : Mustard (*Brassica juncea* L. Czern & Coss.)
           | grown under irrigated conditions |
| Treatment : Spray at 60d after sowing (flowering stage) and 20 days after harvest |
| Design    : Randomized complete block design |

Table 2a. Model of analysis of variance (ANOVA) of data from a 5x2 factorial experiment in randomized complete block design (Experiment 1)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.S.</th>
<th>F. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spray (S)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultivar (C)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction (S x C)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
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<tr>
<td>Total</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
experiment was carried out under non-irrigated conditions. The scheme of the treatments and other details of observations are same as in Experiment 1. The scheme of treatments is given in Table 3 and ANOVA in Table 3a.

3.10.3 Experiment 3

A factorial experiment conducted according to randomized complete block design during winter season of 1999-2000 under irrigated conditions. The aim of the experiment was to investigate the effect of leaf-applied 0, 100 or 200 μL/L ethrel (selected on the basis of Experiment 1) at 60d after sowing (flowering stage) on growth, physiological, biochemical, yield and quality characteristics of mustard (Brassica juncea L.) cultivar Alankar. The cultivar Alankar was selected based on its better performance than PBM 16 in Experiment 1. The plants were grown with 0, 40, 60 and 80 kg N/ha. The N doses were selected on the basis of recommendation for cultivation of mustard. Basal 80 kg N/ha is recommended dose and the other doses 60 and 40 kg N/ha are two sub-optimal doses.

The scheme of the treatments is summarized in the Table 4 and ANOVA is given in Table 4a. All other plant growing cultivation practices, including size of plots, sowing method, seed rate, number of irrigation, weeding and pest control operations were kept same as in Experiment 1.

Sampling was done at 80, 100 and 120 d after sowing to record growth, physiological, biochemical, yield and quality characteristics as described for Experiment 1. Among biochemical characteristics, nitrate reductase activity in leaves was also determined in this experiment.

3.10.4 Experiment 4

This experiment was carried out simultaneously with Experiment 3, but under non-irrigated conditions in the winter season of 1999-2000. The aim of this factorial randomized complete block design experiment was to study the effect of leaf-applied 0, 100 or 200 μL/L ethrel at 60 d after sowing (flowering stage) on mustard (Brassica juncea L.) cultivar Alankar grown with basal 0,
Table 3. Scheme of treatments for Experiment 2 (1998–99)

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alankar</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBM16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Crop: Mustard (*Brassica juncea* L. Czern & Coss.) grown under non-irrigated conditions

Treatment: Spray at 60d after sowing (flowering stage)

Design: Randomized complete block design

Table 3a. Model of analysis of variance (ANOVA) of data from a 5x2 factorial experiment in randomized complete block design (Experiment 2)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.S.</th>
<th>F. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spray (S)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultivar (C)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Interaction (S x C)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4  Scheme of treatments for Experiment 3 (1999–2000)

<table>
<thead>
<tr>
<th>Basal nitrogen levels (kg N/ha)</th>
<th>Ethrel (μL/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>N₀</td>
<td>+</td>
</tr>
<tr>
<td>N₄₀</td>
<td>+</td>
</tr>
<tr>
<td>N₆₀</td>
<td>+</td>
</tr>
<tr>
<td>N₈₀</td>
<td>+</td>
</tr>
<tr>
<td>Crop</td>
<td>Mustard (<em>Brassica juncea</em> L Czern &amp; Coss)</td>
</tr>
<tr>
<td>grown under irrigated conditions</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td><strong>Spray at 60d after sowing (flowering stage)</strong></td>
</tr>
<tr>
<td>Design</td>
<td>Randomized complete block design</td>
</tr>
</tbody>
</table>

Table 4a Model of analysis of variance (ANOVA) of data from a 3x4 factorial experiment in randomized complete block design (Experiment 3)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d f</th>
<th>S S</th>
<th>M S S</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spray (S)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen (N)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction (S x N)</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>Error</td>
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<td></td>
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<tr>
<td>Total</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
40, 60 and 80 kg N/ha on the plant characteristics described for Experiment 3. All practices of cultivation and observation details recorded at various growth stages are similar to Experiment 3. The scheme of treatments is summarized in the Table 5 and ANOVA is given in Table 5a.

Experiment 1 and 2 were conducted separately in one season in view of easy handling of the experiments, but for comparison of effect of ethrel spray treatments under irrigated and non-irrigated conditions analysis of some important data as group comparison was done. Similar analysis was carried out for Experiment 3 and 4.

3.10.5 Experiment 5

This factorial experiment was performed according to randomized complete block design during winter season of 2000-2001. Based on the findings of Experiment 3 and 4, application of 0 and 200 μL/L ethrel or 1mM silver thiosulphate (STS) on mustard (Brassica juncea L.) cultivar Alankar grown under irrigated and non-irrigated conditions was done at 60 d after sowing (flowering stage). A uniform basal application of 80 kg N/ha was given. The aim of this experiment was to confirm the findings on effects of ethrel on plant characteristics as found in Experiment 3 and 4. Because of this reason silver thiosulphate, which inhibits physiological action of ethylene was used in the experiment along with ethrel. Ethrel is a source of ethylene and its effects are manifested through physiological action of ethylene. The scheme of the treatment is summarized in the Table 6 and ANOVA is given in Table 6a.

All other plant growing cultivation practices, including size of plots, sowing methods, seed rate, number of irrigation, weeding and pest control operations were kept same as in Experiment 3 and 4.

Sampling was done at 80, 100, 120 d after sowing to assess the growth performance in terms of leaf area and dry weight per plant, physiological, yield and quality characteristics were studied. The details of the characteristics studied are given in the following pages.
Table 5 Scheme of treatments for Experiment 4 (1999–2000)

<table>
<thead>
<tr>
<th>Basal nitrogen levels (kg N/ha)</th>
<th>Ethrel (µL/L)</th>
<th>Ethrel (µL/L)</th>
<th>Ethrel (µL/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>N₀</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>N₄₀</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N₆₀</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N₈₀</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
| Crop                           | Mustard \(Brassica\ juncea\ \text{L.\ Czern\ &\ Coss.})
|                                | grown under non-irrigated conditions |
| Treatment                      | Spray at 60d after sowing (flowering stage) |
| Design                         | Randomized complete block design |

Table 5a Model of analysis of variance (ANOVA) of data from a 3x4 factorial experiment in randomized block design (Experiment 4)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d f</th>
<th>S.S</th>
<th>M S S</th>
<th>F. value</th>
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<tr>
<td>Replication</td>
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<td></td>
</tr>
<tr>
<td>Treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Spray (S)</td>
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<tr>
<td>Nitrogen (N)</td>
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<td></td>
</tr>
<tr>
<td>Interaction (S x N)</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Scheme of treatments for Experiment 5 (2000–2001)

<table>
<thead>
<tr>
<th>Irrigation</th>
<th>Spray treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Water</td>
</tr>
<tr>
<td>Irrigated</td>
<td>+</td>
</tr>
<tr>
<td>Non-irrigated</td>
<td>+</td>
</tr>
</tbody>
</table>

Crop: Mustard (*Brassica juncea* L. Czern & Coss.) cv. Alankar grown under irrigated and non-irrigated conditions.

Treatment: Spray at 60d after sowing (flowering stage).

Design: Randomized complete block design.

Table 6a. Model of analysis of variance (ANOVA) of data from a 3x2 factorial experiment in randomized complete block design Experiment 5

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.S.</th>
<th>F. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spray (S)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irrigation (I)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction (S x I)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.11 Biometric Observation

The observations were made at 20 days interval after spray treatment i.e., at 80 (pod fill), 100 (pod maturity) and 120 (harvest) d after sowing. At each sampling five plants from each plot were cut at the soil level, washed with water and dried with blotting paper to record growth, physiological and biochemical characteristics. At harvest, 12 plants, equipment to 1m² land area were removed to record the yield characteristics.

3.12 Growth Characteristics

The following growth characteristics were studied at 80, 100 and 120 d after sowing in Experiments 1-4.

1. Plant height
2. Plant leaf area
3. Leaf area index
4. Specific leaf area
5. Specific leaf weight
6. Plant dry weight
7. Dry weight of different plant parts
   (i) Leaf dry weight
   (ii) Stem dry weight
   (iii) Pod dry weight
8. Per cent distribution of dry weight
   (i) Leaf
   (ii) Stem
   (iii) Pod
9. Leaf fresh weight
10. Leaf turgid weight
11. Relative water content in leaf

In Experiment 5, the following growth characteristics were studied.

1. Plant leaf area
2 Plant dry weight

3.12.1 Plant leaf area

Leaf area was determined by gravimetric method. Leaf area of about 10\% of total leaves from each treatment was determined by tracing on a graph sheet and dry weight for these leaves was recorded. Leaf area/plant was computed by using leaf dry weight/plant and dry weight of those leaves for which the area was estimated (Watson, 1958) using the following formula:

\[
\frac{LA_1 \times W_2}{W_1} = LA
\]

LA \_1 = Leaf area of the leaves traced on graph paper
W \_1 = Dry weight of the leaves for which area was traced on graph paper
W \_2 = Total leaf dry weight/plant

3.12.2 Leaf area index

Leaf area index (LAI) was calculated on the formula suggested by Watson (1958):

\[
\frac{\text{Leaf area}}{\text{Ground area}} = \text{LAI}
\]

3.12.3 Specific leaf area

Specific leaf area (SLA) represents the leaf area of unit amount of leaf biomass. This was calculated as leaf area divided by leaf dry weight:

\[
\frac{\text{Leaf area}}{\text{Leaf weight}} = \text{SLA}
\]

3.12.4 Specific leaf weight

Specific leaf weight (SLW) is the measurement of allocation of leaf dry weight per unit of leaf area. It was calculated as leaf dry weight divided by leaf area.
3.12.5 Dry weight

Dry weight of different plant parts was recorded after drying them in an oven for 48 h at 80 °C.

3.12.6 Leaf fresh weight and turgid weight

The sampled plants were divided into different parts like leaf, stem and pod corresponding to different sampling stages. Leaf fresh weight was recorded and samples were placed in water and their turgid weight was recorded accordingly.

3.12.7 Leaf relative water content

Relative water content (RWC) of leaves was expressed as percentage of the water content of the fully turgid leaves and calculated as follows.

\[
\text{Relative water content (\%)} = \frac{W_f - W_d}{W_t - W_d} \times 100
\]

Here, 
- \( W_t \) = Weight of the fully turgid leaves
- \( W_f \) = Fresh weight of the leaves
- \( W_d \) = Dry weight of the leaves

3.13 Physiological Characteristics

Following physiological parameters were studied at 80 and 100 d after sowing in all experiments.

1. Rate of photosynthesis
2. Stomatal conductance
3. Internal CO\(_2\) concentration
4. Transpiration rate
5. Carboxylation efficiency
6. Photosynthetic water use efficiency
7. Plant water use efficiency
In Experiments 3-5, in addition to the above mentioned parameters following characteristics were also studied.

1. 1-Aminocyclopropane carboxylic acid (ACC) content
2. ACC oxidase
3. Ethylene evolution

3.13.1 Rate of photosynthesis

The data on rate of photosynthesis, stomatal conductance, internal CO$_2$ concentration and transpiration rate were measured in fully expanded top leaf of each main axis of plant using the Li COR–6200 Portable photosynthesis system (Nebraska, USA) at 1250 µ mol m$^{-2}$ s$^{-1}$ photosynthetically active radiation at 1100-1200 hours (temperature 23$^\circ$ C, relative humidity 72 %). The care was taken to use leaves of the same age for measurement of photosynthesis in control and treated plants. Each observation was replicated twice for control and treated plants and average of these was taken as a replicate.

3.13.2 Carboxylation efficiency

Carboxylation efficiency was computed by dividing the photosynthesis by internal CO$_2$ concentration and expressed in percentage.

\[
\text{Carboxylation efficiency (\%) } = \frac{\text{Photo}}{\text{Int}} \times 100
\]

3.13.3 Photosynthetic water use efficiency

Photosynthetic water use efficiency is a measure of CO$_2$ assimilation and has direct relation with rubisco activity (Vanden Boogard et al., 1996). It was expressed as the ratio of photosynthesis rate to stomatal conductance (A/gs) to avoid effects of small differences in vapour pressure between measurements (Von Cammerer and Farquhar, 1981).
3.13.4 Plant water use efficiency

Water use efficiency was calculated as the ratio of biomass to cumulative transpiration (Vanden Boogard et al., 1996).

\[
\text{Biomass} \quad \text{Plant water use efficiency} = \frac{\text{Biomass}}{\text{Transpiration}}
\]

3.13.5 Estimation of 1-aminocyclopropane-1-carboxylic acid (ACC)

At sampling times, 1 g of leaf sample was homogenized in 10 ml of 80% ethanol and then extracted under reflux with boiling ethanol for 30 min. It was then filtered through four layers of fine gauze and evaporated to dryness. The process was repeated with the addition of 0.5 ml chloroform. The residue was suspended in 2 ml double-distilled water, and then centrifuged for 20 min at 27000 g (Mc Keon et al. 1982). The ACC content in the aqueous extract was determined by its chemical conversion to ethylene after the addition of NaOCl (Lizada and Yang 1979). ACC content in the extract was estimated assuming that the percentage conversion of ACC to ethylene in the extract was identical to that of ACC added as an internal standard.

3.13.6 Assay of ACC oxidase activity

ACC oxidase activity was measured as ability of leaves to convert exogenous ACC to ethylene. Leaf sample (0.5 g) was cut into small pieces and incubated with 0.5 mL of 5 mM ACC. After flushing with air, the tubes were capped and incubated in light for 1 h under the same conditions used for plant growth (Vioque and Castellano, 1994). The ethylene evolved during incubation was determined on gas chromatograph (Nucon GLC 5700, India), as described in Section 3.11.3

3.13.7 Ethylene evolution

For ethylene measurement leaf material was trimmed to small pieces, weighed and placed in 30 ml tubes, which were stoppered with rubber secure cap and placed in light for 2 h under the same conditions as used for plant
growth. Ethylene content in the gas phase of tubes was determined from 1 ml samples were removed from the tubes and injected into a Nucon GLC 5700 gas chromatograph fitted with a flame ionisation detector and 1.8 m x 4 mm glass column packed with 80-100 mesh porapack-N. The oven temperature was 100 °C. The flow rates of nitrogen and hydrogen were 30 ml min⁻¹, and of oxygen was 300 ml min⁻¹. Ethylene identification was based on the retention time compared with a pure ethylene standard.

3.14 Biochemical Characteristics

Following parameters were studied:

1. Nitrate reductase activity
2. N content
3. N accumulation

Nitrate reductase activity in fresh leaves was estimated and dried plant material, collected at different sampling stages was used for the estimation of N content and its accumulation. The details of the estimation procedure are given in the following pages.

3.14.1 Assay of nitrate reductase activity

Enzyme nitrate reductase catalyses the reduction of nitrate to nitrite.

\[
\text{Nitrate reductase} \\
\text{NO}_3^- + \text{NADH} + \text{H}^+ \longrightarrow \text{NO}_2^- + \text{NAD} + \text{H}_2\text{O}
\]

Leaf nitrate reductase activity (NRA) was estimated \textit{in vivo} by the method of Jaworski (1971), which is based on the reduction of nitrate to nitrite. The nitrite formed was then determined spectrophotometrically.

Fresh leaf sample (200 mg) was transferred to polythene vials, containing 2.5 mL of phosphate buffer (pH 7.0) and 0.5 mL of 0.2 M potassium nitrate solution (Appendix) was added followed by addition of 2.5 mL of 5 % isopropanol (Appendix). Finally, 2 drops of 0.5 % chloramphenicol solution (Appendix) was added to avoid bacterial growth in the medium. These vials were incubated for 2 hours in dark at 30°C.
3.14.1.1 Colour development

Incubated mixture (0.4 mL) was taken in a test tube to which 0.3 mL of 1 % sulphanilamide (Appendix) and 0.02 % naphthylethlenediamine hydrochloride (NED–HCl) were added. The test tube was left for 20 minutes for maximum colour development. The mixture was diluted again to 5 mL with sufficient amount of double distilled water. Then absorbance was read at 540 nm using a blank on spectrophotometer (SL171 Elico, Hyderabad, India).

3.14.1.2 Standard curve for NRA

30 mg of sodium nitrite (NaNO₂) was dissolved in 100 mL double distilled water. From this solution, various amounts was taken in ten different test tubes, viz., 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mL. To these 0.3 mL of 1 % sulphanilamide and 0.02 % NED–HCl were added. The solutions were diluted to 5 mL with double distilled water and the absorbance was read at 540 nm, using a blank on spectrophotometer (SL171 Elico, Hyderabad, India).

3.14.2 Nitrogen content

3.14.2.1 Digestion of plant samples for N content

Dried plant material was powdered and passed through 70- mesh screen and stored in polythene vials. This plant material was used for the estimation of N content.

100 mg of the oven dried powder from each replicate was transferred to a 50 mL Kjeldahl flask to which 2 mL of sulphuric acid was added. The content of the flask was heated on temperature-controlled assembly for about 2 hours to allow complete reduction of nitrates present in the plant material by organic matter itself. As a result, the contents of the flask were turned black. After cooling the flask for about 15 minutes, 0.5 mL of 30 % H₂O₂ was added drop by drop and the solution was heated again until the colour turns from black to light yellow. Again after cooling for 30 minutes an additional 3-4 drops of 30 % H₂O₂ were added, followed by heating for another 15 minutes.
The process was repeated until the contents of the flask turned colourless. The peroxide digested material was transferred from Kjeldahl flask to 100 mL volumetric flask with three washings of double distilled water. The volume of the flask was made up of to the mark with double distilled water. This peroxide digested material was used for the estimation of N content.

3.14.2.2 Estimation of nitrogen

Nitrogen was estimated according to Lindner (1944). A 10 mL aliquot of the digested material was taken in 50 mL volumetric flask. To this, 2 mL of 2.5 N NaOH and 1 mL of 10% sodium silicate solution were added which neutralizes excess of acid and prevents turbidity. The volume of the solution was made up to the mark with double distilled water. In a 10 mL graduated test tube, 5 mL of this solution was taken and 0.5 mL of Nessler’s reagent was added. The final volume was made up with double distilled water. The contents of the tube were allowed to stand for 5 minutes for maximum colour development and absorbance was read at 525 nm on spectrophotometer (SL171 Elico, Hyderabad, India).

3.14.2.2.1 Standard curve for nitrogen

50 mg ammonium sulphate was dissolved in 1 litre double distilled water. From this solution, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml were pipetted to ten different test tubes. The solution in each test tube was diluted to 5 mL with double distilled water. In each test tube, 0.5 mL Nessler’s reagent was added and after 5 minutes, the absorbance was read at 525 nm on spectrophotometer (SL171 Elico, Hyderabad, India). A blank was run with each set of determination. Standard curve was plotted using different concentrations of ammonium sulphate solution versus absorbance and with the help of this standard curve the amount of nitrogen present in the sample was determined.
3.14.3 N accumulation

Nitrogen content of the plant at different sampling stages and their respective dry matter at these stages were used (as a product) to calculate the N accumulation.

3.15 Yield Characteristics

Following parameters were recorded at harvest.

1. Number of pods per plant
2. Number of seeds per pod
3. 1000 seed weight
4. Seed yield
5. Biological yield
6. Harvest index
7. Oil yield

In addition to the above parameters, following parameters were also included in the study in Experiments 3-5 because these experiments included nitrogen as a treatment.

1. Seed N
2. Nitrogen harvest index
3. Nitrogen yield potential

3.15.1 Number of pods per plant

At harvest, 12 plants from each treatment equivalent to 1 m$^2$ land were removed. The pods were collected and counted.

3.15.2 Number of seeds per pod

Seeds of 12 pods from each plant in a treatment were collected and counted.

3.15.3 1000 seed weight

From the produce of the plot, 1000 seeds were randomly drawn and the weight was recorded.
3.15.4 Seed yield

Total seeds from one-meter square area of the plot were cleaned and weighed to compute the seed yield.

3.15.5 Biological yield

Total biological yield from one-meter square area was recorded from sun-dried samples before threshing.

3.15.6 Harvest index

Harvest index was computed by dividing the seed yield with biological yield and expressed in percentage.

\[
\text{Harvest index (\%) = \frac{\text{Seed yield}}{\text{Biological yield}}} \times 100
\]

3.15.7 Seed N content per plant

Seed N content was determined as a product of N concentration and the dry weight of the seed.

3.15.8 Nitrogen harvest index

Nitrogen harvest index is a measure of distribution of nitrogen from vegetative part to seed. This was calculated by dividing the seed N with plant N.

\[
\text{Nitrogen harvest index} = \frac{\text{Seed N}}{\text{Plant N}}
\]

3.15.9 Nitrogen yield potential

Nitrogen yield potential denotes the efficiency of plants to mobilize total N contained in plants to seed during pod fill. This was calculated as a product of nitrogen harvest index and seed N i.e.

\[
\text{Nitrogen yield merit} = \text{Nitrogen harvest index} \times \text{Seed N}
\]
3.15.10 Oil yield

The per cent oil content in seeds when multiplied with seed yield gave the oil yield.

3.16 Quality Characteristics

The seed samples were crushed to get a fine meal for extracting the oil after separating them from extraneous material. The oil was analysed for following quality parameters.

1. Oil content
2. Acid value
3. Iodine value
4. Saponification value

3.16.1 Determination of oil content

25 g of ground seeds meal was transferred to a Soxhlet apparatus and sufficient quantity of petroleum ether was added. The apparatus was kept on a hot water bath running at 60 °C for about 6 h, for extraction of oil. Petroleum ether from the extracted oil was evaporated after some time. The extracted oil was expressed as a percentage by mass of the seeds and was calculated by the following formula:

$$\text{Oil content (\%) = \frac{m_0}{m_S} \times 100}$$

Here, $m_0$ = Sum of the mass of oil

$m_S$ = Seed sample mass

3.16.2 Determination of acid value

Acid value of oil is the amounts of potassium hydroxide spend to neutralize free acid in one gram of oil. It was determined by the following method (Anonymous, 1970).

2 g of oil was dissolved in 50 mL solvent mixture of 95 % alcohol and diethyl ether (1:1) in a 250 mL conical flask. Titration was carried out with 0.1
N potassium hydroxide. Phenolphthalein was used as an indicator and the amount of mL ‘a’ of 0.1 N NaOH required was noted. The acid value was calculated by the following formula.

\[
\text{Acid value} = \frac{a \times 0.05661 \times 1000}{W}
\]

Here, \(a\) = ml of 0.1N KOH used in titration

\(W\) = weight of oil

**3.16.3 Determination of iodine value**

Iodine value of oil is the number of gm of iodine absorbed by 100 gm of oil and expressed as the weight of iodine. It was determined by using iodine monochloride method described below (Anonymous, 1970).

Oil (2 gram) was taken in a dry ground neck flask to which 10 mL carbon tetrachloride and 20 mL iodine monochloride solution were added. The flask was stopper and allowed to stand in a dark place for about 30 minutes. After 30 minutes, 15 mL potassium iodide and 100 mL double distilled water was poured into it with proper shaking. Titration was carried out with 0.1 N sodium thiosulphate (Na\(_2\)S\(_2\)O\(_3\)) solution using starch solution as an indicator. Number of mL ‘a’ of sodium thiosulphate used was noted. For blank, similar operation was put in practice without the oil and the number of ml ‘b’ of 0.1 N sodium thiosulphate solution used was noted. Iodine value was calculated by the following formula (Anonymous, 1970).

\[
\text{Iodine value} = \frac{(b - a) \times 0.01269 \times 100}{W}
\]

Here, \(a\) = number of mL of 0.1 N Na\(_2\)S\(_2\)O\(_3\) solution used in the sample

\(b\) = number of mL of 0.1 N Na\(_2\)S\(_2\)O\(_3\) solution used in blank

\(W\) = weight of oil
3.16.4 Determination of saponification value

Saponification value of oil is the amount of mg of KOH consumed by 1gm of the oil to neutralize the fatty acid resulting from complete hydrolysis.

2 g of oil was taken in a 250 mL conical flask to which 25 mL of 0.5 N KOH was added. The flask was attached with reflux condenser and heated on water bath for about 1h with frequent rotation of the contents of the flask. The excess of alkali was titrated with 0.5 N HCl. The number of mL (a) of 0.5 N HCl was noted. A similar practice was repeated without oil and the number of mL (b) of 0.5 N HCl required was noted (Anonymous, 1970). Saponification value was calculated by the following formula:

\[
\text{Saponification value} = \frac{(b - a) \times 0.02805 \times 1000}{W}
\]

Where a and b are number of mL of 0.05 N HCl used in the sample and blank titration respectively, and W is weight of oil

3.17 Statistical Analysis

All the experimental data were subjected to statistical analysis by adopting analysis of variance techniques according to the design of the experiments (Gomez and Gomez, 1984) and the significance of the results were determined at 5% levels of probability. Pooled analysis of Experiments 1 and 2 and Experiments 3 and 4 was performed from a split plot design to evaluate the effect of combinations of factors. If the data were found significant, Least Significant Difference (LSD) was calculated. Correlation values between various traits were also worked out.