CHAPTER 3

MATERIALS AND METHODS


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MATERIALS AND METHODS

Six field experiments were conducted on the farm of the Aligarh Muslim University, Aligarh. Out of these, two experiments were conducted on summer moong (*Vigna radiata* L. Wilczek) var. T 44 in the "zaid" (summer) season of 1992 and 1993 and two experiments each on mustard (*Brassica juncea* L.) var. Varuna and wheat (*Triticum aestivum* L.) var. HD 2004 in the "rabi" (winter) season of 1992-93 and 1993-94.

3.1 Agro climatic conditions

Aligarh is situated in western Uttar Pradesh, about 125 km from New Delhi. This small industrial town, having an area of 5,024 sq km, is situated at 27°52' N latitude, 78°51' E longitude and 187.45 m altitude. It has a semi arid and sub tropical climate with hot dry summers (April June) and cold winters (middle of October to end of March) constituting the "rabi" season. The mean temperature for December and January, the coldest months, is about 15°C and 13°C and the extreme minimum record for any single day is 2°C and 0.5°C respectively. The summer is hot, the average temperature for May is 34.5°C and for June 34°C whereas, the extreme maximum record is 45°C and 45.5°C respectively. The average annual rainfall is 847.3 mm. More than 85% of the total rainfall occurs during June to September and the rest
in winter which is useful for "rabi" crops. The meteorological data for the period of these experiments were obtained from the Meteorological Observatory, Aligarh Muslim University, Aligarh. Various types of soil are found in different areas of this district such as sandy, loamy, sandy loam and clayey loam.

3.2 Soil characteristics

Before starting each experiment, small soil samples were collected at a depth of 15 cm from every experimental plot. These were mixed thoroughly to get a composite sample. The soil from this composite sample was analysed in the Soil Chemistry Laboratory of the Indian Agricultural Research Institute, New Delhi for physico-chemical properties (Table 1). In addition, soil samples were also taken along with plant samples to assess soil pH.

3.3 Preparation of the field

Prior to each experiment, the field was thoroughly ploughed to ensure maximum aeration. Plots of 10 sqm size were prepared in which sufficient organic manure was added for maintaining fertility of the soil keeping the type of crop in view. A uniform recommended basal dose of nitrogen, phosphorus and potassium was also applied for each crop.

3.4 Seed treatment

Authentic seeds of moong, mustard and wheat were obtained from the National Seed Corporation Ltd. I.A.R.I.,
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sandy-loam</th>
<th>Sandy-loam</th>
<th>Sandy-loam</th>
<th>Sandy-loam</th>
<th>Sandy-loam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size distribution</td>
<td>73.20</td>
<td>73.16</td>
<td>73.00</td>
<td>72.86</td>
<td>73.00</td>
</tr>
<tr>
<td>Sand %</td>
<td>7.86</td>
<td>7.84</td>
<td>7.85</td>
<td>7.86</td>
<td>7.84</td>
</tr>
<tr>
<td>Silt %</td>
<td>19.43</td>
<td>18.75</td>
<td>19.36</td>
<td>18.76</td>
<td>18.78</td>
</tr>
<tr>
<td>Clay %</td>
<td>8.3</td>
<td>8.14</td>
<td>8.2</td>
<td>8.18</td>
<td>8.2</td>
</tr>
<tr>
<td>pH (1:2)</td>
<td>7.04</td>
<td>6.54</td>
<td>6.54</td>
<td>6.54</td>
<td>6.54</td>
</tr>
<tr>
<td>Conductivity (m mhos/cm)</td>
<td>0.46</td>
<td>0.48</td>
<td>0.48</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td>Available nitrogen (kg N/ha)</td>
<td>223.34</td>
<td>230.35</td>
<td>226.46</td>
<td>234.56</td>
<td>236.66</td>
</tr>
<tr>
<td>Available phosphorus (kg P/ha)</td>
<td>18.33</td>
<td>18.34</td>
<td>19.44</td>
<td>18.86</td>
<td>18.89</td>
</tr>
<tr>
<td>Available potassium (kg K/ha)</td>
<td>690.64</td>
<td>688.55</td>
<td>681.66</td>
<td>684.78</td>
<td>675.98</td>
</tr>
</tbody>
</table>
New Delhi and their viability was tested by standard methods. The seeds were surface sterilised and soaked in water (control) or in appropriate pyridoxine hydrochloride solution. In Experiments 1 and 4 on summer moong, seeds were inoculated with Rhizobium according to a modification of the method given by Subba Rao (1972). Rhizobium culture for moong was obtained from the Government seed store, Aligarh. The inoculum was prepared by dissolving 400 g colourless gum-arabic (coating material) and 100 g sugar in 1 litre warm water. The solution was allowed to cool and a packet of Rhizobium culture (containing 200 g bacterial culture in peat) was added to it and mixed well, resulting in a muddy solution. It was sufficient to inoculate 10 kg seeds of moong. Seeds were vigorously mixed with the inoculum until they were evenly covered and moistened by it. These inoculated seeds were spread on a clean blotting paper in shade to let the coating get hard. Thereafter, they were sown in the field.

3.5 Experiment 1-6

The details of the six trials performed to investigate the response, if any of summer moong, mustard and wheat to basal application of calcium are given below.

3.5.1 Experiments 1, 2 and 3

The aim of these three field trials was to investigate the effect of basal application of calcium and pre-sowing seed treatment with pyridoxine as well as of
their interaction on the performance of summer moong (Experiment 1), mustard (Experiment 2) and wheat (Experiment 3) with regard to their growth, yield and quality parameters. The design of each of these experiments was factorial randomised. The physico chemical properties of the soil of the field are given in Table 1.

Four basal doses of calcium (0, 10, 20 and 30 kg/ha) for summer moong and 0, 20, 40 and 60 kg/ha for mustard and wheat were given in the form of gypsum (CaSO$_4$.2H$_2$O). The seed treatments included two controls, viz. (1) Un soaked (US), (2) water soaked (WS) for all three crops. One aqueous pyridoxine (PY) solution (0.3%) was taken for soaking the summer moong while two concentrations of aqueous pyridoxine solution (0.01% and 0.02%) and designated as PY$_1$ and PY$_2$ taken for mustard and wheat. The details of these treatments are given in Tables 2, 3 and 4. There were thus twelve treatments in all for summer moong and sixteen treatments for mustard and wheat, each replicated three times. The seeds were soaked in pyridoxine solution or in water for 4, 8 and 10 h for summer moong mustard and wheat respectively (Samiullah et al., 1985). Seeds of summer moong were inoculated with Rhizobium before being sown.

Sowing was done in furrows at the rate of 20 kg/ha (summer moong), 10 kg/ha (mustard) and 90 kg/ha (wheat) on 15 April, 8 October and 6 December 1992 in 10 sqm. plots. The furrows were kept 3 cm, 22.5 cm and 22 cm apart. The seed number per row was kept approximately uniform in each row.
Table 2: Scheme of treatments for Experiment 1 on summer moong.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Basal calcium dose (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Unsoaked (US)</td>
<td>+</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
</tr>
<tr>
<td>0.3% pyridoxine (PY)</td>
<td>+</td>
</tr>
</tbody>
</table>

N.B. (1) Seeds were soaked for 4 h and then treated with rhizobium inoculum.

(2) A uniform basal dose of 10 kg N, 30 kg P and 35 kg K/ha was applied.

(3) The design of the experiment was factorial randomised.
Table 3: Scheme of treatments for Experiment 2 on mustard.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Basal calcium dose (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Unsoaked (US)</td>
<td>+</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
</tr>
<tr>
<td>0.01% pyridoxine (PY₁)</td>
<td>+</td>
</tr>
<tr>
<td>0.02% pyridoxine (PY₂)</td>
<td>+</td>
</tr>
</tbody>
</table>

N.B. (1) Seeds were soaked for 8 h.
(2) A uniform basal doses of 90 kg N, 30 kg P, 30 kg K/ha was applied.
(3) The design of the experiment was factorial randomised.
Table 4: Scheme of treatments for Experiment 3 on wheat.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Basal calcium dose (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Unsoaked (US)</td>
<td>+</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
</tr>
<tr>
<td>0.01% pyridoxine (PY₁)</td>
<td>+</td>
</tr>
<tr>
<td>0.02% pyridoxine (PY₂)</td>
<td>+</td>
</tr>
</tbody>
</table>

N.B. (1) Seeds were soaked for 12 h.

(2) A uniform basal doses of 120 kg N, 30 kg P, and 50 kg K/ha was applied.

(3) The design of the experiment was factorial randomised.
recommended basal doses of 10 kg N, 30 kg P and 35 kg K/ha for moong, 90 kg N, 30 kg P and 30 kg K/ha for mustard and 120 kg N, 30 kg P and 50 kg K/ha for wheat were applied before sowing in the form of urea, monocalcium superphosphate and muriate of potash respectively. The field was irrigated three times for summer moong and mustard and twice for wheat between sowing and harvesting. Weeding was done when it was required.

The plants of summer moong were sampled at 15, 30 and 45 DAS and of mustard and wheat at 50, 70 and 90 DAS for growth analysis. Crop growth rate (CGR) relative growth rate (RGR) and net assimilation rate (NAR) were calculated for 15-30 and 30-45 DAS for summer moong and 50-70 and 70-90 DAS for mustard and wheat crops. Yield and quality parameters were studied at harvest (67 DAS for summer moong, 120 DAS for mustard and 140 DAS for wheat).

5.5.2 Growth parameters

The following growth parameters were studied at 15, 30 and 45 DAS for summer moong and at 50, 70 and 90 DAS for mustard and wheat:

a) Height/plant
b) Fresh weight/plant
c) Dry weight/plant
d) Leaf number/plant
e) Leaf area index (LAI)
f) Root nodule number/plant (only in summer moong)
g) Root length (only in summer moong and mustard)
h) Crop growth rate (CGR)
i) Relative growth rate (RGR)
j) Net assimilation rate (NAR)

3.5.3 Yield and quality parameters

At harvest, the following parameters were studied:

a) Ear number/plant (only in wheat)
b) Ear length (only in wheat)
c) Spikelet number/ear (only in wheat)
d) Grain number/ear (only in wheat)
e) Pod number/plant (only in summer moong and mustard)
g) Seed number/pod (only in summer moong and mustard)
g) 1,000 seed/grain weight
h) Seed/grain yield/ha
i) Oil percentage - (only in mustard)
j) Oil yield (only in mustard)
k) seed protein content (only in summer moong and wheat)
l) Seed protein yield/ha (only in summer moong and wheat)
m) Seed carbohydrate content (only in wheat)
n) Carbohydrate yield/ha (only in wheat)

3.5.4 Biochemical and chemical parameters

The following characteristics were estimated at various growth stages of each crop.

a) Pyridoxine content of seeds/grains before sowing
b) Leaf nitrate reductase activity (NRA)
c) Computed N, P, K and Ca status of plants

3.6 Experiments 4, 5 and 6

These trials were planned on the basis of the results of Experiments 1, 2 and 3 respectively. One observation worthy of consideration was the similarity of the growth, yield and quality response of the two controls (US and WS) taken in Experiments 1-3. It was, therefore, decided to retain only one control in the subsequent experiments. One aqueous pyridoxine (PY) solution (0.03%) was retain same as in experiment 1 for soaking the summer moong while one concentration of aqueous pyridoxine solution (0.04%) designated as PY_3 was added and (0.01%) concentration was deleted from experiments 5 and 6. Among the calcium treatments, the data, particularly concerning seed/grain yield, revealed that the application of calcium proved beneficial for each of the three crops, for example 10 kg Ca/ha proved optimum for summer moong in Experiment 1. Similarly, 40 kg Ca/ha (at par with 60 kg Ca/ha) proved the best dose for mustard (Experiment 2) and, for wheat (Experiment 3), 20 kg Ca/ha proved optimum. However, as the source of applied calcium was gypsum (CaSO_4·2H_2O) that contains sulphur also, the observed beneficial effect could not be assigned unequivocally to calcium alone. To remove this lacuna, it was decided to reschedule the basal doses of calcium for Experiments 4, 5 and 6 as under:
Experiment 4 (summer moong) : (1) control (Ca$_0$S$_0$), (2) 8 kg S/ha as K$_2$SO$_4$ (Ca$_0$S$_8$) and (3) 10 kg Ca + 8 kg S/ha as gypsum (Ca$_{10}$S$_8$). Experiment 5 (mustard) : (1) control (Ca$_0$S$_0$) (2) 32 kg S/ha (Ca$_0$S$_{32}$) and (3) 48 kg S/ha, both as (NH$_4$)$_2$SO$_4$, (Ca$_0$S$_{48}$), (4) 40 kg Ca/ha + 32 kg S/ha (Ca$_{40}$S$_{32}$) and (5) 60 kg Ca/ha + 48 kg S/ha (Ca$_{60}$S$_{48}$) both applied as gypsum. Experiment 6 (wheat) : (1) control (Ca$_0$S$_0$), (2) 16 kg S/ha (Ca$_{16}$S$_{16}$), (3) 32 kg S/ha (Ca$_0$S$_{32}$), both as (NH$_4$)$_2$SO$_4$ (4) 20 kg Ca/ha + 16 kg S/ha (Ca$_{20}$S$_{16}$) and (5) 40 kg Ca/ha + 32 kg S/ha (Ca$_{40}$S$_{32}$) both applied as gypsum. The design of the three experiments was factorial randomised and the recommended basal doses and sources of N, P and K were retained. Thus, in all, there were six treatments in Experiment 4 and fifteen treatments each in Experiments 5 and 6 which were replicated three times. It may be added that, in Experiment 4, treatments Ca$_0$S$_8$ contained 19.5 kg K/ha as K$_2$SO$_4$ in Experiment 5, Ca$_0$S$_{32}$ and Ca$_0$S$_{48}$ contained 28 and 42 kg N/ha as (NH$_4$)$_2$SO$_4$ in Experiment 6, Ca$_{16}$S$_{16}$ and Ca$_0$S$_{32}$ contained 14 and 28 kg N/ha as (NH$_4$)$_2$SO$_4$. This quantity of potassium and nitrogen was, therefore, with held at the time of application of basal muriate of potash and urea in these treatments (Tables 5, 6 and 7). All the agricultural practices and sampling techniques employed in Experiments 4, 5 and 6 as well as the parameters studied were similar to those in Experiments 1, 2 and 3 respectively.
### Table 5: Scheme of treatments for Experiment 4 on summer moong.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Basal calcium/sulphur dose (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Ca}_0\text{S}_0$</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
</tr>
<tr>
<td>0.3% pyridoxine (PY)</td>
<td>+</td>
</tr>
</tbody>
</table>

**N.B.**

1. Seeds were soaked for 4 h and then treated with rhizobium.
2. In basal treatments, the amounts of N, P and K were kept uniform at 10 kg, 30 kg and 35 kg/ha respectively.
3. For applying sulphur without calcium, gypsum was replaced by potassium sulphate, adjusting the dose of basal potassium accordingly.
4. The design of the experiment was factorial randomised.
Table 6: Scheme of treatments for Experiment 5 on mustard.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Calcium/sulphur dose (kg/ha)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Ca}_0\text{S}_0$</td>
<td>$\text{Ca}<em>{40}\text{S}</em>{32}$</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.02% pyridoxine (PY$_1$)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.04% pyridoxine (PY$_2$)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

N.B.  (1) Seeds were soaked for 8 h.

(2) The amount of N, P and K was kept constant for all the treatments @ 90, 30 and 30 kg/ha respectively.

(3) For applying sulphur without calcium, gypsum was replaced by ammonium sulphate, adjusting the dose of basal nitrogen accordingly.

(4) The design of the experiment was factorial randomised.
Table 7: Scheme of treatments for Experiment 6 on wheat.

<table>
<thead>
<tr>
<th>Soaking treatment</th>
<th>Basal calcium/sulphur dose (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca_{0}S_{0}</td>
</tr>
<tr>
<td>Water soaked (WS)</td>
<td>+</td>
</tr>
<tr>
<td>0.03% pyridoxine (PY₁)</td>
<td>+</td>
</tr>
<tr>
<td>0.04% pyridoxine (PY₂)</td>
<td>+</td>
</tr>
</tbody>
</table>

N.B. (1) Seeds were soaked for 12 h.

(2) In the basal treatments amount of N, P and K was kept constant for all the treatments @ 120 kg N, 30 kg P and 50 kg K/ha respectively.

(3) For applying sulphur without calcium, gypsum was replaced by ammonium sulphate, adjusting the dose of basal nitrogen accordingly.

(4) The design of the experiment was factorial randomised.
3.7 Details of physiological parameters

The following well recognised parameters were determined for each crop.

3.7.1 Leaf area index (LAI)

Leaves of known total area were weighed and the average dry weight per unit area was used to estimate the total leaf area per plant and then leaf area index was calculated by using the formula suggested by Watson (1958).

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

3.7.2 Crop growth rate (CGR)

Crop growth rate was calculated by using the formula suggested by Watson (1952).

\[
\text{CGR} = \frac{\text{dw}}{\text{dt}} \times \frac{1}{\text{P}}
\]

where \( \text{dw} \) = difference in dry weight at a given time
\( \text{dt} \) = time interval
\( \text{P} \) = land area

3.7.3 Relative growth rate (RGR)

Relative growth rate was calculated by using the formula of Watson (1952).

\[
\text{RGR} = \frac{\text{In} W_2 - \text{In} W_1}{t_2 - t_1}
\]

where \( W_1 \) = dry weight of plant at I growth stage
\( t_1 \) = days to sampling at I growth stage
\( W_2 \) = dry weight of plant at II growth stage
\( t_2 \) = days to sampling at II growth stage
3.7.4 Net assimilation rate (NAR)

NAR was calculated according to Milthorpe and Moorby (1979).

\[
\text{NAR} = \frac{(W_2-W_1)}{(t_2-t_1)} \times \frac{\ln L_2 - \ln L_1}{L_2 - L_1}
\]

i.e. \[
\text{NAR} = \frac{W_2-W_1}{t_2-t_1} \times \frac{2.303 (\log_{10} L_2 - \log_{10} L_1)}{(L_2 - L_1)}
\]

where, \( W_1 \) = dry weight of whole plant at I growth stage
\( L_1 \) = Leaf area of whole plant at I growth stage
\( t_1 \) = days to sampling at I growth stage
\( W_2 \) = dry weight of whole plant at II growth stage
\( L_2 \) = Leaf area of whole plant at II growth stage
\( t_2 \) = days to sampling at II growth stage
\( \ln \) = logarithm to base e
\( \log_{10} \) = logarithm to base 10.

3.7.5 Harvest index (HI)

It was calculated by the following formula :

\[
\text{H.I.} = \frac{\text{seed yield}}{\text{Total biomass}} \times 100
\]

3.8 Biochemical and chemical analysis

(i) Pyridoxine content of samples of seeds used in all the six experiments was estimated on dry weight basis before sowing in the field.

(ii) Nitrate reductase activity (NAR) in leaves was measured at various stages of growth of the three crops on
fresh weight basis.

(iii) Nitrogen, phosphorus, potassium and calcium content in different parts of plant was estimated at various stages of growth on dry weight basis in all the six experiments.

(iv) Protein content of seeds was estimated at harvest for assessing seed quality of summer moong and wheat.

(v) Carbohydrate content of seeds was estimated at harvest for assessing seed quality of wheat.

(vi) Oil content of seeds was estimated at harvest for assessing seed quality of mustard.

3.8.1 Estimation of pyridoxine

Seeds were dried and powdered. The powder was sieved and pyridoxine content was estimated colorimetrically, according to the method of Hochberg et al. (1944ab, which is described below:

3.8.1.1 Preparation of seed extract

Weighed seed powder (1 g) was taken in a 20 ml calibrated test tube. To this, 10 ml of 4 N hydrochloric acid was added. The test tube was placed in a water bath and heated for 1 h. The contents of the tube were stirred occasionally which helped in hydrolysing the bound pyridoxine as well as its extraction. The solution was cooled and the pH was adjusted at 3.0 with 1 N sodium
hydroxide and 1 N hydrochloric acid. At this pH, 3 ml of buffer (sodium citrate) was mixed followed by the addition of 2.5 g of Fuller's earth.

The tube was stoppered and shaken occasionally for 5 min. The suspension was centrifuged and the supernatant discarded. The residue was washed with 15 ml of acidulated water. 5 ml of 2 N sodium hydroxide was added to the residue and the final volume was made upto 20 ml with distilled water. The suspension was dispersed for 3 min by frequent inversions of the tube which was thereafter centrifuged. With 10 ml of the eluate was mixed 50 ml of isopropanol and it was again centrifuged. The clear supernatant was decanted and its pH adjusted to 5.0-7.0 by using a few drops of 12 N hydrochloric acid. This extract was used for pyridoxine estimation.

3.8.1.2 Colour development and pyridoxine estimation

The following tubes were set up in order to estimate pyridoxine content in the seeds:

Test tube 1: 6 ml test extract + 2 ml ammonia-ammonium chloride solution + 1 ml boric acid solution.

Test tube 2: 6 ml test extract + 2 ml ammonia-ammonium chloride solution + 1 ml distilled water.

Test tube 3: 6 ml test extract + 2 ml ammonia-ammonium chloride solution + 1 ml of standard pyridoxine hydrochloride solution containing 10 µg of the vitamin.
In each test tube, 1 ml of 2,6-dichloroquinone chloroimide solution was added. Test tube 1 acted as the blank. The optical density was read at 660 nm on a "Spectronic-20" colorimeter exactly after 1 min of addition of 2,6 dichloroquinone chloroimide reagent. The pyridoxine content of seeds was calculated by using the following formula:

\[
\frac{L_2}{L_3 - L_2} \times \frac{10}{6} \times \frac{60}{10} \times \frac{18.5}{W} = \text{ug pyridoxine/g seed powder}
\]

In the above equation:

- \(L_2\) represents optical density of the solution present in test tube 2.
- \(L_3 - L_2\) represents increase in optical density due to the 10 ug pyridoxine added in test tube 3.
- \(W\) stands for weight of seed powder used.

\[
\frac{60 \times 18.5}{10}
\]

is used for dilution factor.

3.8.2. Estimation of NRA

NRA was estimated in fresh leaf pieces. Random samples of leaves from each plot were taken and cut into small pieces. The enzyme activity was determined according to the method of Jaworski (1971) which is described briefly below:

500 mg leaf pieces were weighed and placed in polythene vials. To each, 2.5 ml of phosphate buffer pH 7.0 and 0.5 ml of 0.2 M potassium nitrate solution were added,
followed by addition of 2.5 ml of 5% isopropanol. Lastly, two drops of chloramphenicol solution were added to avoid bacterial growth in the medium. These vials were incubated for 2 h in dark at 30°C.

3.8.2.1 Colour development

0.4 ml of incubated mixture was taken in a test tube to which 0.3 ml of 1% sulphanilamide and 0.02% N-1-nepthyl ethylene diamine hydrochloride (NED-HCl) were added. The solution was left for 20 min for maximum colour development.

It was diluted to 5 ml with sufficient amount of distilled water and the optical density was read at 540 nm, using a "Spectronic-20" colorimeter. A blank consisting of 4.4 ml of distilled water plus 0.3 ml each of sulphanilamide and NED-HCl was used simultaneously for comparison.

A standard curve was plotted by taking graded dilutions of potassium nitrate of known strength from a standard aqueous solution of this salt. The optical density of the samples was compared with this calibrated curve and NRA was expressed as n mol NO₂/g/h in fresh leaf tissue.

3.8.3 NPK and Ca content of plants

Plants were dug out from the soil and were separated into leaves, rachis, stem and root at various stages of growth. At harvest, pods/ear were collected. Later, their dry weights were determined after drying to a constant weight at 80°C. The total amount of nitrogen, phosphorus, potassium and calcium accumulated in the plant parts of
respective crops, viz. root, stem, leaf, pod, pod wall, ear and grain at different sampling stages was calculated on the basis of their N, P, K and Ca contents and dry weight. This represents the status of these nutrients (mg/plant) in plants. The details of NPK and Ca estimation in different plant part are discussed below:

3.8.3.1 Digestion of plant material for estimation of NPK and Ca

The powdered dried plant parts were digested according to Lindner (1944) for the estimation of nitrogen, phosphorus and potassium. To start with, 100 mg of dry powder was taken in a 50 ml Kjeldahl flask. To this, 2 ml of conc. sulphuric acid was added and the mixture heated for 2 h which turned the contents black. After cooling for 15 min, 0.5 ml of chemically pure 30% hydrogen peroxide was added drop by drop. The solution was again heated for about 30 min till the colour became light yellow. It was then cooled and 3-4 drops of hydrogen peroxide were added and again heated for about 15 min to get a clear solution. Excess of hydrogen peroxide was avoided as it could oxidise ammonia in the absence of organic matter. The peroxide digested material was transferred to a 100 ml volumetric flask with three or four washings with distilled water and the volume was made upto the mark.

3.8.3.1.1 Estimation of nitrogen

The method of Lindner (1944), adopted for the estimation of nitrogen in the samples is briefly described below:
A 10 ml aliquot of the peroxide digested material was taken in a 50 ml volumetric flask. To it, 2 ml of 2.5 N sodium hydroxide and 1 ml of 10% sodium silicate solution were added to neutralise excess of acid and to prevent turbidity respectively. The volume of the solution was made upto the mark with distilled water. In a 10 ml graduated test tube, 5 ml aliquot of this solution was taken and 0.5 ml Nessler's reagent was added and stirred. The final volume (10 ml) was made up with distilled water. After waiting for 5 min to get optimum colour development, the optical density of the solution was determined at 525 nm with a "Spectronic-20" colorimeter. A blank, consisting of distilled water and Nessler's reagent, was run simultaneously. A standard curve, taking known dilutions of a standard ammonium sulphate solution, was plotted. The reading of each sample was compared with this calibration curve and nitrogen content in different parts of plants was expressed in terms of percentage on dry weight basis.

3.8.3.1.2 Estimation of phosphorus

Total phosphorus in the sulphuric acid peroxide digest was estimated by the method of Fiske and Subba Rao (1925). A 5 ml aliquot was taken in a 10 ml graduated test tube and 1 ml of molybdic acid (2.5% ammonium molybdate in 10 N sulphuric acid) was added carefully followed by the addition of 0.4 ml of 1-amino-2 nephthol-4-sulphonic acid. The colour turned blue and the volume was made upto 10 ml.
with distilled water. Solution was shaken, kept for 5 min and then transferred to a colorimetric tube. The optical density was read at 620 nm on a "Spectronic-20" colorimeter. A blank was run simultaneously with each determination. A standard curve was prepared by using known concentrations of monobasic potassium phosphate solution. The reading of samples was compared with this curve and phosphorus content in different parts of plant was expressed in terms of percentage on dry weight basis.

3.8.3.1.3 Estimation of potassium

Potassium content was estimated flame photometrically. A 10 ml aliquot of the digest was taken and it was read by using the filter for potassium. A blank was run side by side. The readings were compared with a calibration curve plotted with the help of known dilutions of a standard potassium sulphate solution. The potassium content in different parts of plants was expressed as percentage on dry weight basis.

3.8.3.1.4 Estimation of calcium

50 mg of oven-dried powder of each of the various plant parts was taken in a 50 ml volumetric flask. In this flask, 2 ml concentration nitric acid (HNO₃) was added and it was heated on an electric hot plate till the appearance of brown effervescence. When the effervescence stopped "TAM" (Tri acid mixture) solution was added till a clear solution
was obtained. This mixture of three acids, viz. nitric acid, sulphuric acid and perchloric acid (TAM) was prepared in the ratio of 10:5:4. The hole material was allowed to dry on the hot plate. 50 ml of double distilled water was added, shaken and transferred into another 50 ml volumetric flask with three washings with double distilled water. The final volume was made up to the mark. The calcium in digested samples was estimated with the help of a flame photometer directly. A standard curve, taking known dilutions of a standard CaCO₃ solutions, was plotted. The reading of each sample was compared with this calibration curve and calcium in different parts of plants was expressed in terms of percentage on dry weight basis.

3.8.4 Estimation of seed/grain protein

The protein of seeds/grain was extracted according to Yih and Clark (1965) and estimated by the method of Lowry et al. (1951). Sufficient amount of seed powder was spread over a sheet of paper and dried overnight in an oven at 80°C. The dried samples were cooled in a dessicator for about 5 min before weighing. 50 mg of each sample was taken and transferred to a mortar. 1 ml of cold 5% trichloroacetic acid was added to it. The powder was ground well and the homogenate was collected in a centrifuge tube with repeated washings with trichloroacetic acid. The volume was made up to 5 ml with 5% trichloroacetic acid. It was kept for 1 h to allow the complete precipitation of proteins. The homogenate
was then centrifuged at 4,000 rpm for 15 min and the supernatant was discarded. To the residue, 5 ml of 1 N sodium hydroxide solution was added and shaken well for complete mixing. It was kept for half an hour on a water bath at 60°C to dissolve the precipitated protein completely. After cooling for 15 min, the mixture was centrifuged at 4,000 rpm for 15 min and the supernatant, containing the protein, was collected. It was then diluted with appropriate quantity of water and used for estimation of total protein in the seed/grain.

3.8.4.1 Colour development

1 ml of the diluted aliquot was taken in a test tube. To it, 5 ml of reagent B (Appendix) was added and left for 10 min. Later, 0.5 ml of Folin reagent was added with immediate mixing and kept for half an hour for optimum colour development. The optical density of each sample was measured at 660 nm on a "spectronic-20" colorimeter. A blank, containing distilled water, reagent B and Folin reagent, was used simultaneously with each sample. The reading was compared with a calibration curve obtained by using known dilutions of a standard egg albumin solution.

3.8.4.2 Computation of protein yield

The protein yield/ha was calculated by multiplying the seed/grain yield with its protein concentration.
3.8.5 Estimation of grain carbohydrate

Soluble and insoluble carbohydrates were extracted according to the method of Yih and Clark (1965) and estimated by the method of Dubois et al. (1956). These are briefly described below:

50 mg of powder of the grain sample was taken in a centrifuge tube and to it 5 ml of 80% alcohol was added and heated on a water bath for 10 min. After cooling, the sample was centrifuged at 4,000 rpm for 10 min and the supernatant was taken in a 25 ml volumetric flask for the estimation of soluble carbohydrates. The residue was kept for estimation of insoluble carbohydrates. This residue was washed twice with 5 ml of 80% alcohol and the washings were added to the flask containing the supernatant. The volume was made up to the mark with 80% alcohol. 1 ml of the alcohol extract was taken in a 10 ml test tube and dried on a water bath. When the alcohol was completely evaporated, the test tube was taken out from the water bath and cooled. After cooling 2 ml of distilled water was added to each test tube.

3.8.5.1 Estimation of soluble carbohydrate

To the supernatant of the alcohol extract 1 ml of 5% phenol was added followed by 5 ml conc. sulphuric acid. The colour turned yellowish orange. After cooling for half an hour, it was transferred to a colorimetric tube and the optical density was read at 490 nm on a "Spectronic-20" colorimeter. A blank was run with each sample. The soluble
carbohydrate content of each sample was obtained by comparing its optical density with a calibration curve plotted by taking known dilutions of a standard solution of chemically pure glucose.

3.8.5.2 Estimation of insoluble carbohydrates

To residue kept after centrifugation with 80% alcohol was used for the determination of insoluble carbohydrates by the following procedure:

5 ml of 1.5 N sulphuric acid was added to the residue and heated on a water bath for about two hours. After cooling, it was transferred to a centrifuge tube and centrifuged at 4,000 rpm for 10 min. The extract was then collected in a 25 ml volumetric flask.

The residue was washed twice with distilled water and the washing was added to the above volumetric flask. The volume was made up to the mark with distilled water. The insoluble carbohydrate content was determined using the same method as in the case of soluble carbohydrate.

3.8.5.3 Total carbohydrate

The values for soluble and insoluble fractions were added together to get the total carbohydrate content of the grain.

3.8.5.4 Computation of carbohydrate yield

The carbohydrate yield was computed by multiplying
the grain yield with the total carbohydrate concentration of the grain.

3.8.6 Determination of seed oil content

As mentioned earlier, oil content was estimated in mustard seeds only. In order to extract the oil, grinding of seed was done until the formation of a fine meal.

20 g meal of ground seeds was transferred to a Soxhlet apparatus and sufficient quantity of pure petroleum ether was added. The apparatus was kept in a hot water bath, running at 60°C, for about 6 h for complete extraction of the oil. The petroleum ether was evaporated from the extract. The percentage of the extract oil was calculated by the following formula:

\[
\frac{m \times 100}{m_0}
\]

where, \( m \) is the mass of oil and \( m_0 \) is mass of seed powder.

3.8.6.1 Computation of oil yield

The oil yield of the crop was calculated by multiplying its total seed yield with its seed oil percentage.

3.9 Statistical analysis

The experimental data were statistically analysed by adopting analysis of variance technique according to Panse and Sukhatme (1985). In applying the 'f' tests, the error
due to replicates was also determined. When 'f' value was found to be significant at the 5 per cent level of probability, critical difference (C.D.) was calculated. The models of the analysis of variance (ANOVA) are given in Table 8.

Table 8: Models of analysis of variance (ANOVA) of Experiments 1-6.

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