MATERIALS AND METHODS
# Materials and Methods

## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Experimental Material</td>
<td>52</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Nomenclature</td>
<td>52</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Botanical Description</td>
<td>53</td>
</tr>
<tr>
<td>3.1.2.1</td>
<td>Genomic Relationships of <em>Brassica juncea</em></td>
<td>54</td>
</tr>
<tr>
<td>3.1.2.2</td>
<td>Canopy Orientation in Mustard</td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td>Experimental Site</td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td>Agro-Climatic Conditions of Aligarh</td>
<td>55</td>
</tr>
<tr>
<td>3.4</td>
<td>Soil Characteristics</td>
<td>56</td>
</tr>
<tr>
<td>3.5</td>
<td>Experimental Lay-Out and Experimentation</td>
<td>56</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Experiment 1</td>
<td>57</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Experiment 2</td>
<td>58</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Experiment 3</td>
<td>59</td>
</tr>
<tr>
<td>3.5.4</td>
<td>Plant sampling</td>
<td>61</td>
</tr>
<tr>
<td>3.5.5</td>
<td>Chemicals</td>
<td>61</td>
</tr>
<tr>
<td>3.5.6</td>
<td>Biometric Observations</td>
<td>62</td>
</tr>
<tr>
<td>3.5.6.1</td>
<td>Growth Characteristics</td>
<td>62</td>
</tr>
<tr>
<td>3.5.6.1.1</td>
<td>Leaf number per plant</td>
<td>62</td>
</tr>
<tr>
<td>3.5.6.1.2</td>
<td>Fresh mass per plant</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.3</td>
<td>Dry mass per plant</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.4</td>
<td>Leaf area per plant</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.5</td>
<td>Leaf mass ratio</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.6</td>
<td>Stem mass ratio</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.7</td>
<td>Leaf area ratio</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.8</td>
<td>Crop growth rate</td>
<td>63</td>
</tr>
<tr>
<td>3.5.6.1.9</td>
<td>Relative growth rate</td>
<td>64</td>
</tr>
<tr>
<td>3.5.6.1.10</td>
<td>Unit leaf rate</td>
<td>65</td>
</tr>
<tr>
<td>3.5.6.2</td>
<td>Photosynthetic Characteristics</td>
<td>65</td>
</tr>
<tr>
<td>3.5.6.2.1</td>
<td>Chlorophyll content</td>
<td>66</td>
</tr>
<tr>
<td>3.5.6.2.1.1</td>
<td>Extraction</td>
<td>66</td>
</tr>
<tr>
<td>3.5.6.2.1.2</td>
<td>Estimation</td>
<td>66</td>
</tr>
<tr>
<td>3.5.6.2.1.3</td>
<td>Calculation for chlorophyll content</td>
<td>66</td>
</tr>
<tr>
<td>3.5.6.2.2</td>
<td>Assay of carbonic anhydrase activity</td>
<td>67</td>
</tr>
<tr>
<td>3.5.6.2.2.1</td>
<td>Estimation</td>
<td>67</td>
</tr>
<tr>
<td>3.5.6.2.3</td>
<td>Net photosynthetic rate, stomatal conductance and intercellular CO₂ concentration</td>
<td>68</td>
</tr>
<tr>
<td>3.5.6.2.4</td>
<td>Intrinsic water-use efficiency</td>
<td>68</td>
</tr>
<tr>
<td>3.5.6.3</td>
<td>N Assimilation</td>
<td>68</td>
</tr>
<tr>
<td>3.5.6.3.1</td>
<td>Assay of nitrate reductase activity</td>
<td>69</td>
</tr>
<tr>
<td>3.5.6.3.1.1</td>
<td>Standard curve for nitrate reductase</td>
<td>70</td>
</tr>
</tbody>
</table>
3.5.6.3.2 Assay of nitrite reductase activity
3.5.6.3.2.1 Standard curve for nitrite reductase
3.5.6.3.3 Assay of glutamine synthetase activity
3.5.6.3.3.1 Standard curve for glutamine synthetase
3.5.6.3.4 Plant nitrogen concentration
3.5.6.3.4.1 Standard curve for nitrogen
3.5.6.3.5 Plant N Content
3.5.6.4 Biochemical Characteristics
3.5.6.4.1 Plant carbon concentration
3.5.6.4.2 Plant protein concentration
3.5.6.4.2.1 Standard curve for total protein
3.5.6.4.3 Plant carbohydrate concentration
3.5.6.4.3.1 Standard curve for carbohydrate concentration
3.5.6.4.4.1 Carbon : Nitrogen ratio
3.5.6.5 Ethylene Biosynthesis
3.5.6.5.1 Assay of ACC synthase activity
3.5.6.5.2 Ethylene evolution
3.5.6.6 Yield Characteristics
3.5.6.6.1 Pod number per plant
3.5.6.6.2 Seed number per pod
3.5.6.6.3 1000 seed weight
3.5.6.6.4 Seed yield
3.5.6.6.5 Biological yield
3.5.6.6.6 Harvest index
3.5.6.7 Statistical analysis
MATERIAL AND METHODS

The chapter deals with the techniques and methodology used for the experimentation and estimation of various parameters during the course of the present study.

3.1 Experimental Material

Seeds of Indian mustard (Brassica juncea L. Czern & Coss.) cv. Alankar were obtained from Regional Research Centre, Kanpur, India and used as the experimental material.

3.1.1 Nomenclature

The oleiferous Brassicas grown in India are divided into four groups.

1. Brown mustard: commonly known as rai, raya or laha (Brassica juncea L. Czern & Coss.)

2. Sarson
   a. Yellow sarson: Brassica campestris L. var. Sarson Prain
   b. Brown sarson: Brassica campestris L. var. Dichotoma Watt

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).
These do not fall under any of the four groups. These are moreover grown to a limited extent. Mustard (*Brassica juncea* L. Czern & Coss.) is the dominant species grown in India (Prakash, 1980).

### 3.1.2 Botanical Description

Rape and mustard include annual herbs. Roots in general, are long and tapering. Toria is more or less a surface feeder but brown sarson has long roots with limited lateral spread enabling its successful cultivation under drier conditions. The height of the stem varies from 0.45 m (in some varieties of Toria) to 1.90 m (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 ovary. In brown sarson and toria, the ovary is bicarpellary, whereas in the case of yellow sarson, it may also be tri-or tetra-carpellary.

The fruit is siliqua. The pods are two-valved, three-valved or four-valved, depending upon the number of carpels in the ovary. The flowers begin to open from 8.00 h and continue upto 12 noon.
Plate 1: (A) mustard field and (B) Leaves in lower layers of axis of mustard
(*Brassica juncea* L.).
3.1.2.1 Genomic Relationships of *Brassica juncea*

The modern understanding of genomic relationships among the *Brassica* species and cytological evidence show that *Brassica napa* (n = 10; A), *Brassica nigra* (n = 8; B) and *Brassica oleracea* (n = 9; C) are primary species, and *Brassica juncea* (n = 18; AB) is an amphidiploid resulting from a cross between *Brassica napa* and *Brassica nigra* (Morinaga, 1934).

3.1.2.2 Canopy Orientation in Mustard

Canopy structure refers to the amount and organization of above-ground plant material, including the size, shape and orientation of plant organs such as leaves, stems, flowers and fruits (Norman and Campbell, 1989). Thus, canopy structure has a major influence on the exchange of mass and energy between plants and their environment. Like other plant communities, mustard also has its own unique spatial pattern displaying photosynthetic surfaces and hence for intercepting the photosynthetic photo flux. Leaf orientation is the angle formed clockwise from north to the horizontal projection of the axis. It is random for many canopies and is important in determining interception of photosynthetic photon flux, arise at an angle of 30-40° in brown mustard and toria and 10-20° in yellow sarson, thereby, give the plant a pyramidal shape and cause shading effect on the lower leaves (Plate I A).
3.2 Experimental Site

Three field experiments were conducted during “rabi” (winter) season from 2000-2003 at the Experimental Field, Aligarh Muslim University, Aligarh, India. The experimental period stretches from October to March. Aligarh is situated at 27°52’N, 78°51’E and 187.4 m altitude above sea level in the mid of Doab—the land between the Ganga and Yamuna rivers at a distance of 130 km southeast of Delhi on the Delhi-Howrah rail route.

3.3 Agro-Climatic Conditions of Aligarh

Aligarh experiences semi arid and subtropical climate, with hot dry summer and cold winters. The winter varies from the middle of October till the end of March.

The temperature in December and January reaches 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 June. In this season maximum temperature sometimes reaches to 46°C in the month of June.

The mean annual rainfall is about 847.3 mm. More than 85% of the total downpour is delivered during a short span of four months from June to September. The remaining rain showers are received during winter. Winter rainfall is useful for mustard crop.
3.4 Soil Characteristics

Before the beginning of each experiment, soil samples from various spots of the experimental field were collected randomly at a depth of 15cm. A composite sample was prepared after thoroughly mixing the collected samples for the analysis of N availability. Generally, the soil was sandy loam (Alfisols with Ustochrept type). Moisture content of soil collected from various sites in the experimental field used for the three experiments at different sampling times is given in Table 1.

3.5 Experimental Lay-Out and Experimentation

The treatments in each experiment were arranged in a randomized block design with three replications. The individual plot size was 10m$^2$ (5 x 2m). Calendar of field operations for the experiments (Table 2) shows time of experimentation, treatment and data collection. The environmental conditions during the sampling times for the Experiment 1, Experiment 2 and Experiment 3 are given in Figure 1, Figure 2 and Figure 3, respectively. The seeds were sown by the usual behind the plough method at a rate of 10kg/ha. A distance of 30cm between rows and 15cm between plants in each row was maintained. After establishment of the crop, i.e. after 12DAS, seedlings were thinned to maintain a uniform plant population of 12plants/m$^2$. In order to check the aphid contagion, if any, insecticidal spray of Dimecron-100 was
Table 1: Moisture content (%) of soil collected from various sites in the experimental field at 0, 40, 60, 80, 100 and 120d after sowing (DAS) corresponding to sowing, pre-flowering, post-flowering, pod-fill, pod-maturity and harvest stages, respectively of plant cycle. The values represent mean ± S.E. (n=10)

<table>
<thead>
<tr>
<th>Sampling time (DAS)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.60±1.5</td>
<td>12.66±1.8</td>
<td>12.85±1.1</td>
</tr>
<tr>
<td>40</td>
<td>11.33±1.5</td>
<td>11.00±1.2</td>
<td>11.72±1.1</td>
</tr>
<tr>
<td>60</td>
<td>9.22±0.9</td>
<td>9.70±0.8</td>
<td>10.03±0.9</td>
</tr>
<tr>
<td>80</td>
<td>8.61±0.6</td>
<td>8.36±0.7</td>
<td>8.71±0.6</td>
</tr>
<tr>
<td>100</td>
<td>6.33±0.4</td>
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<td>120</td>
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<td>4.90±0.5</td>
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<tr>
<td>Operations</td>
<td>Date of operation</td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>-----------------------------</td>
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<tr>
<td>1. Preparation tillage</td>
<td></td>
<td></td>
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</tr>
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<td>A. Ploughing</td>
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<td>02.10.2001</td>
</tr>
<tr>
<td>B. Leveling</td>
<td></td>
<td>06.10.2000</td>
<td>07.10.2001</td>
</tr>
<tr>
<td>2. Layout and sowing</td>
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<td></td>
</tr>
<tr>
<td>A. Layout</td>
<td></td>
<td>10.10.2000</td>
<td>09.10.2001</td>
</tr>
<tr>
<td>3. Treatments</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A. Defoliation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B. Nitrogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Top dressing N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. First dose</td>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5. Weeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Sampling</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D. Fourth sampling</td>
<td></td>
<td>20.01.2001</td>
<td>19.01.2002</td>
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<tr>
<td>7. Harvesting</td>
<td></td>
<td>25.03.2001</td>
<td>27.03.2002</td>
</tr>
</tbody>
</table>
Figure 1. Variation in photosynthetically active radiation, temperature, humidity, and atmospheric CO$_2$ concentration at 40, 60, 80 and 100 days after sowing (DAS). Sampling times are shown on the x-axis.

- **Atmospheric CO$_2$** (µ mol mol$^{-1}$)
- **Temperature** (°C)
- **Humidity** (%)
- **Photosynthetically active radiation** (µ mol m$^{-2}$ s$^{-1}$)
Figure 2. Variation in photosynthetically active radiation, temperature, humidity and atmospheric CO₂ concentration at 40, 60, 80 and 100 days after sowing (DAS) sampling times of Experiment 2.
Figure 3 Variation in photosynthetically active radiation, temperature, humidity, and atmospheric CO₂ concentration at 40, 60, 80, and 100 days after sowing (DAS).
done with a hand spray. Weeding was done twice during the entire crop season to keep the experimental field free of weeds. Before sowing 20litre/m² irrigation was given to all plots. Thereafter, this amount of irrigation was done at 50DAS. Soil analysis for N was done prior to each experiment, and is presented in Figure 4. Experiments 2 and 3 employ application of N and, therefore, available soil N content was taken into consideration for deciding levels of N application and maintaining the treatments.

3.5.1 Experiment 1

The experiment was conducted in the winter season of 2000-2001. Seeds were sown on 12th October, 2000 and harvesting was done on 25th March, 2001. The aim of the experiment was to observe the effects of time of defoliation of 50% lower leaves on plant axis on growth and physiological characteristics, N assimilation and yield characteristics. There were 12 leaves on the plant axis at 40DAS. Out of total leaves present on the plant axis at 40DAS, 50% of leaf number on the lower half axis was removed by excising leaf blades either at 40 (pre-flowering) or 60 (post-flowering) DAS. In control plants the leaves were left intact. The treatments were arranged in a randomized block design. The scheme of treatments is given in Table 3 and ANOVA model in Table 4. The degree of defoliation treatment was selected on the basis of earlier experience of N. A. Khan and associates. They found that
Figure 4: Available soil N of the experimental field used for Experiment 1, Experiment 2 and Experiment 3. The values represent an average of ten soil samples (Mean ± S.E.)
Table 3: Scheme of treatments for Experiment 1.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>No defoliation control</td>
<td>No defoliation done (leaves left intact)</td>
</tr>
<tr>
<td>Defoliation at 40DAS</td>
<td>Defoliation of 50% leaves on lower layers of plant axis done at 40DAS (pre-flowering)</td>
</tr>
<tr>
<td>Defoliation at 60DAS</td>
<td>Defoliation of 50% leaves on lower layers of plant axis done at 60DAS (post-flowering)</td>
</tr>
</tbody>
</table>

Crop: Mustard (*Brassica juncea* L. Czem & Coss.) cv. Alankar

Experimental design: Randomized block design

Plants were treated with a uniform soil application of 120kg N/ha as urea, 30kg P/ha as single superphosphate and 180kg K/ha as muriate of potash
**Table 4: ANOVA model for Experiment 1.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>MSS</th>
<th>F-value</th>
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<tr>
<td>Replication</td>
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<td></td>
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<tr>
<td>Treatments</td>
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</tr>
<tr>
<td>Error</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td></td>
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</tbody>
</table>
defoliation of 50% lower leaves at 40DAS brings about beneficial effects on plant growth and yield. Moreover, removal of 50% upper leaves had detrimental effects on the growth and physiology of plant. However, no study was conducted on the comparison of stage of defoliation on plant growth, physiology and yield. This study was conducted to generate the information on the comparison of the effects of defoliation either at 40 or 60DAS. Each treatment was replicated three times. The plants were grown with a uniform soil application of 120kg N/ha as urea 30kg P/ha as single super phosphate and 180kg K/ha as muriate of potash at the time of sowing so as the nutrients may not be the limiting factor. The available soil N was 130kg/ha (Figure 4).

3.5.2 Experiment 2

The experiment was conducted during the winter season of 2001-2002. The sowing of the seeds was done on 11th October, 2001 and the crop was harvested on 27th March, 2002. The aim of the experiment was to examine and examine the efficacy of defoliated and non-defoliated plants in the acquisition of N when they were grown with different N levels. Of total number of leaves on the axis of plant 50% on lower axis was defoliated at 40DAS (selected on the basis of findings of Experiment 1) and the plants were treated with soil application of N as urea at the rate of 0, 60, 100 or 150kg N/ha at the time of sowing. In control group of plants leaves were not defoliated and left intact, but
with the similar rates of N application. The treatments were arranged in a complete randomized block design. Since the available soil N was 100kg/ha (Figure 4), the available soil N may also be designated as 100, 160, 200 and 250kg N/ha. Sufficient P (30kg P/ha) and K (180kg K/ha) were added as single super phosphate and muriate of potash, respectively so as to be non-limiting to plant growth. The standard plant cultivation practices were followed. Scheme of treatments is given in Table 5 and ANOVA model for the experiment in Table 6.

3.5.3 Experiment 3

Experiment 3 was conducted during the winter season of 2002-2003. Sowing of seeds was done on 10th October, 2002 and produce was harvested on 26th March, 2003. The available soil N for this experiment was 100kg N/ha (Figure 4). In this experiment N was given as single basal application of 150kg N/ha at the time of sowing or as split in two doses; 100kg N/ha given at the time of sowing and 50kg N/ha at 40 or 60DAS. The basal application of 150kg N/ha was also split as; 75kg N/ha given at the time of sowing and rest 75kg N/ha at 40 or 60DAS. These N applications were given to defoliated (50% lower leaves as in earlier experiments) and no defoliated control plants. Thus the treatments in respect of soil treated with N were: BN100+N50(40d) [soil treated with 100kg N/ha at the time of sowing and 50kg N/ha at 40DAS], BN100+N50(60d) [soil treated with 100kg N/ha at the time of
Table 5: Scheme of treatments for Experiment 2.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Description</th>
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<td>No defoliation</td>
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<tr>
<td>N levels (kg/ha)</td>
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<td>0</td>
<td>Plants were treated with soil-applied 0kg N/ha and no defoliation done</td>
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<tr>
<td>60</td>
<td>Plants were treated with soil-applied 60kg N/ha and no defoliation done</td>
</tr>
<tr>
<td>100</td>
<td>Plants were treated with soil-applied 100kg N/ha and no defoliation done</td>
</tr>
<tr>
<td>150</td>
<td>Plants were treated with soil-applied 150kg N/ha and no defoliation done</td>
</tr>
<tr>
<td>Defoliation</td>
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<td>N levels (kg/ha)</td>
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<tr>
<td>0</td>
<td>Plants were treated with soil-applied 0kg N/ha and defoliation of 50% leaves on lower layers of plant axis done at 40DAS</td>
</tr>
<tr>
<td>60</td>
<td>Plants were treated with soil-applied 60kg N/ha and defoliation of 50% leaves on lower layers of plant axis done at 40DAS</td>
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<tr>
<td>100</td>
<td>Plants were treated with soil-applied 100kg N/ha and defoliation of 50% leaves on lower layers of plant axis done at 40DAS</td>
</tr>
<tr>
<td>150</td>
<td>Plants were treated with soil-applied 150kg N/ha and defoliation of 50% leaves on lower layers of plant axis done at 40DAS</td>
</tr>
</tbody>
</table>

Crop: Mustard (*Brassica juncea* L. Czem & Coss.) cv. Alankar

Experimental design: Randomized block design

Source of N: Urea

Plants were treated with a uniform soil application of 30kg P/ha as single superphosphate and 180kg K/ha as muriate of potash
Table 6: ANOVA model for Experiment 2.

<table>
<thead>
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<th>Source of variation</th>
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sowing and 50kg N/ha at 60DAS], BN75+N75(40d) [soil treated with 75kg N/ha at the time of sowing and 75kg N/ha at 40DAS], 
BN75+N75(60d) [soil treated with 75kg N/ha at the time of sowing and 75kg N/ha at 60DAS] and BN150 [soil treated with 150kg N/ha at the 
time of sowing]. Basal application of 150kg N/ha (BN150) was selected 
on the basis of Experiment 1 in which it proved most effective in 
producing the effects in comparison to other N treatments. The 
treatments were arranged in a complete randomized block design. These 
treatments can also be described in terms of available soil N as: 
N200+N50(40d) [available N 200kg/ha at the time sowing and 50kg 
N/ha given at 40DAS], BN200+BN50(60d) [available N 200kg/ha at the 
time of sowing and 50kg N/ha given at 60DAS], N175+N75(40d) 
[available N 175kg/ha at the time of sowing and 75kg N/ha given at 
40DAS], N175+N75(60d) [available N 175kg/ha at the time of sowing 
and 75kg N/ha given at 60DAS] and N250 [total available soil N 
250kg/ha at the time of sowing]. Sufficient P (30kg P/ha) and K (180kg 
K/ha) were added as single super phosphate and muriate of potash, 
respectively so as to be non-limiting to plant growth. Scheme of 
treatments is given in Table 7 and ANOVA model for the experiment in 
Table 8.

The observations recorded and sampling times were similar as 
described for Experiment 1.
Table 7: Scheme of treatment for Experiment 3.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No defoliation</strong></td>
<td></td>
</tr>
<tr>
<td>N levels (kg/ha)</td>
<td></td>
</tr>
<tr>
<td>BN150</td>
<td>Plants treated with single basal application of 150kg N/ha at the time of sowing and no defoliation done</td>
</tr>
<tr>
<td>BN100+N50(40d)</td>
<td>Plants treated with 100kg N/ha given at the time of sowing and 50kg N/ha at 40DAS (pre-flowering) and no defoliation done</td>
</tr>
<tr>
<td>BN75+N75(40d)</td>
<td>Plants treated with 75kg N/ha given at the time of sowing and 75kg N/ha at 40DAS (pre-flowering) and no defoliation done</td>
</tr>
<tr>
<td>BN100+N50(60d)</td>
<td>Plants treated with 100kg N/ha given at the time of sowing and 50kg N/ha at 60DAS (post-flowering) and no defoliation done</td>
</tr>
<tr>
<td>BN75+N75(60d)</td>
<td>Plants treated with 75kg N/ha given at the time of sowing and 75kg N/ha at 40DAS (pre-flowering) and no defoliation done</td>
</tr>
<tr>
<td><strong>Defoliation</strong></td>
<td></td>
</tr>
<tr>
<td>N levels (kg/ha)</td>
<td></td>
</tr>
<tr>
<td>BN150</td>
<td>Plants were treated with single basal application of 150kg N/ha at the time of sowing and defoliation of 50% leaves on lower layers on plant axis done at 40DAS (pre-flowering)</td>
</tr>
<tr>
<td>BN100+N50(40d)</td>
<td>Plants treated with 100kg N/ha given at the time of sowing and 50kg N/ha at 40DAS (pre-flowering) and defoliation of 50% leaves on lower layers on plant axis done at 40DAS</td>
</tr>
<tr>
<td>BN75+N75(40d)</td>
<td>Plants were treated 75kg N/ha given at the time of sowing and 75kg N/ha at 40DAS (pre-flowering) and defoliation of 50% leaves on lower layers on plant axis done at 40DAS</td>
</tr>
<tr>
<td>BN100+N50(60d)</td>
<td>Plants treated with 100kg N/ha given at the time of sowing and 50kg N/ha at 60DAS (post-flowering) and defoliation of 50% leaves on lower layers on plant axis done at 40DAS</td>
</tr>
<tr>
<td>BN75+N75(60d)</td>
<td>Plants were treated 75kg N/ha given at the time of sowing and 75kg N/ha at 60DAS (post-flowering) and defoliation of 50% leaves on lower layers on plant axis done at 40DAS</td>
</tr>
</tbody>
</table>

Crop: Mustard (*Brassica juncea* L. Czern & Coss.) cv. Alankar
Experimental design: Randomized block design
Source of N: Urea
Plants were treated with a uniform soil application of 30kg P/ha as single superphosphate and 180kg K/ha as muriate of potash
Table 8: Model of ANOVA, Experiment 3.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>MSS</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
<td>9</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>
3.5.4 Plant sampling

The data on growth and photosynthetic characteristics, N assimilation, biochemical characteristics and ethylene biosynthesis were recorded at 40, 60, 80 and 100DAS. At maturity (120DAS) yield characteristics were noted. Crop growth rate, relative growth rate and unit leaf rate were calculated for the period 80-100DAS. The growth stage at 40, 60, 80 and 100DAS are described as pre-flowering, post-flowering, pod fill and pod maturity, respectively (Khan et al., 1998; Khan and Samiullah, 2003). At each sampling time, five plants from each treatment and replication were uprooted, brought to laboratory in polythene bags for determination of various characteristics. The procedures adopted and methodologies used in the determination of characteristics are described in detail under separate headings in the following pages.

Plants were harvested by cutting at the ground level from each plot. The harvested plants were sun-dried, threshed to collect the seeds. The details are given in the following pages under the heading yield characteristics.

3.5.5 Chemicals

Reagents and chemicals used in the study were of Analytical Grade obtained from Sigma or s.d. fine Chemicals.
3.5.6 Biometric Observations

As mentioned earlier observations were carried out at 40, 60, 80 and 100d after sowing and at maturity (120DAS).

3.5.6.1 Growth Characteristics

The following growth characteristics were studied.

1. Leaf number per plant
2. Leaf area per plant
3. Fresh mass per plant
4. Dry mass per plant
5. Leaf dry mass per plant
6. Stem dry mass per plant
7. Pod dry mass per plant
8. Leaf mass ratio
9. Stem mass ratio
10. Leaf area ratio
11. Crop growth rate
12. Relative growth rate
13. Unit leaf rate

3.5.6.1.1 Leaf number per plant

Number of functional leaves on plant axis was counted and recorded as leaf number per plant.
3.5.6.1.2 Fresh mass per plant

Plants were uprooted carefully from the plots, washed to remove dust, if any and fresh mass was recorded.

3.5.6.1.3 Dry mass per plant

The plants of which fresh mass was taken were separated into leaf, stem and pod, and were dried separately in hot-air oven at 80°C till constant weight. The dried material was weighed on electrical balance and the weight was recorded as dry weight of different plant parts as well as their sum as plant dry mass.

3.5.6.1.4 Leaf area per plant

Leaf area of functional plant leaves was determined with a LA21 leaf area meter (Systronics, Ahmedabad, India)

3.5.6.1.5 Leaf mass ratio

Leaf mass ratio was calculated as the ratio of leaf dry mass per plant to plant dry mass.

3.5.6.1.6 Stem mass ratio

Stem mass ratio was calculated as the ratio of stem dry mass per plant to plant dry mass.

3.5.6.1.7 Leaf area ratio

Leaf area ratio was represented as leaf area of unit leaf weight.

3.5.6.1.8 Crop growth rate

Dry matter accumulation per plant per unit land area in a unit time
is expressed as crop growth rate (CGR). It was calculated by using the formula proposed by Watson (1952).

\[ \text{CGR} = \frac{\text{dw}}{\text{dt}} \]

where \( \text{dw} \) = difference in dry mass in given time/plant

\( \text{dt} \) = time interval

### 3.5.6.1.9 Relative growth rate

Relative growth rate (RGR) is the increase in dry mass in unit time interval in relation to the initial mass. It was calculated by the formula proposed by Radford (1967).

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

i.e. \( \text{RGR} = \frac{2.303 (\log_{10} W_2 - \log_{10} W_1)}{t_2 - t_1} \)

where \( W_1 \) = Dry mass of plant at growth stage I

\( W_2 \) = Dry mass of plant at growth stage II

\( t_1 \) = Days to sampling at growth stage I

\( t_2 \) = Days to sampling at growth stage II

\( \ln \) = Logarithm to base e

\( \log_{10} \) = Logarithm to base 10.
3.5.6.1.10 Unit leaf rate

Unit leaf rate (ULR) is the increase in weight per unit leaf area in unit time. It was calculated by using the formula proposed by Milthorpe and Moorby (1979).

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

where

- \(W_1\) = Dry weight per plant at growth stage I
- \(W_2\) = Dry weight per plant at growth stage II
- \(t_1\) = Days to sampling at growth stage I
- \(t_2\) = Days to sampling at growth stage II
- \(L_1\) = Leaf area per plant at growth stage I
- \(L_2\) = Leaf area per plant at growth stage II
- \(\ln\) = Logarithm to base e
- \(\log_{10}\) = Logarithm to base 10

3.5.6.2 Photosynthetic Characteristics

The following photosynthetic characteristics were observed:

1. Chlorophyll content
2. Carbonic anhydrase activity
3. Net photosynthetic rate
4. Stomatal conductance
5. Intercellular CO\(_2\) concentration
6. Intrinsic water-use efficiency

3.5.6.2.1 Chlorophyll content

Total chlorophyll was extracted by adopting the method of Hiscox and Isrelstam (1979) by using dimethyl sulphoxide as extraction medium, and estimated by the method of Arnon (1949).

3.5.6.2.1.1 Extraction

Fresh leaves (100 mg) were cut into small pieces and collected in test tubes containing 7.0 ml of dimethyl sulphoxide (DMSO). The test tubes were covered with black paper and incubated at 45°C for 40 minutes for extraction. The reaction mixture was transferred to a graduated tube and the final volume was made up to 10.0 ml with DMSO. The chlorophyll content in the extraction medium was estimated immediately.

3.5.6.2.1.2 Estimation

3.0 ml of the chlorophyll extract was transferred to a cuvette and the absorbance was read at 645 and 663nm on SL164 UV-Vis spectrophotometer (Elico, Hyderabad, India).

3.5.6.2.1.3 Calculation for chlorophyll content

Total chlorophyll content was calculated according to the equation given by Arnon (1949).

\[
\text{Total Chlorophyll (mg g}^{-1} \text{ leaf fresh mass) =} \frac{V}{1000 \times W} \left[20.2 (\text{OD}_{645}) + 8.02 (\text{OD}_{663})\right]
\]
where, \( V \) = volume of the extract

\( W \) = mass of the leaf tissue taken

3.5.6.2.2 Assay of carbonic anhydrase activity

Carbonic anhydrase (CA) facilitates the supply of \( \text{CO}_2 \) to the carboxylation sites. It catalyzes the reversible hydration of carbon dioxide (Raven, 1995; Khan et al., 2004).

\[
\text{CA} \quad \text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}^+ + \text{HCO}_3^-
\]

3.5.6.2.2.1 Estimation

Carbonic anhydrase activity was estimated by adopting the method of Dwivedi and Randhava (1974). Leaves used for photosynthesis determination were used for the enzyme assay. Leaves were cut into small pieces (2-3mm length) in 10ml 0.2M cystein in a Petri dish at 0-4°C. The solution adhering at the leaf surface was removed with the help of blotting paper followed by immediate transfer of leaves to a test tube having 4ml of phosphate buffer (pH 6.8). Four ml of 0.2M sodium bicarbonate in 0.02M sodium hydroxide solution and 0.2ml 0.002% bromothymol blue indicator were added to the tubes. The tubes were kept at 4°C for 20 minutes.

\( \text{CO}_2 \) liberated during catalytic action of enzyme on sodium bicarbonate was estimated with titration of the reaction mixture against 0.05N hydrochloric acid, using methyl red as an indicator. The control
reaction mixture was also titrated against 0.05N hydrochloric acid. The difference of the sample and control readings was noted for the calculation of the enzyme activity.

3.5.6.2.3 Net photosynthetic rate, stomatal conductance and intercellular CO₂ concentration

The net photosynthetic rate, stomatal conductance and intercellular CO₂ concentration were measured in fully expanded uppermost leaves of four plants in each treatment using infra red gas analyzer LiCOR-6200 portable photosynthesis system (Nebraska, USA). The fully expanded leaves used for measurements included new leaves emerged following defoliation treatment. The measurements were done between 1100-1200 h at saturating light intensity. The values for photosynthetically active radiation (PAR), temperature, humidity and atmospheric CO₂ concentration at each sampling time is shown in Figures 1-3.

3.5.6.2.4 Intrinsic water-use efficiency

Intrinsic water-use efficiency was calculated according to the formula proposed by Dudley (1996).

\[
WUE = \frac{\text{Net photosynthetic rate}}{\text{Stomatal conductance}}
\]

3.5.6.3 N Assimilation

Following characteristics were determined to determine N
assimilation.

1. Nitrate reductase activity
2. Nitrite reductase activity
3. Glutamine synthetase activity
4. Plant N concentration
5. Plant N content

Activities of nitrate reductase, nitrite reductase and glutamine synthetase were determined in leaves that were used for photosynthetic measurements.

3.5.6.3.1 Assay of nitrate reductase activity

Nitrate reductase (NR) catalyses the reduction of nitrate to nitrite.

\[
\text{NO}_3^- + \text{NADH} + \text{H}^+ \xrightarrow{\text{NR}} \text{NO}_2^- + \text{NAD} + \text{H}_2\text{O}
\]

The enzyme activity was estimated by the method proposed by Jaworski (1971), which is based on the reduction of nitrate to nitrite and the nitrite formed was determined spectrophotometrically.

200mg leaf tissue were taken in polythene vials and to each vial 2.5ml of phosphate buffer (pH 7.5) and 0.5ml of 0.2M potassium nitrate solution were added, followed by the addition of 2.5ml of 5% isopropanol. In order to avoid bacterial growth, 2 drops of chloramphenicol solution were added to the medium. Each vial was incubated for 2 h in dark at 30°C. After incubation 0.4ml of the reaction
mixture was taken in different test tubes. To each test tube 0.3 ml each of 1% sulphanilamide and 0.02% napthylethylenediamine dihydrochloride (NED-HCl) were added. The test tubes were kept for 20 minutes for maximum colour development. The reaction mixture in each test tube was diluted to make the volume 5 ml with double distilled water. The optical density of the solution was read at 540 nm on SL-164 UV-VIS spectrophotometer (Elico, Hyderabad, India) using a blank simultaneously.

3.5.6.3.1.1 Standard curve for nitrate reductase

30 mg sodium nitrite was dissolved in 100 ml double distilled water. From this solution, 0.8 ml was taken and again diluted to 100 ml. From this diluted solution ten concentrations viz., 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 ml were taken in separate test tubes. To each test tube, 0.3 ml each of 1% sulphanilamide and 0.02% NED-HCl were added. The solutions were diluted to 5 ml and optical density was read at 540 nm using blank on SL-164 UV-VIS spectrophotometer (Elico, Hyderabad, India). A standard curve was plotted using the selected concentrations of sodium nitrite versus optical density. The optical density of the sample was compared with a calibrated curve and nitrate reductase activity was calculated.

3.5.6.3.2 Assay of nitrite reductase activity

Reduction of nitrite to ammonia is catalyzed by nitrite reductase
Nitrite is directly reduced to ammonia without the liberation of free intermediates by nitrite reductase.

\[
\text{NO}_2^- + 6e^- \xrightarrow{\text{NiR}} \text{NH}_4^+
\]

The nitrite reductase activity was assayed using methyl viologen as reductant adopting the procedure detailed by Lillo (1984). The extraction was done at 4°C.

5g leaf tissue was homogenized in 50 ml Tris-HCl buffer (pH 7.5) in a blender for 3 minutes to force the homogenate to flow through multilayered cheese cloth. The filtrate was used as source for the enzyme.

A reaction mixture was prepared by mixing 6.25ml of Tris-HCl buffer, 2ml of sodium nitrite, 2ml methyl viologen solution and 14.75 ml double distilled water. A 0.3ml of enzyme extract was taken in different test tubes and 1.5ml of reaction mixture was added to it. To start the reaction, 0.2ml of freshly prepared 0.29M dithionite sodium bicarbonate solution was added and the mixture was incubated at 30°C for 15 minutes. The reaction was stopped by vigorous shaking on vortex mixer until the blue colour disappeared. From this 20μL aliquot was taken in another test tube and 1.0ml sulphanilamide followed by 1.0ml NED-HCl reagents was added. The volume was made 5.0ml by adding sufficient double distilled water. The solution was left as such for 30
minutes. After 30 minutes the optical density was read at 540nm on SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*) using a blank.

### 3.5.6.3.2.1 Standard curve for nitrite reductase

851mg potassium nitrite was dissolved in 100ml double distilled water. From this 10ml solution was taken in a separate flask and diluted to 100ml with double distilled water. Different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1.0ml were taken in separate test tubes and the volume was made 2ml by adding sufficient double distilled water. To each test tube 1ml each of sulphanilamide and 0.02% NED-HCl were added and were left as such for 30 minutes. The optical density was read at 540nm using a blank on SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*). A standard curve was plotted using concentrations of potassium nitrite versus optical density to calculate the enzyme activity.

### 3.5.6.3.3 Assay of glutamine synthetase activity

Glutamine synthetase is considered to have a primary role in the assimilation of ammonium into amino-acids (Miflin and Lea, 1977). It catalyzes the synthesis of glutamine.

\[
\begin{align*}
\text{L-glutamate} + \text{NH}_3 + \text{ATP} \rightarrow & \text{L-glutamine} + \text{ADP} + \text{Pi} \\
\text{Glutamine} + \text{NH}_2\text{OH} \rightarrow & \gamma\text{-glutamyl hydroyxamate} + \text{NH}_4^-
\end{align*}
\]

It also catalyzes the formation of \(\gamma\)-glutamyl hydroxamate by the transferase reaction.
The enzyme activity in the presence of Mn$^{++}$ represents the total glutamine synthetase activity.

Glutamine synthetase activity was estimated by the method proposed by Farnden and Robertson (1980). 1.0g leaf material was homogenized in 5ml of 50mM imidazole-acetate buffer (pH 7.8) containing 0.5mM EDTA, 1mM dithrothreitol, 2mM MnCl$_2$ and 20% glycerol. The extract was centrifuged at 10,000rpm at 4°C on CPR24 centrifuge (*Remi, New Delhi, India*) for 3 minutes. For the purification, the enzyme was precipitated with (NH$_4$)$_2$SO$_4$ at 60% saturation. The precipitate was re-suspended in extraction buffer. The extract was desalted over Sephadex G25.

To 0.2ml enzyme extract, 2.0ml 0.2M L-glutamine, 0.5 ml of 20 mM sodium arsenate 0.3 ml 2 mM MnCl$_2$ were added followed by the addition of 0.5ml 1mM ADP and 50mM hydroxylamine. To set a blank, 2ml of 20 mM Tris-HCl buffer instead of glutamine was added. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 1.0ml ferric chloride to the reaction mixture. The intensity of brown colour developed was measured at 540nm on SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*). A blank was run simultaneously with each set of determination.

3.5.6.3.3.1 Standard curve for glutamine synthetase

0.006g of 10mM $\gamma$-glutamyl hydroxamate was dissolved in 4ml
double distilled water. From this solution, different concentrations of γ-glutamyl hydroxamate e.g. 0.2, 0.4, 0.6, 0.8 and 1.0ml were added to 4ml of Tris-HCl buffer. The volume was maintained upto 5.0ml by adding sufficient amount of double distilled water. 1ml of ferric chloride was added to each concentration for colour development. The intensity of colour was measured at 540nm on SL-164 UV-VIS spectrophotometer (Elico, Hyderabad, India). A standard curve was plotted using the selected concentrations of γ-glutamyl hydroxamate and optical density. The optical density of the sample was compared with that of standard to calculate the quantity of γ-glutamyl hydroxamate.

3.5.6.3.4 Plant nitrogen concentration

Plant nitrogen content was estimated by Kjeldahl digestion method of Lindner (1944).

Oven dried plant material was ground in mortar and pestle to prepare a fine powder. 100mg of powder was transferred to a 50ml Kjeldahl flask to which 2ml sulphuric acid was added. The content of the flask was heated on temperature-controlled assembly for 2h to allow complete reduction of nitrates in the plant material by the organic matter itself. As a result the content of the flask turned black. After cooling the flask for about 15 minutes, 0.5ml of 30% H₂O₂ was added drop by drop and the solution was heated again until the colour turned to light yellow. After cooling for about 30 minutes additional 3-4 drops of H₂O₂ were
added followed by heating for another 15 minutes. The process was repeated till the light yellow colour turned colourless. The digested leaf material was transferred to 100ml volumetric flask with three washings with double distilled water. The volume of the flask was maintained up to the mark.

A 10ml aliquot of the digested material was taken in 50ml volumetric flask. To the flask, 2ml of 2.5N NaOH and 1ml of 10% sodium silicate solution were added to neutralize the excess of acid and prevent turbidity. The volume of the solution was made up to the mark with double distilled water. In a 10ml graduated test tube 5ml of the solution was taken and 0.5ml of Nessler reagent was added. The final volume was maintained with double distilled water. The content of the tube was allowed to stand for 5 minutes for maximum colour development. The intensity of the solution was read at 525nm on SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*).

### 3.5.6.3.4.1 Standard curve for nitrogen

50mg ammonium sulphate was dissolved in water to get 1000ml solution. From this solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0ml were taken in ten different test tubes. The solution in each test tube was diluted to 5ml with double distilled water. In each test tube 0.5ml Nessler reagent was added. After 5 minutes, the intensity of colour was read at 525nm. A blank was run simultaneously with each set
of determination.

Standard curve was plotted using different concentrations of ammonium sulphate solution versus optical density and with the help of the standard curve, the amount of nitrogen present in the sample was determined on dry weight basis.

3.5.6.3.5 Plant N Content

Plant N content was calculated as a product of N concentration and plant dry weight.

3.5.6.4 Biochemical Characteristics

The dried plant material collected at different sampling stages was used for the estimation of following biochemical characteristics.

1. Plant carbon concentration
2. Plant protein content
3. Plant carbohydrate content
4. Carbon:nitrogen ratio

3.5.6.4.1 Plant carbon concentration

Carbon content was estimated according to the digestion method proposed by Walkley and Black (1934).

Five g of the dried plant material was taken in 500ml conical flask and 10ml of 1N potassium dichromate solution and 20ml of concentrated sulphuric acid were added. After shaking for about 2 minutes, the flask was left as such for about 30 minutes for the mixture to react. Following
this, 200ml of double distilled water, 10ml of 85% ortho-phosphoric acid and 1ml of diphenyl-amine indicator were added. A deep violet colour was developed, which was titrated against 0.5N ferrous ammonium sulphate solution till the colour changed to purple and finally green. A blank was also run without the sample simultaneously. Percentage of the total organic carbon was calculated as follows:

\[
\text{Carbon (\%) = \frac{\text{Blank titration} - \text{Sample titration}}{\text{Weight of the sample (g)}} \times 0.033 \times 100 \times N}
\]

Where, \(N\) is the normality of ferrous ammonium sulphate solution used.

3.5.6.4.2 Plant Protein concentration

Protein concentration was estimated by the method of Lowry et al. (1951).

Plant material was ground to a fine powder in a mortar and pestle. 500mg of sample was further ground in 5-10ml of 5% trichloroacetic acid solution. 0.1ml and 0.2ml of each sample were taken in two test tubes and the volume was made 1ml in each test tube with double distilled water. 5ml of reagent C\(^1\) was added to each test tube including blank and centrifuged at 4000rpm. Then 0.5ml of reagent D\(^2\) was added to each test tube and mixed well. The mixture was incubated at room temperature for 30 minutes in the dark for colour development.

---

\(^1\) Reagent C: Prepared by mixing 50ml of Reagent A (2% sodium carbonate and 0.1N NaOH in 1:1 ratio) and 1ml of reagent B (0.5% copper sulphate and 1% potassium sodium tartrate in 1:1 ratio).

\(^2\) Reagent D: Prepared by mixing 50ml of 2% sodium carbonate solution and 1ml of reagent B.
intensity of blue colour developed was read at 660nm using a blank on SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*).

### 3.5.6.4.2.1 Standard curve for total protein

50mg of Bovine serum albumin was dissolved in double distilled water in a 50ml volumetric flask and the volume was maintained. From this solution, 10ml was taken and diluted to 50ml in another 50ml volumetric flask. 1ml of this solution contained 200μg protein. Different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1.0ml from this solution were taken to different test tubes and the volume was maintained to 1ml. 5ml of reagent C was added, mixed well and allowed to stand for 10 minutes. Then 0.5 ml of reagent D was added to each test tube as in sample and incubated at room temperature in the dark for 30 minutes for maximum colour development. The colour intensity was read at 660nm using SL-164 UV-VIS spectrophotometer (*Elico, Hyderabad, India*).

Standard curve was plotted using different concentrations of the working standard versus optical density. With the help of this standard curve the amount of protein present in the sample was calculated.

### 3.5.6.4.3 Plant carbohydrate concentration

Carbohydrate concentration was extracted following method of Yih and Clark (1965) and estimated by the method of Dubois *et al.* (1956).

Oven dried plant material was ground to fine powder in a mortar
and pestle. 50mg of this powder was taken in a centrifuge tubes to which 5ml of 1.5N H₂SO₄ was added. The mixture was heated on a water bath for about 2 h. After cooling, the solution was centrifuged at 4000 rpm for 10 minutes. The extract was collected in 25ml volumetric flask. The residue was washed twice with double distilled water followed by the centrifugation and collection of same washings in the same flasks. 1.0ml of this solution was taken to which 1ml of 5% aqueous phenol was added followed by the addition of 5ml concentrated H₂SO₄. The colour developed was yellow orange. After 30 minutes, the optical density was read at 490nm on SL-164 UV-VIS spectrophotometer (Elico, Hyderabad, India) using a blank sample.

3.5.6.4.3.1 Standard curve for carbohydrate concentration

100mg of glucose was dissolved in 100ml double distilled water. From this 10ml solution was taken in another 100ml volumetric flask and diluted to 100ml with double distilled water. Different concentrations such as 0.2, 0.4, 0.6, 0.8 and 1.0ml of the solution were taken in different test tubes and the volume was made 1ml with double distilled water. To each test tube 1ml of 5% phenol was added followed by the addition of 5ml of concentrated H₂SO₄. After the colour development, the mixture was left as such for 30 minutes and optical density was read at 490nm using a blank on SL-164 UV-VIS spectrophotometer (Elico, Hyderabad, India).
A standard curve was plotted using different concentrations versus optical density. With the help of the curve carbohydrate content in the sample was calculated.

3.5.6.4.4.1 Carbon:Nitrogen ratio

The carbon:nitrogen ratio was calculated by dividing plant carbon concentration by plant nitrogen concentration.

3.5.6.5 Ethylene Biosynthesis

For ethylene biosynthesis activity of ACC synthase and ethylene evolution were determined.

3.5.6.5.1 Assay of ACC synthase activity

ACC synthase activity was measured adopting the methods of Avni et al. (1994) and Woeste et al. (1999). Leaf tissue (5g) was ground in 100mM N-2 hydroxyethy-piperazine-N-2 ethanesulfonic acid buffer (pH 8.0) containing 4mM dithiothretol 2.5mM pyridoxal phosphate and 25% polyvinyl polypyrrolodone. After thorough homogenization, the preparation was centrifuged at 12,000 g for 15 minutes on CPR 24 centrifuge (Remi, New Delhi, India). 1ml of the supernatant was placed in a 30ml tube and 100ml of 5mM AdoMet was added. This was incubated for 1 h at 22°C. The ACC formed was determined by its conversion to ethylene by addition of 100μL of 20mM HgCl₂, followed by 100μL of 1:1 saturated mixture of NaOH : NaOCl. The tubes were capped immediately after addition of NaOH/NaOCl and incubated on ice
for 10 minutes. For control set, AdoMet was not added. Ethylene evolution was monitored on a gas chromatograph.

3.5.6.5.2 Ethylene evolution

A 5ml of gas phase was removed with a syringe and ethylene was measured on a gas chromatograph GC 5700 (Nucon, New Delhi, India) equipped with 1.8m Porapack N (80/100 mesh) column, a flame ionization detector. Nitrogen was used as carrier gas. The flow rate of nitrogen, hydrogen and oxygen were 30, 30 and 300ml/min respectively. The oven temperature was 100°C. The detector was at 150°C. Ethylene identification was based on the retention time and quantified comparing with the peaks from standard ethylene concentrations.

3.5.6.6 Yield Characteristics

Yield is the final manifestation of morphological, physiological and biochemical traits of a crop, which are dependent upon various environmental factors. One m² area of each plot was marked for the purpose of harvest analyses and seed yield.

At harvest following parameters were recorded:

1. Pod number per plant
2. Seed number per pod
3. 1000 seed weight
4. Seed yield
5. Biological yield
6. Harvest index

3.5.6.6.1 Pod number per plant

At harvest 25 plants from each plot were removed. The pods were collected and counted.

3.5.6.6.2 Seed number per pod

The number of seeds from 25 pods from each replicate was counted.

3.5.6.6.3 1000 seed weight

From the produce of each treatment, 1000 seeds were randomly collected with four replications and the weight was recorded.

3.5.6.6.4 Seed yield

The total seeds from 1 m² area of each plot were cleared, sun dried and weight to compute seed yield. Seed yield was transformed in one hectare field using the number of plants in one square meter field area.

3.5.6.6.5 Biological yield

The total biomass from 1 m² area of the plot was recorded from sun dried plants before threshing.

3.5.6.6.6 Harvest index

Harvest index (HI) was computed by dividing the seed yield by biological yield.

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HI = \frac{\text{Seed yield}}{\text{Biological yield}} \times 100
\]
3.5.7 Statistical analysis

All experimental data were statistically analyzed using analysis of variance (ANOVA) and Least Significant Difference (LSD) was calculated for the significant data to compare the treatments mean. Standard error mean of data for soil N analysis and different measurements for environmental conditions during sampling times was calculated. Index of relationships among different characteristics in Experiment 1 and Experiment 3 was calculated. The index number for any treatment was expressed as a percentage relative to the maximum value obtained in a treatment. The data were analyzed as described by Gomez and Gomez (1984) with the help of Microsoft Excel.