CHAPTER - THREE

MATERIALS AND METHODS
The details of materials used and techniques adopted during the course of present investigations are presented in this chapter.

3.1 EXPERIMENTAL MATERIAL

Rapeseed-mustard (*Brassica juncea* cv. Pusa Jai Kisan and *Brassica campestris* cv. Pusa Gold) were used as experimental materials. Seed material of above cultivars was obtained from National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi.

3.2 CHEMICALS

All the chemicals used were of AR or GR quality. Most of the chemicals were the products of BDH, IDPL, E. Merck, Aldrich-Sigma, Qualigen, S.d.fines and Loba.

3.3 EXPERIMENTAL SITE

The field experiments were conducted at the experimental field of Hamdard University, New Delhi, India.
3.4 SOIL CHARACTERISTICS

Soil samples were collected from various randomly chosen spots spread over the entire experimental field before sowing of the experimental crops and subjected to mechanical and chemical analysis for studying physical and chemical characteristics of soil. Data obtained on particle size and chemical characteristics for the 0-30 cm soil layer and physical constants for soil are presented in table 1.

3.5 CLIMATE AND WEATHER CONDITIONS

New Delhi is situated 28.38° N latitude and 77.11° E longitude at an altitude of 228 m above the mean sea level. It has a semiarid and subtropical climate with extremes of hot weather in summer and cold weather in winter. There is gradual decrease in daily temperature from October to January reaching as low as 8-10°C. There is also a gradual increase in temperature from February to April. Occasionally, frost do occur during December to January period.

The mean annual rainfall is about 650 mm of which about 80% is received during a short span of three months from July to September. Winter showers sometimes are accompanied with high wind velocity and hale storm. The annual pan evaporation is about 850 mm with mean daily evaporation ranging from as low as 2.2 mm in the month of January to as high as 16.0 mm in the month of June. The relative humidity reaches maximum in January and minimum in April during the rabi season.
TABLE 1. PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE SOIL OF EXPERIMENTAL FIELD

<table>
<thead>
<tr>
<th>PARTICULAR</th>
<th>VALUES</th>
<th>METHODS EMPLOYED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mechanical analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Sand (%)</td>
<td>63.8</td>
<td>Hydrometer method</td>
</tr>
<tr>
<td>ii. Silt (%)</td>
<td>18.8</td>
<td>(Bouyoucos, 1962)</td>
</tr>
<tr>
<td>iii. Clay (%)</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>2. Chemical composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Organic carbon (%)</td>
<td>0.39</td>
<td>Walky and Black, 1934</td>
</tr>
<tr>
<td>ii. Available P (μ g g$^{-1}$ soil)</td>
<td>48.0</td>
<td>Olsen et al., 1964</td>
</tr>
<tr>
<td>iii. Available K (μ g g$^{-1}$ soil)</td>
<td>110.2</td>
<td>Flame photometric method (Jackson, 1967)</td>
</tr>
<tr>
<td>iv. Available S (%)</td>
<td>0.001</td>
<td>(William and Steinbergs, 1959)</td>
</tr>
<tr>
<td>v. Available N (%)</td>
<td>0.069</td>
<td>(Subbiah and Asija, 1959)</td>
</tr>
<tr>
<td>vi. pH (Soil:water) (1 : 2.5)</td>
<td>7.8</td>
<td>Control Dynamic pH meter (Jackson, 1967)</td>
</tr>
</tbody>
</table>
3.6 EXPERIMENTAL DETAILS

The details of the experiments are as follows:

3.6.1 TREATMENTS

The fertilizer treatments in 1995-96 included three levels of sulphur viz., 0, 40 and 60 kg ha\(^{-1}\) and two levels of nitrogen viz., 60 and 100 kg ha\(^{-1}\) in the following combinations:

- \(T_1\) : \(S_0 N_{100}\)
- \(T_2\) : \(S_{40} N_{60}\)
- \(T_3\) : \(S_{40} N_{100}\)
- \(T_4\) : \(S_{60} N_{100}\)

Treatments, \(T_2\), \(T_3\) and \(T_4\) were applied in two and three splits, designated as \(S_2\) and \(S_3\). Two splits consisted of 1/2+1/2 dose of S and N, while three splits consisted of 1/2, 1/4 and 1/4 dose of S and N. The treatment, \(T_1\) was applied in two splits.

The combinations of S and N in 1996-97 experiment were as follows:

- \(T_3\) : \(S_{40} N_{100}\)
- \(T_4\) : \(S_{60} N_{100}\)
- \(T_5\) : \(S_{60} N_{150}\)

In the treatment \(T_3\), S was applied in single basal dose (\(S_1\)), two (\(S_2\)) and three splits (\(S_3\)), while N in two and three splits. The treatment, \(T_4\) was applied in two equal splits. In the treatment \(T_5\), S was applied in two equal splits and N in three equal splits.
3.6.2 EXPERIMENTAL DESIGN AND LAYOUT OF THE FIELD EXPERIMENTS

The field experiments were laid out in randomized block design with three replications of each treatment. The individual plot size was 16 m² (4m x 4m) having 10 rows with a row to row distance of 30 cm and plant to plant distance of 15 cm.

3.6.3 CHARACTERS OF THE GENOTYPES USED

3.6.3.1 BRASSICA JUNCEA CV. PUSA JAI KISAN (V₁)

This variety has been developed by the National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi through tissue culture method. The crop duration is 115-118 days. Plant height is about 180 cm. Seed size is bold (about 6g/1000 seed) and colour is blackish-brown. Average yield per hectare is 20 quintals.

3.6.3.2 BRASSICA CAMPESTRIS CV. PUSA GOLD (V₂)

This variety has been developed by the Division of Genetics and National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi through inter-varietal hybridization. It is early maturing variety with a life span of 100-105 days. Plant height is about 140 cm. Pods are tetralocular and contain 30-45 seeds. Seeds are medium bold and bright yellow in colour. Average yield is 18 q ha⁻¹ and oil content is 45%.
3.6.4 CULTURAL OPERATIONS

Various field operations carried out during the experimental period is presented in Table 2.

3.6.4.1 PREPARATORY TILLAGE

The fields were ploughed and levelled prior to sowing. The plots were then made with proper bunds along with necessary irrigation channels. During both the years of experimentation, a pre-sowing irrigation was given.

3.6.4.2 FERTILIZER APPLICATIONS

The recommended doses of phosphorus and potassium, at the rate of 40 kg ha⁻¹ each, were given to all plots as basal dressings. The basal dressing also included the first split of nitrogen and sulphur. Second dose of nitrogen and sulphur was given at 40 days after sowing and third dose after 55 days of sowing. The sources of nitrogen, sulphur, potassium and phosphorus were urea, gypsum, muriate of potash and single super phosphate, respectively.

3.6.4.3 SOWING

Seeds were sown at the rate of 6 kg ha⁻¹ in rows, with inter row distance of 30 cm.

3.6.4.4 THINNING

After two weeks of sowing, seedlings were thinned to keep an intra-row spacing of 15 cm.
<table>
<thead>
<tr>
<th>OPERATIONS</th>
<th>DATE OF OPERATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1995-96</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1. PREPARATORY TILLAGE</td>
<td></td>
</tr>
<tr>
<td>a. Ploughing</td>
<td>02-10-95</td>
</tr>
<tr>
<td>b. Dishing twice and leveling</td>
<td>06-10-95</td>
</tr>
<tr>
<td>2. LAYOUT AND SOWING</td>
<td></td>
</tr>
<tr>
<td>a. Layout</td>
<td>10-10-95</td>
</tr>
<tr>
<td>b. Sowing</td>
<td>12-10-95</td>
</tr>
<tr>
<td>3. FERTILIZER APPLICATION</td>
<td></td>
</tr>
<tr>
<td>a. First dose</td>
<td>11-10-95</td>
</tr>
<tr>
<td>b. Second dose</td>
<td>20-11-95</td>
</tr>
<tr>
<td>c. Third dose</td>
<td>05-12-95</td>
</tr>
<tr>
<td>4. THINNING</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31-10-95</td>
</tr>
<tr>
<td>5. WEEDING</td>
<td></td>
</tr>
<tr>
<td>a. Basalin (pre-pl incorporation)</td>
<td>10-10-95</td>
</tr>
<tr>
<td>b. Manual weeding</td>
<td></td>
</tr>
<tr>
<td>I.</td>
<td>30-10-95</td>
</tr>
<tr>
<td>II.</td>
<td>15-11-95</td>
</tr>
<tr>
<td>6. PLANT PROTECTION</td>
<td></td>
</tr>
<tr>
<td>a. Dusting of BHC (10%)</td>
<td>11-10-95</td>
</tr>
<tr>
<td>b. Spraying of Metasystox (1.0%)</td>
<td>10-12-95</td>
</tr>
<tr>
<td>7. SAMPLING</td>
<td></td>
</tr>
<tr>
<td>a. First sampling</td>
<td>13-11-95</td>
</tr>
<tr>
<td>b. Second sampling</td>
<td>29-11-95</td>
</tr>
<tr>
<td>c. Third sampling</td>
<td>15-12-95</td>
</tr>
<tr>
<td>d. Fourth sampling</td>
<td>31-12-95</td>
</tr>
<tr>
<td>e. Fifth sampling</td>
<td>16-01-96</td>
</tr>
<tr>
<td>f. Sixth sampling</td>
<td>31-01-96</td>
</tr>
<tr>
<td>8. HARVESTING</td>
<td></td>
</tr>
<tr>
<td>Brassica juncea cv. Pusa Jai Kisan</td>
<td>25-02-96</td>
</tr>
<tr>
<td>Brassica campestris cv. Pusa Gold</td>
<td>18-02-96</td>
</tr>
<tr>
<td>9. THRESHING</td>
<td></td>
</tr>
<tr>
<td>Brassica juncea cv. Pusa Jai Kisan</td>
<td>05-03-96</td>
</tr>
<tr>
<td>Brassica campestris cv. Pusa Gold</td>
<td>30-02-96</td>
</tr>
</tbody>
</table>
3.6.4.5 PLANT PROTECTION

In order to keep the experimental field free of weeds, pre-plant incorporation of 
basailin (fluchloralin) at the rate of 10 kg a.i. ha\(^{-1}\) was done. Hand weeding was done 
twice during the entire crop season. In order to control aphids, spraying of Metasystox 
(1.0 \%) was done at the time of fruiting.

3.6.4.6 IRRIGATION

One uniform irrigation was done before sowing and subsequently, whenever needed.

3.6.4.7 HARVESTING AND THRESHING

Plants from the 1 m\(^2\) area were harvested by cutting at the ground level and were 
allowed for sun drying \textit{in situ}. After sun drying, threshing was done, seeds were cleared 
and seed yield recorded.

3.7 BIOMETRIC OBSERVATIONS

The experimental plot had 10 rows with row to row spacing of 30 cm. One m\(^2\) area of each plot was earmarked permanently with wooden pegs for the purpose of 
harvest, dry matter yield and analysis of seed yield and its components. The remaining 
area (except the border rows) was used for taking periodic plant samples. The sampling 
was done at 30, 45, 60, 75, 90, 105 days after sowing and at harvest (HR). Three plants 
were taken from each plot, randomly. The samples were cut at root-shoot junction and 
brought to the laboratory, where analysis of growth parameters was carried out using 
appropriate protocols.
3.7.1 GROWTH PARAMETERS AND PHYSIOLOGICAL ANALYSIS

3.7.1.1 PLANT STAND

In each plot, the population count was made at 30 days after sowing from a spot of one meter square. The number of plants in one meter square area was 18.

3.7.1.2 DRY MATTER

Three plants at random were sampled from each plot after 30 days of sowing at regular interval of 15 days during different stages of growth. The plants so selected were cut at root shoot junction and divided into various plant parts like leaves, stem and pods, etc. according to the stage of growth. The separated plant parts were dried in hot air oven at 65 ± 2°C for 48 hours and later were weighed on top pan balance. The final constant weight was recorded as dry matter, g. pl⁻¹.

3.7.1.3 LEAF AREA

Leaf area of green leaves of the plants, sampled for analysis of growth parameters, was measured with leaf area meter (Model LI COR 3000, USA).

3.7.1.4 LEAF AREA INDEX (LAI)

Leaf area index expresses the ratio of leaf surface (one side only) to the ground area occupied by the crop. It was calculated using the following equation:

\[ \text{LAI} = \frac{(L_{A_2} + L_{A_1})}{2 \cdot (1/G_{A})} \]

Where,
- \( L_A \) = Leaf area
- \( G_A \) = Ground area
3.7.1.5 LEAF AREA DURATION (LAD)

Leaf area duration expresses the magnitude and persistence of leaf area or leafiness during the period of crop growth. It was calculated using the following equation and expressed in days:

\[ \text{LAD} = \frac{(\text{LA}_2 + \text{LA}_1)(T_2 - T_1)}{2} \]

Where,

\[ \text{LA} = \text{Leaf area} \]
\[ T = \text{Time} \]

3.7.1.6 CROP GROWTH RATE (CGR)

Crop growth rate (CGR) is the dry matter accumulation per unit of land area per unit of time. It was measured employing following equation and expressed in g m\(^{-2}\) day\(^{-1}\).

\[ \text{CGR} = \frac{W_2 - W_1}{T_2 - T_1} \]

Where,

\[ W_1 = \text{weight of dry matter m}^2\text{ at the time } T_1 \]
\[ W_2 = \text{weight of dry matter m}^2\text{ at the time } T_2 \]

3.7.1.7 RATE OF PHOTOSYNTHESIS

The rate of photosynthesis of green leaves was recorded throughout the growth period at periodic intervals using Infra Red Gas Analyzer, IRGA (Model LI COR 6200, USA).
3.7.1.8 ESTIMATION OF CHLOROPHYLL

Chlorophyll a, b and total chlorophyll were extracted by non-maceration of tissue using dimethylsulphoxide (DMSO) by the method of Hiscox and Israelstam (1979).

3.7.1.8.1 CHLOROPHYLL EXTRACTION

Freshly harvested leaves (100 mg) were finely chopped and collected in test tubes containing 7.0 ml DMSO. The test tubes were covered with black paper and incubated at 65°C for 40 minutes. The reaction mixture was transferred to a graduated tube and the final volume was made up to 10.0 ml by adding DMSO. The chlorophyll content was then measured immediately or the reaction mixture without leaves was stored at 4°C in dark until analyzed.

3.7.1.8.2 CHLOROPHYLL ESTIMATION

3.0 ml sample of chlorophyll extract was transferred to cuvette and the absorbance was taken at 645 and 663 nm on uv-vis spectrophotometer (Model DU 640B, Beckman, USA). The chlorophyll content was expressed as mg g⁻¹ fw.

3.7.1.8.3 CALCULATION OF CHLOROPHYLL CONTENT

Chlorophyll content was calculated following the equation given by Arnon (1949).

\[
\text{Chlorophyll a (mg g}^{-1} \text{ fw) = } \frac{(12.7 \times \text{OD}_{663}) - (2.69 \times \text{OD}_{645}) \times V}{1000 \times W} \\
\text{Chlorophyll b (mg g}^{-1} \text{ fw) = } \frac{(22.9 \times \text{OD}_{645}) - (4.68 \times \text{OD}_{603}) \times V}{1000 \times W}
\]

Where, \( V \) = volume of the extract
\( W \) = weight of the tissue taken
3.7.1.9 IN VIVO NITRATE REDUCTASE (NR) ASSAY

The in vivo assay of NR activity in the leaves was done according to the procedure of Hageman and Hucklesby (1971).

The leaves were cut into 2 mm slices and placed in ice cold incubation medium containing 3.0 ml of 0.2 M potassium phosphate buffer (pH-6.8) and 3.0 ml of 0.4 M KNO₃ solution. The tubes were evacuated with a vacuum pump and then incubated in a water bath at 33°C for one hour under dark conditions. At the end of incubation period, tubes were placed in boiling water bath for 5 minutes to stop the enzyme activity and the complete leaching of the nitrite in the medium.

Nitrite was estimated by the method of Evans and Nason (1953). 0.2 ml of the aliquot from reaction mixture was taken and 1.0 ml each of 1.0 per cent sulphanilamide in 1N-HCl and 0.02 per cent N-(1-Naphthyl)-ethylene diammonium dichloride (NEDD) in distilled water were added. The pink colour due to diazotization was allowed to develop for 30 minutes after which the volume was made upto 6.0 ml with water. The absorbance was read at 540 nm, using uv-vis spectrophotometer (Model DU 640B, Beckman, USA). The calibration curve was prepared using sodium nitrite solution. The enzyme activity was expressed as μmole NO₃ g⁻¹ fw h⁻¹.

3.7.1.10 IN VITRO ATP-SULPHUROLASE ASSAY

In vitro ATP-sulphurylase assay was done following the method of Wilson and Bandurski (1958).
3.7.1.10.1 EXTRACTION

Extracts were prepared by grinding 1 gm of plant material in 10 ml of 0.1 M Tris-HCl (pH-8.0) containing 2.0 mM MgCl₂, 100 mM KCl, 10 mM DTE and 10 mM ouabain in a glass homogenizer cooled with ice. The homogenate was centrifuged for 10 minutes at 10,000 rpm at 4°C.

3.7.1.10.2 COLORIMETRIC MOLYBDOLYSIS ASSAY

3.7.1.10.2.1 Preparation of reagents

A. REACTION MIXTURE

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified water (Phosphate &amp; sulphate free)</td>
<td>8 ml</td>
</tr>
<tr>
<td>0.4 mM Tris-Buffer (pH - 8.0)</td>
<td>3 ml</td>
</tr>
<tr>
<td>40 mM MgCl₂.6H₂O</td>
<td>4 ml</td>
</tr>
<tr>
<td>Na₂ATP</td>
<td>45 mg</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>40 mg</td>
</tr>
<tr>
<td>Inorganic Pyrophosphatase solution</td>
<td>20 µl</td>
</tr>
<tr>
<td>(Sigma ; Package of 514 units mg⁻¹ protein</td>
<td></td>
</tr>
<tr>
<td>dissolved in 200 µl water)</td>
<td></td>
</tr>
</tbody>
</table>

B. STOP SOLUTION (pH 4.0)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Sodium acetate</td>
<td>18 ml</td>
</tr>
<tr>
<td>0.5 M Acetic acid</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

C. REDUCING SOLUTION

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-amino-2-naphthol-4-sulphonic acid</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Na₂SO₃.7H₂O</td>
<td>3.0 gm</td>
</tr>
<tr>
<td>Na₂S₂O₃</td>
<td>6.0 gm</td>
</tr>
</tbody>
</table>

The above chemicals were mixed in a mortar and stored at 2°C in the dark. 0.25 gm of this mixture was prepared fresh in 10 ml double distilled water.
3.7.1.10.2.2 Assay Procedure

The assay was started by adding 0.1 ml of extract to 0.4 ml of the reaction mixture. Incubation was done at 30°C for 10 minutes. The reaction was stopped by adding 1 ml of stop solution. Control was prepared by substituting NaCl in place of sodium molybdate.

\[
\text{ATP-SULPHURYLASE} \quad \text{ATP} + \text{SO}_4^{2-} \rightleftharpoons \text{APS} + \text{PPi}
\]

\[
\text{INORGANIC PYROPHOSPHATASE} \quad \text{PPi} \rightarrow 2\text{Pi}
\]

The inorganic phosphate was estimated by the procedure of Fiske and Subba Row (1925). 1.0 ml of 5N H₂SO₄, 0.5 ml of 2.5 % ammonium molybdate and 0.1 ml of reducing solution were added to the aliquot. The volume was made upto 10 ml by adding double distilled water. After lapse of 20 minutes, the absorbance was measured at 660 nm using uv-vis spectrophotometer (Model DU 640B, Beckman, USA). The calibration curve was prepared using KH₂PO₄ solution. The enzyme activity was expressed as \( \mu \) mole Pi mg⁻¹ protein min⁻¹.

3.7.1.11 ESTIMATION OF SOLUBLE PROTEIN CONTENT

Soluble protein content was estimated in leaves by the method given by Bradford (1976).

3.7.1.11.1 Preparation of Bradford's Reagent

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commassie Brilliant Blue G-250</td>
<td>100 mg</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>50 ml</td>
</tr>
<tr>
<td>O-phosphoric acid (85%, w/v)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Final volume with double distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Final concentrations in the reagent were 0.01 % (w/v) Commassie Brilliant Blue G-250, 4.75 % (w/v) ethanol and 8.5 % (w/v) o-phosphoric acid.

3.7.1.11.2 Procedure

To 0.5 ml of the crude extract, 1 ml of 10 % TCA was added and allowed to age for 30 minutes in ice. After centrifugation at 5,000 rpm for 5 minutes, the pellet obtained was washed with 1 ml of 5 % TCA. The precipitate was then dissolved in 0.1N NaOH. To 0.1 ml of aliquot, 5.0 ml Bradford’s reagent was added and mixed vigorously. A blue colour develop within 2 minutes. The absorbance was taken at 595 nm with uv-vis spectrophotometer (Model DU 640B, Beckman, USA). Calibration curve was drawn using different concentrations of bovine serum albumin, treated similarly as that of aliquots to calculate protein content. The protein content was expressed as mg g⁻¹ fw.

3.7.2 Chemical Analysis of Dry Matter

3.7.2.1 Estimation of Reduced Nitrogen

The method followed for reduced nitrogen estimation was given by Linder (1944). It consisted of the two main steps: (i) the acid digestion of plant material, and (ii) the determined of nitrogen as ammonia content in digested samples.

3.7.2.1.1 Digestion

The plant samples were digested according to a modified version of Kjeldahl procedure. 100 mg finely ground samples dried at 80°C were taken in digested tubes of 75 ml capacity. The 4.0 ml of acid mixture and a digestion tablet was added in each digestion tube. The acid mixture consisted of concentrated sulphuric and o-phosphoric acid of AR quality in the ratio of 2:1. Each digestion
tablet contained 1.5 g of potassium sulphate and 0.0075 g of selenium as catalyst. The digestion was carried out on a Tecator digestion unit at a temperature between 250 to 300°C. After the sample was near to complete digestion, 1.0 ml of 30 % (v/w) hydrogen peroxide was added drop by drop to each tube. Again tubes were kept for some time on the digestion unit till the digested solution became colourless. Subsequent to digestion, the volume were made upto 75 ml with distilled water. 40 samples were digested at a time and with each of 40 samples there was one check material of known nitrogen content.

3.7.2.1.2 Determination of Nitrogen

3.7.2.1.2.1 Preparation of Nessler's reagent

Solution A : 100 gm NaOH in 400 ml double distilled water

Solution B : 100 gm HgI₂ + 70 gm KI in 400 ml double distilled water

Solution A was mixed with cooled solution B with continuous stirring. The volume was made upto 1000 ml with double distilled water and mixture was kept at room temperature for 24 hours. The solution was filtered off to make it free from visible suspension or precipitates. Clear solution was used as Nessler's reagent.

3.7.2.1.2.2 Procedure

To 0.5 ml of aliquot, 2.0 ml of Nessler's reagent was added and final volume was made upto 5.0 ml by adding 2.5 ml double distilled water. The mixture was kept for 20 minutes at room temperature for colour development. Absorbance was taken at 440 nm using uv-vis spectrophotometer (Model DU-640B, Beckman, USA). Calibration curve was prepared from acidic ammonium sulphate solutions of different concentrations to calculate nitrogen content. The N content was expressed on percentage N concentration basis.
3.7.2.2 ESTIMATION OF TOTAL SULPHUR

The estimation of total sulphur in plant samples was carried out by Turbidimetric method of Chesnin and Yien (1950). The method consisted of two main steps:

(i) digestion, and (ii) turbidity development.

3.7.2.2.1 DIGESTION

100 mg finely ground samples dried at 80°C, were taken in digestion tubes of 75 ml capacity. The 4.0 ml of acid mixture and 0.0075 g of selenium dioxide (used as catalyst) was added in each digestion tube. The acid mixture consisted of concentrated nitric acid and perchloric acid of AR quality in the ratio of 1:1. The digestion was carried out on a Tecator digestion unit at a temperature between 250 to 300°C till the digested solutions became colourless. 40 samples were digested at a time and with each of 40 samples there was one check material of known sulphur content. Subsequent to digestion, the volume were made up to 75 ml with distilled water. Care was taken to check the interference of silica by filtering the contents of the tubes.

3.7.2.2.2 DETERMINATION OF SULPHUR

A suitable aliquot was pipetted out from the digested solutions for turbidity development in 25 ml volumetric flasks. Turbidity was developed by adding 2.5 ml gum acacia (0.25%) solution, 1 g barium chloride (BaCl₂, sieved through 40-60 mesh) and the volume was made up with double distilled water to the mark. Contents of 25 ml volumetric flasks were thoroughly shaken till BaCl₂ was completely dissolved. Turbidity was allowed to develop for 2 minutes. The value were recorded at 415 nm, with uv-vis spectrophotometer (Model DU-640B, Beckman, USA) within 10 minutes
after the turbidity development. The amount of sulphate was calculated with the help of a calibration curve prepared afresh with a series of $K_2SO_4$ solutions, treated similar to that of aliquots, described as above. The S content was expressed in percentage S concentration basis.

3.7.2.3 ESTIMATION OF OIL CONTENT

Oil content is defined as the whole of the substances extractable by n-hexane under specified conditions. The oil content was estimated by two methods:

(ii) Rapid Gravimetric Determination  (ii) Pulse Nuclear Magnetic Resonance Method.

3.7.2.3.1 RAPID GRAVIMETRIC DETERMINATION

The rapid gravimetric determination of oil content was done by cold percolation as described by Kartha and Sethi (1957).

0.3 gm of seed was weighed and transferred to a glass mortar. 0.2 gm each of glass powder (Pyrex glass washed with concentrated hydrochloric acid) and anhydrous sodium sulphate were added and the mixture reduced to fine powder. The mixture was transferred to a small glass percolator, 20 cm long and 1.5 cm in diameter. The mortar and pestle were washed twice with 0.5 gm of anhydrous sodium sulphate and the washings were also packed over the seed powder. Finally, the mortar and pestle were washed with 3 ml of freshly distilled petroleum ether (b.p. 70-90°C) and this was transferred to the packed meal powder. This initial 3 ml of solvent served to wet the mixture. The mixture was allowed to remain as such for 5 minutes and then percolation started by adding 7 ml of solvent on the top of the column. The extract was collected in a weighed disk containing four one-inch square-strips of filter paper. The solvent was evaporated by keeping the disk in an oven at 96-100°C for half an hour and disk was re-weighed. The difference in the weight of disk before
extraction and after extraction of oil was the weight of extracted oil. The oil content was expressed in percentage.

3.7.2.3.2 **PULSE NUCLEAR MAGNETIC RESONANCE (NMR) METHOD**

The rapid determination of oil content by pulse NMR technique was done with the help of the instrument procured from Bruckle Company, Minispec PO 201 (Analyser Model). This was done at Nuclear Research Laboratory, IARI, following the method developed by Tiwari *et al.* (1974).

3.7.2.3.2.1 **Preparation of calibration curve**

To determine the quantity of the oil in seeds, a calibration curve was drawn by recording the NMR signals of the different quantities of pure oil from the same crop. The signals were measured after the $\pi/2$ pulse on the mode 1, keeping the pulse separation (repetition rate) $D + D_4$, 10 sec and delay time $D_3$, 250 $\mu$ sec. Since the digital voltmeter reading becomes nonlinear above 2200, the quantity of seed in the amplifier was adjusted so that the digital voltmeter reading for the samples under analysis did not exceed 2200. When analysis of per cent oil content in the seed was performed, the quantity of the sample and the seed in amplifier were adjusted to keep the signal in the linear portion of the calibration curve, preferably above 500 for less error in the analysis. At the time of the observation for the calibration curve, the NMR signal of a standard was also measured. The quantity of the standard was such that it gave the signal in the region of 1500. The quantity of the sample was similar to that of the standard. The glass tubes used for drawing the calibration curve and measuring the standard and sample signals were identical.
3.7.2.3.2 Analysis of oil in samples

The samples to be analyzed were cleaned and dried at 60°C. The NMR signals of the dried samples were recorded at the same setting of the spectrometer that was used for drawing the calibration curve. The signal of the standard was also recorded after every 10 samples. The sample signal was corrected by multiplying it with the ratio of the standard signal taken at the time of drawing the calibration curve to its value observed at the time of sample analysis. The quantity of oil in the sample can be read against its corrected signal from the calibration curve.

3.7.2.4 DETERMINATION OF FATTY ACID COMPOSITION

3.7.2.4.1 Extraction of Oil

The extraction of oil was done using soxhlet. The solvent was petroleum ether (b.p. 60-80°C).

3.7.2.4.2 Preparation of Methyl Esters of Fatty Acids

The methyl esters of fatty acids present in oil were prepared by esterification method proposed by Morrison and Smith (1964) with slight modification.

0.1 ml of oil was taken in air-tight, capped glass tubes. 0.5 ml of 0.5N sodium methoxide (prepared in methanol) was added. The tubes were capped and checked that they were air-tight. The contents of the tubes were mixed thoroughly and heated in boiling water for 10 minutes. The tubes were cooled in cold water. Two drops of BF₃ (prepared in 14% methanol) was added to the solution in each tube, and then these were heated for 10 minutes. After cooling in cold water, 5 ml of hexane was added in each tube and mixed thoroughly. After about 10 minutes, the content of the tubes get separated in two layers. The upper layer containing methyl esters was taken out, concentrated and kept in separate vials.
3.7.2.4.3 Determination of Fatty Acid Methyl Ester (FAME)

The fatty acid methyl esters were determined with a gas chromatograph (Model Perkin Elmer 8700, USA) equipped with a flame ionisation detector (FID). A stainless steel packed column, 2 m long, containing 3% OV-17 on chromosorb (WHP 100-200 mesh) was used. The following experimental conditions were maintained:

- **Oven Temperature**: 215°C
- **Injector Temperature**: 240°C
- **Detector Temperature**: 240°C
- **Isothermal Time**: 15 min.
- **Carrier Gas**: Nitrogen
- **Carrier Gas Flow**: 30 ml min⁻¹

The sample (0.2 μl) was injected. The retention time and percent area was noted.

3.7.2.4.4 Calculation of Fatty Acids Content in Oil

The fatty acid content was calculated as follows:

Peak area (%) of total fatty acid = 100 - peak area (%) of solvent

Quantity of individual fatty acid (%) = \( \frac{\text{Peak area} \text{ (%) of individual fatty acid}}{\text{Peak area} \text{ (%) of total fatty acid}} \times 100 \)

3.7.3 Yield Attributes

3.7.3.1 Number of Siliquae per Plant

The siliquae from 3 plant samples collected from each plot were separated from the plant and counted. Results were expressed as number of siliquae per plant.
3.7.3.2 NUMBER OF SEEDS PER SILIQUE

The number of seeds of 25 siliquae from each treatment were counted and calculated on per siliqua basis.

3.7.3.3 1000-SEEDS WEIGHT

From the produce of the plot, 1000 seeds were randomly drawn and the weight was recorded. Results were expressed as one thousand seeds weight in gm.

3.7.3.4 HARVEST ANALYSIS

3.7.3.4.1 SEED YIELD

The total grain produce from one meter square area was cleaned and weighed to compute the seed yield in g. m$^{-2}$ and t ha$^{-1}$.

3.7.3.4.2 BIOLOGICAL YIELD

Total biological yield from the one meter square area was recorded before threshing. Biological yield was represented as g. m$^{-2}$.

3.7.3.4.3 OIL YIELD

The per cent oil content when multiplied with seed yield gave the oil yield. The oil yield was expressed in t ha$^{-1}$.

3.7.3.4.4 PROTEIN CONCENTRATION IN SEEDS

Protein concentration in seeds was determined by multiplying the nitrogen percentage in seed with the factor, 6.25 (A.O.A.C., 1960) and expressed in percentage.
3.7.3.4.5 Harvest index (HI)

Harvest index was computed by dividing the economic yield (seed yield, g. m\(^{-2}\)) by the biological yield (g. m\(^{-2}\)) and expressed in percentage (Donald and Hamblin, 1976).

\[
HI = \frac{\text{Seed yield (g. m}^{-2}\text{)}}{\text{Biological Yield (g. m}^{-2}\text{)}} \times 100
\]

3.7.3.4.6 Nitrogen Harvest Index (N-HI)

Nitrogen harvest index was computed by dividing the seed nitrogen (g. m\(^{-2}\)) by the total nitrogen (g. m\(^{-2}\)) at harvest and expressed in percentage (Austin et al., 1977).

\[
N\text{-HI} = \frac{\text{Seed nitrogen (g. m}^{-2}\text{)}}{\text{Total nitrogen (g. m}^{-2}\text{)}} \times 100
\]

3.7.3.4.7 Sulfur Harvest Index (S-HI)

Sulfur harvest index was computed by dividing the seed sulphur (g. m\(^{-2}\)) by the total sulphur (g. m\(^{-2}\)) and expressed in percentage.

\[
S\text{-HI} = \frac{\text{Seed sulphur (g. m}^{-2}\text{)}}{\text{Total sulphur (g. m}^{-2}\text{)}} \times 100
\]

3.8 Statistical Analysis

Data collected from experiments were analyzed statistically by using Analysis of Variance Technique as described by Cochran and Cox (1957). Suitable graphical presentation and figure of relevant data were given at appropriate places. The procedure and formula, described by Snedecor and Cohran (1968), were adopted for correlation and regression study.