CHAPTER III

MATERIALS AND METHODS
3. MATERIALS AND METHODS

Field experiments during summer seasons of 2003, 2004 and 2005 were conducted in the old alluvial soil zone of West Bengal, India, for agronomical physiological and biochemical studies in mungbean [*Vigna radiata* (L) Wilczek] in old alluvial soil of Burdwan for i) screening of best variety, ii) screening of combined dose of biofertilizer and chemical fertilizer, and iii) screen of best time for the application of biofertilizer. Experiments were conducted in the Crop Research and Seed multiplication Farm of the University of Burdwan, West Bengal, India. The farm is situated at 87°50'51"E latitude and 23°15'12"N longitude with an average altitude of 30 meter above the mean sea level.

The materials used and methods followed during the course of studies are described below:

**Table 3.1.: Chemical composition of the experimental soil**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Values</th>
<th>Methods used</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Soil pH</td>
<td>Electronics pH meter in 1:2.5 soil water suspension as described by Jackson (1972)</td>
<td></td>
</tr>
<tr>
<td>ii) Organic carbon(%)</td>
<td>Volumetric method (Walkley and Black, 1947) as described by Muhr <em>et al.</em> (1965)</td>
<td></td>
</tr>
<tr>
<td>iii) Total nitrogen(%)</td>
<td>Kjeldahl method described by Jackson (1972)</td>
<td></td>
</tr>
<tr>
<td>iv) Total phosphorus(kg ha⁻¹)</td>
<td>As described by Saxena (1998)</td>
<td></td>
</tr>
<tr>
<td>v) Total potash (kg ha⁻¹)</td>
<td>Flame photometer method as described by Saxena (1998)</td>
<td></td>
</tr>
</tbody>
</table>
3.2. Climatic condition

University Farm is situated under monsoon-humid climate zone having a little extreme weather condition. The average annual rain fall varies from 1600-1500 mm of which maximum occurs in July-August. But owing to porous nature, medium texture and high organic matter content in soil, the soil moisture received through rainfall or irrigation water is not preserved in the sub-soil for a longer period for successful crop production, particularly during rainless days. The details of the climatic condition of the crop growing period (from 2003-2005) have been presented in the Table 3.3.

Weekly mean maximum temperature in the crop season varied from 24.2°C to 37.1°C in 2003, 27.9°C to 42°C in 2004 and 29.9°C to 40.2°C in 2005. The mean minimum temperature varied from 10°C to 20°C in 2003, 11°C to 21°C in 2004 and 21.2°C to 27.8°C in 2005.

Total rain fall received during the crop season in 2003, 2004 and 2005 were 12.0 mm, 10.4 mm and 66.5 mm respectively.

Relative humidity in 2003, 2004 and 2005 in early February were 64%, 66.4%, 58.9% and in late June were 86%, 91% and 80% respectively.

The mean sunshine hours day\(^{-1}\) varied from 5.8 hrs.day\(^{-1}\) during early February to 9 hrs.day\(^{-1}\) during the late June of 2003. The mean sunshine hours in 2004 and 2005 during the same time periods were between 5.5 hrs.day\(^{-1}\) to 10.7 hrs.day\(^{-1}\) and 6.1 hrs.day\(^{-1}\) to 11.0 hrs.day\(^{-1}\) respectively.
Table 3.2.: Climatic condition of experimental station pertaining to the period (2003, 2004 and 2005) of experimentation

<table>
<thead>
<tr>
<th>Month</th>
<th>Standard week</th>
<th>Maximum Temperature (°C)</th>
<th>Minimum Temperature (°C)</th>
<th>Total Rainfall (mm)</th>
<th>Mean Relative humidity (%)</th>
<th>Mean Sunshine hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb.</td>
<td>First</td>
<td>37.1</td>
<td>24.5</td>
<td>40.2</td>
<td>16.6</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>29.5</td>
<td>26.3</td>
<td>36.7</td>
<td>21.2</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>30.0</td>
<td>28.6</td>
<td>37.4</td>
<td>18.6</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>Fourth</td>
<td>31.2</td>
<td>30.6</td>
<td>34.5</td>
<td>15.3</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>Fifth</td>
<td>29.8</td>
<td>34.0</td>
<td>29.9</td>
<td>17.1</td>
<td>19.0</td>
</tr>
<tr>
<td>Mar.</td>
<td>First</td>
<td>33.1</td>
<td>24.5</td>
<td>34.1</td>
<td>14.5</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>29.8</td>
<td>26.3</td>
<td>31.0</td>
<td>16.3</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>30.4</td>
<td>28.2</td>
<td>33.2</td>
<td>12.1</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>Fourth</td>
<td>31.4</td>
<td>30.6</td>
<td>29.9</td>
<td>15.0</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>Fifth</td>
<td>32.6</td>
<td>34.0</td>
<td>31.6</td>
<td>14.2</td>
<td>19.0</td>
</tr>
<tr>
<td>Apr.</td>
<td>First</td>
<td>36.2</td>
<td>33.3</td>
<td>32.8</td>
<td>14.3</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>37.0</td>
<td>31.3</td>
<td>33.9</td>
<td>11.9</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>28.8</td>
<td>32.1</td>
<td>35.4</td>
<td>14.4</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>Fourth</td>
<td>27.2</td>
<td>29.4</td>
<td>31.0</td>
<td>13.6</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>Fifth</td>
<td>29.9</td>
<td>33.7</td>
<td>37.0</td>
<td>12.2</td>
<td>11.0</td>
</tr>
<tr>
<td>May.</td>
<td>First</td>
<td>27.1</td>
<td>31.7</td>
<td>34.5</td>
<td>20.0</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>28.2</td>
<td>30.4</td>
<td>37.3</td>
<td>13.6</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>31.0</td>
<td>29.8</td>
<td>37.6</td>
<td>18.0</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>Fourth</td>
<td>35.0</td>
<td>36.2</td>
<td>37.6</td>
<td>14.0</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>Fifth</td>
<td>29.1</td>
<td>34.1</td>
<td>39.1</td>
<td>11.6</td>
<td>19.1</td>
</tr>
<tr>
<td>Jun.</td>
<td>First</td>
<td>24.2</td>
<td>33.9</td>
<td>40.2</td>
<td>17.1</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>26.3</td>
<td>31.5</td>
<td>36.7</td>
<td>11.0</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>31.0</td>
<td>42.0</td>
<td>37.4</td>
<td>12.4</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>Fourth</td>
<td>30.5</td>
<td>35.2</td>
<td>34.5</td>
<td>13.5</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>Fifth</td>
<td>26.7</td>
<td>30.7</td>
<td>29.9</td>
<td>10.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

3.3. Experimental details

3.3.1. Title of the experiment

"Development of agrotechnology in old alluvial soil zone of Burdwan district, West Bengal for sustainable soil health and crop yield of mungbean (Vigna radiata(L) Wilczek)."

[50]
3.3.2. Experimental design

Field experiments were conducted in randomized block design replicated thrice. The layout of field experiments for the year 2003, 2004 and 2005 have been presented in Figs. 1, 2, and 3 respectively along with other necessary information in Table 3.3. Treatment combination along with biofertilizer application time and method of field experiments for 2003, 2004 and 2005 have presented in Table 3.4.

Table 3.3: Experimental design

<table>
<thead>
<tr>
<th>Year</th>
<th>Design</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of replication</td>
<td>Randomized block design</td>
<td>Randomized block design</td>
<td>Randomized block design</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>No. of main plot treatment (irrigation)</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>No. of sub plot treatment (plant growth regulator)</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>No. of treatment combination</td>
<td>18</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Size of each sub plot</td>
<td>5m × 3m</td>
<td>5m × 3m</td>
<td>5m × 4m</td>
</tr>
<tr>
<td></td>
<td>Season of cultivation</td>
<td>Spring to Summer</td>
<td>Spring to Summer</td>
<td>Spring to Summer</td>
</tr>
<tr>
<td></td>
<td>Variety</td>
<td>NORMAL, PDM-11, PDM-54, PDM-139, PUSA-9531, PANT MUNG</td>
<td>PDM-54</td>
<td>PDM-54</td>
</tr>
<tr>
<td></td>
<td>Spacing: Row to Row:</td>
<td>30 cm</td>
<td>30 cm</td>
<td>30 cm</td>
</tr>
<tr>
<td></td>
<td>Plant to Plant:</td>
<td>15 cm</td>
<td>15 cm</td>
<td>15 cm</td>
</tr>
</tbody>
</table>

[51]
Figure 1: Layout of the experiment 2003

V₁ = Normal (local variety); V₂ = PDM-11; V₃ = PDM-54; V₄ = PDM 139; V₅ = PUSA-9531; V₆ = PANT MUNG
Varietal performance of PDM-54 in 2003 under recommended dose of chemical fertilizer (60 days after sowing)

Varietal performance of PANT MUNG in 2003 under recommended dose of chemical fertilizer (60 days after sowing)
Varietal performance of PDM-139 in 2003 under recommended dose of chemical fertilizer (60 days after sowing)

Varietal performance of PDM-9531 in 2003 under recommended dose of chemical fertilizer (60 days after sowing)
Pods of PDM-54 and PDM-139 under the trial of 2003 under recommended dose of chemical fertilizer

Varietal performance of PDM-54 in 2003 under recommended dose of chemical fertilizer (60 days after sowing)
Varietal performance of PDM-11 in 2003 under recommended dose of chemical fertilizer (60 days after sowing)

Pods of different varieties in 2003
Figure 2: Layout of the experiment 2004

T₁ = 20% N-less + 25% P-less + K-normal; T₂ = 30% N-less + 25% P-less + K-normal;
T₃ = 40% N-less + 25% P-less + K-normal; T₄ = 50% N-less + 25% P-less + K-normal;
T₅ = 60% N-less + 25% P-less + K-normal; T₆ = N : P : K - 20 : 40 : 20
Preparation of experimental field (2005)

Application of chemical fertilizer in reduced dose: (2004)

- $T_1 = 20\%$-less nitrogen + $25\%$-less phosphate + potassium normal
- $T_2 = 30\%$-less nitrogen + $25\%$-less phosphate + potassium normal
- $T_3 = 40\%$-less nitrogen + $25\%$-less phosphate + potassium normal
- $T_4 = 50\%$-less nitrogen + $25\%$-less phosphate + potassium normal
- $T_5 = 60\%$-less nitrogen + $25\%$-less phosphate + potassium normal
- $T_6 =$ Recommended dose: N : P : K = 20 : 40 : 20
Crop on 30 DAS of 2004 under 30%-less nitrogen and 25%-less phosphate and normal potassium + Rhizobium inoculation (basal)

Crop on 30 DAS of 2004 under 40%-less nitrogen and 25%-less phosphate and normal potassium + Rhizobium inoculation (basal)
Crop on 35 DAS of 2004 under 20%-less nitrogen and 25%-less phosphate and normal potassium + Rhizobium inoculation (basal)

Crop on 35 DAS of 2004 under 50%-less nitrogen and 25%-less phosphate and normal potassium + Rhizobium inoculation (basal)
Crop in the field on 40 DAS (2004, closed view)

Overview of the entire crop field (2004)
Figure 3: Layout of the experiment 2005

\[ T_1 = \text{As basal 1.5 kg ha}^{-1} + 21 \text{ DAS 0.75 kg ha}^{-1} \text{ spray} + 42 \text{ DAS 0.75 kg ha}^{-1} \text{ spray}; \]
\[ T_2 = \text{Only basal application 3 kg ha}^{-1}; \]
\[ T_3 = \text{As basal 1.5 kg ha}^{-1} + 21 \text{ DAS 1.5 kg ha}^{-1} \text{ soil application}; \]
\[ T_4 = \text{As basal 1.5 kg ha}^{-1} + 21 \text{ DAS 1.5 kg ha}^{-1} \text{ spray}; \]
\[ T_5 = \text{As basal 0.75 kg ha}^{-1} + 21 \text{ DAS 1.5 kg ha}^{-1} \text{ soil application} + 42 \text{ DAS 0.75 kg ha}^{-1} \text{ soil application}; \]
Supervisors of the research work during preparation of experimental field (2005)

Sowing of seeds in experimental field (2005)
Drying of biofertilizer after mixing with charcoal

Sowing of seeds (2005)
Crop in the experimental field (10 DAS, 2005)

Crop in the experimental field (15 DAS, 2005)
Overview of crop (all the varieties) in the field (2005)

Data recording view of the research student (2005)
The plots were demarcated by ridges on all sides and to facilitate irrigation evenly irrigation channels were made.

3.3.3. Details of experimental treatments

Critical growth stages of mungbean, namely NORMAL(T1), PDM-11(T2), PDM-54(T3), PDM-139(T4), PUSHA-9531(T5) and PANT MUNG (T6).

Table 3.4.: Calendar of operations

<table>
<thead>
<tr>
<th>Operation</th>
<th>Date</th>
<th>Details of operations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Land preparation</td>
<td>2.03.03</td>
<td>Two harrow followed by one laddering to make the soil well pulverized and make the land free of clods and weeds.</td>
</tr>
<tr>
<td>2. Lay-out</td>
<td>03.03.03</td>
<td>Lay-out was done according to the given plan (Fig 1, 2, &amp; 3).</td>
</tr>
<tr>
<td>3. Chemical fertilizer application</td>
<td>10.03.03</td>
<td>First year (2003): nitrogen @ 20 kg ha(^{-1}) as urea and phosphorus @ 40 kg ha(^{-1}) as DAP, and potass @ 20 kg ha(^{-1}) at MOP were supplies as basal application in each plot just before sowing. This was done after the lay-out of the experiment. Amount was same for each plot. Second year (2004): nitrogen was reduced in different rate such as 25 %, 30 %, 40 %, 50 %, 60 % and normal recommended dose (N: P: K=20: 40: 20) phosphorus was reduced at constant rate 25 %. Potassium remain same. Third year (2005): taking best reduction dose of 30 % nitrogen and 25 % phosphorus reduction.</td>
</tr>
<tr>
<td>4. Seed inoculation and sowing</td>
<td>03.03.03</td>
<td>Mung bean was sown continuously on line at 30 cm row to row distance. Seed rate was 4 kg ha(^{-1}). Before sowing seed inoculation has been done for 12 hours with \textit{Rhizobium} biofertilizer at shade.</td>
</tr>
<tr>
<td>5. Thinning</td>
<td>25.03.03</td>
<td>Thinning was made keeping 10 cm plant to plant distance within the rows.</td>
</tr>
<tr>
<td>6. Intercultural operation</td>
<td>28.03.03</td>
<td>Simultaneous hoeing and weeding was made to keep the plot free from weeds.</td>
</tr>
<tr>
<td></td>
<td>28.04.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.03.04</td>
<td></td>
</tr>
<tr>
<td>7. Irrigation</td>
<td>18.03.03</td>
<td>The crop was irrigated as per treatment.</td>
</tr>
<tr>
<td></td>
<td>02.04.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.04.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>05.05.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>02.05.04</td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Operation</th>
<th>Date</th>
<th>Details of operations</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. Plant protection</td>
<td>27.04.03</td>
<td>Not applied</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insecticide (Vazrn) was spray to keep the plot free from insect.</td>
</tr>
<tr>
<td>9. Leaves and plat collection</td>
<td>24.03.03</td>
<td>Not applied</td>
</tr>
<tr>
<td></td>
<td>12.04.04</td>
<td>Leaves and plant collection for agronomic and biochemical analysis at 20 DAS, 40 DAS and 60 DAS.</td>
</tr>
<tr>
<td></td>
<td>04.05.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.05.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.03.05</td>
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</tr>
<tr>
<td></td>
<td>10.04.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.04.05</td>
<td></td>
</tr>
<tr>
<td>10. Harvesting</td>
<td>16.06.03</td>
<td>Not applied</td>
</tr>
<tr>
<td></td>
<td>19.06.04</td>
<td>Harvesting was made after the crop matured.</td>
</tr>
<tr>
<td></td>
<td>06.06.05</td>
<td>The crop was harvested with sickle in the morning hours to avoid shattering of the capsules. Earmarked (except ring line) portion plants were tied in bundles and tagged with corresponding treatments.</td>
</tr>
<tr>
<td>11. Threshing</td>
<td>23.06.04</td>
<td>Not applied</td>
</tr>
<tr>
<td></td>
<td>11.06.05</td>
<td>The harvested bundles were stacked for 5 days, sun-dried and then threshed. After sun-drying and cleaning of the threshed mung bean grain, weighing was made to have yield (kg ha⁻¹) of the respective treatments.</td>
</tr>
</tbody>
</table>

3.4. Methods of recording observations

The observations for the morpho-physiological growth attributes, yield attributes and yield were taken at various stages of crop growth from the area earmarked for destructive sampling. Border rows and plants around each plot were not taken into consideration for observation(s) and yield estimation to avoid border effect.

3.4.1. Length of shoot

The plants from each plot were randomly selected and height of these plants was scaled from ground level to the tip of the stem on three dates(20DAS, 40DAS and 60DAS). Average of the ten plants height was taken into consideration.

3.4.2. Length of root

Similarly, ten randomly selected plants from each plot were uprooted with much care to avoid any tear-out of roots. Roots were scaled and average of ten was taken.

[53]
3.4.3. Yield components, their associate characters and yield

3.4.3.1. Pod length and circumference

Ten number of capsules were selected randomly from upper, middle and lower portion of plant from each plot to measure length and circumference with ribbon. Then the ribbon length was scaled and final average length and circumference of capsules were tabulated.

3.4.3.2. Number of grains per pod

Ten capsules of different sizes were randomly selected at maturity from each plot and they were excised carefully. Total number of seeds was counted and the average was tabulated.

3.4.3.3. Test weight of grain

After threshing, cleaning and sun drying of composite seed sample from each plot was taken and 1000 seeds were counted separately for each treatment and weighed to record test weight.

3.4.3.4. Grain yield

After threshing, cleaning and sun drying of composite seed sample from 1.0m² area of each plot was taken and total seeds were weighed to record as grain yield.

3.5. Leaf Area Index (LAI)

The representative green leaf lamina from each treatment were taken randomly from destructive samples and the area of leaves was measured by cm graph paper. Leaves were then dried in a hot air oven at 80°C till constant weights were obtained and then weights were recorded. The ratio of area/weight of the measured leaves was used to determine the leaf area
indices (Kemp, 1960). Since LAI is the area of leaf surface per unit of land surface (Watson, 1952), it (LAI) was obtained by multiplying this ratio of area/weight with the dry weight of green leaves produced per unit area (square meter) of land surface.

3.6. Leaf Area Ratio (LAR)

Leaf area ratio is the ratio of leaf area and total biomass. It can be calculated with the following formula:

\[
\text{LAR} = \frac{\text{LA}}{\text{W}} \text{ (m}^2 \text{ g}^{-1})
\]

where \( \text{LA} = \) Leaf area in \( \text{m}^2 \)

\( \text{W} = \) Dry weight of plant

3.7. Leaf Area Duration (LAD)

Leaf area duration is the integrated leaf area index (LAI) over time. It can be calculated with the following formula:

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

where \( \text{LAI}_1 \) and \( \text{LAI}_2 \) are the initial and final leaf area index respectively over a specified period of time \( T_1 \) and \( T_2 \).

3.8. Crop Growth Rate (CGR)

Crop growth rate during the period of two growth stages was determined with the following formula:

\[
\text{CGR} = \frac{W_2 - W_1}{t_2 - t_1} \text{ g m}^{-2} \text{ day}^{-1}
\]

[55]
where \( W_2 \) and \( W_1 \) are the final and initial total dry weights of above ground plant parts per unit land area (m\(^2\)) at the consecutive time \( t_2 \) and \( t_1 \) respectively.

### 3.9. Net Assimilation Rate (NAR)

Net assimilation rate was determined by using the following formula given by Watson (1952):

\[
\text{NAR} = \frac{W_2 - W_1}{t_2 - t_1} \times \frac{L_2 - L_1}{\log L_2 - \log L_1} \text{ g m}^{-2} \text{ day}^{-1}
\]

where \( W_2 \) and \( W_1 \) are the final and initial dry weights of aerial plant parts per unit area at the time \( t_1 \) and \( t_2 \) respectively and \( L_2 \) and \( L_1 \) are the final and initial leaf area indices at respective times.

### 3.10. Biomass

Ten randomly selected plants from each plot were uprooted with much care to avoid any tear-out of roots and oven dried at 80°C for 24 hours and subsequently dry weight was taken.

### 3.11. Estimation of chlorophyll level from leaf

For chlorophyll (a, b and total) estimation, physiologically active leaves (3\(^{rd}\) or 4\(^{th}\) from the top) were collected randomly from 5-plants of a plot to represent the sample. The method of Arnon (1949) was followed for the chlorophyll estimation.

**Materials required**

1. Acetone

2. Whatmann filter paper No. 1
3. Funnel stands
4. Mortar and pestle
5. Volumetric flask (250 ml), and

**Procedure**

80% acetone was prepared 100 mg of fresh leaf material from mixed representative sample was accurately weighed (excluding mid ribs) on electronic monopan balance and transferred to mortar and pestle for grinding. 2 ml of 80% acetone was then added in it and leaves were thoroughly macerated. A homogenous paste was made. Chlorophyll extract was poured in a funnel having Whatmann filter paper No. 1 and collected in volumetric flask. Every care was taken to collect all the extract from the mortar. The green extract was gradually obtained by adding 2 ml of acetone (80%) every time. Three to four washings were given and extraction continued until leachate became colourless. Volume was made to 10 ml with 80% acetone. The optical density (OD) of the chlorophyll extract was recorded on spectrophotometer using 645, 652 and 663 nm wavelengths. OD values of duplicate samples were taken.

Following formulae were used for calculation of chlorophyll a, b and total chlorophyll:

\[
\text{Chlorophyll-a (mg g}^{-1}\text{ FW)} = [12.7 \times D_{663} - 2.69 \times D_{645}] \times V/1000 \times W
\]

\[
\text{Chlorophyll-b (mg g}^{-1}\text{ FW)} = [22.9 \times D_{645} - 4.68 \times D_{663}] \times V/1000 \times W
\]

\[
\text{Total Chlorophyll (mg g}^{-1}\text{ FW)} = D_{652} \times 1000/345 \times V/1000 \times W
\]

where D = Optical density, V = Final volume of 80% acetone (25 ml), W = Fresh wt. Sample taken (1.0 g), FW = Fresh weight
3.12. Estimation of total soluble sugar

The method of Mc. Cready et al (1950) was followed to give a blue-green solution having absorbance maximum at 630 nm was measured. Plotting absorbance vs. concentration of D-glucose drew a standard curve.

Materials required

1. 0.2% anthrone solution: 200 mg of anthrone was dissolved immediately before use in 100 ml conc. H₂SO₄ under refrigeration condition.

2. Standard glucose: Stock – dissolved 100 mg glucose in 100 ml distilled water. Working standard of 10 ml stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.

3. Glass wares: Test tubes, beakers, pipettes, reagent bottles, measuring cylinder, centrifuge tube.

4. Miscellaneous materials: Test tube rack mortar and pestle

Procedure

1. Preparation of different standard sugar solutions. (for calibration curve): First the standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8, 1.0 ml of the working standard. ‘0’ serves as the blank. The final volume was made up to 1 ml in all the tubes. The 4 ml of anthrone regent was added. The mixture was heated for 8 minutes in a boiling water bath. It was cooled rapidly and green to dark green colour at 630 nm. A standard curve was drawn by using Microsoft Excel and the amount of sugar present in the sample tube was calculated.
2. Preparation of the sample: About 1.0 g of fresh leaves weighted and crushed with a pestle in a mortar first with 3 ml and then with 7 ml distilled water. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected. 1 ml from this solution was pipetted out in a test tube, 4 ml freshly prepared 0.2 % anthrone reagent was added in it and incubated for 10 minutes at 100 °C. This was allowed to cool to room temperature.

3.13. Estimation of amino acid

For the estimation of amino acid the method of Moore and Stein (1948)

Materials required

1. 0.3 % ninhydrine: 300 mg of ninhydrine was dissolved in 100 ml of 90 % ethanol.
2. Standard amino acid: Glycine solution (1mg/ml).
3. 90 % ethanol
4. Glass wares: Test tubes, beakers, macro and micro pipettes, reagent bottles, measuring cylinder, cuvettes and centrifuge tubes.
5. Equipments: Spectrophotometers, incubator, chemical balance.

Procedure

1. Preparation of different standard amino acid (glycine) solutions (for calibration curve): A set of standard solution including the blank was
prepared into test tubes with different aliquots of stock solution (glycine) along with water using micro and micropipettes respectively. 1 ml ninhydrine reagent was added to each of the above 10 test tube and incubate for 15 minutes at 100°C in a water bath. It was allowed to cool to room temperature. Then 5 ml of 90% ethanol was added to each test tube and mixed well.

2. Preparation of the sample: 10 mg plant material was weighed out accurately and crushed with a pestle in a mortar with 10 ml of distilled water. This was transferred to a centrifuge tube and centrifuged for 10 minutes at 5000 rpm. Now 0.5 ml from the supernatant liquid was pipetted out in another test tube, 1 ml ninhydrine reagent and 5ml of 90 % ethanol was added in it. It was mixed well.

3. Measurement of O.D: O.D of the standard amino acid (glycine) solution was measured as well as the unknown sample solution at 570 nm.

4. Calibration curve: A plot of the absorbance against the concentration is drawn using micro-soft excel.

5. Calculation: From the calibration curve corresponding to the measured absorbance, the concentration of the unknown sample was found to be x mg/ml.


Estimation of protein was performed by following the method of Lowry et al, (1951).
Materials required

1. Preparation of protein reagent-A:
   a) 2 gm of Na₂CO₃ and 400 mg of NaOH were dissolved in 100 ml of distilled water.
   b) 1 gm of CuSO₄ was dissolved in 100 ml distilled water.
   c) 2 gm of sodium-potassium-tartarate was dissolved in 100 ml of distilled water.
   d) Protein reagent A (mixture): a: b: c = 100 : 1 : 1

2. Preparation of protein reagent –B: The purchased Folin-phenol solution was diluted in the ratio of 1:1 with distilled water.

3. Standard protein: BSA (1 mg/ml)

4. Glass wares: Test tubes, beakers, macro and micropipettes, reagent bottles.

5. Equipments: Spectrophotometer, incubator, chemical balance, centrifuge.

6. Miscellaneous materials: Test tube rack, mortar and pestle.

Procedure

1. Preparation of different standard protein solutions(for calibration curve): A set of standard solutions including the blank was prepared into test tubes with different aliquots of stock solution (protein) along with water using micro and macro pipettes respectively 0.9 ml of reagent a was added to each of the 10 test tube and incubated for 15 minutes at room temperature. Then 0.1 ml of reagent B was added and allowed to stand for 30 minutes. After some time 5 ml of distilled water was added to each test tube and mixed well.
2. Preparation of the sample: 10 mg of the plant material was weighed out and crushed first with 3 ml and then 7 ml of distilled water. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 1000 rpm. 0.25 ml from the supernatant liquid was pipetted out in a test tube and added the protein reagents.

3. Calibration curve: A plot of the absorbance against the concentration is drown using micro-soft excel.

4. Calculation: from the calibration curve corresponding to the measured absorbance at 620 nm, the concentration of protein was calculated to be X mg/ml.

3.15. Estimation of ascorbic acid

Estimation of ascorbic acid was done by following the method of Harris and Ray (1935).

Materials required

1. Preparation of 2% oxalic acid solution: 20 g of oxalic acid was dissolved in distilled water and diluted to 1 litre.

2. Preparation of dye (2, 6-dichlorophenol indophenol blue) solution: Accurately 100 mg of the sodium salt of the dye stuff was weighed out in a 250 ml volumetric flask, dissolve with 100 ml hot distilled water and make up the volume upto the mark with the warm water (stored in refrigerator).

3. Preparation of standard ascorbic acid solution: (100 mg/L): Weighed 25 mg A.R 1-ascorbic acid accurately and made up the volume up to the mark with 2 % oxalic acid solution.
**Procedure**

1. Standardization of the dye stuff solution: 10 ml of the standard ascorbic acid solution was pipetted out in a 100 ml conical flask and titrated the solution with the dye stuff solution adding from the burette until the solution turned pink and stable for 15 seconds.

2. Preparation of sample: 1g of the sample was weighed out and crushed with 10 ml of 2% oxalic acid solution in a mortar. It was transferred in a centrifuged tube and centrifuge for 10 minutes at 1000 rpm. Then the supernatant liquid transferred in a 250 ml volumetric flask. The pup was washed with 2% oxalic acid, collected the washing in the flask and made upto the volume with the same oxalic acid solution.

3. Estimation of ascorbic acid: 10 ml of the sample was pipetted out in a 100 ml conical flask and titrated the solution with the dye stuff solution adding from the burette until the reactions turned pink and stable for 15 second.

3.16. **Estimation of phenol**

Phenol was estimated by following the method of Malik and Singh (1980).

**Materials required**

1. \( \text{Na}_2\text{CO}_3 \) solution (20%): 20g of \( \text{Na}_2\text{CO}_3 \) was dissolved in 100 ml of distilled water.

2. Folin-phenol reagent: The supplied Folin-phenol solution was diluted in the ratio of 1:1 with distilled water.
3. Stock standard solution of catechol (1mg/ml): 50ml of catechol was accurately weighted out in a 50 ml volumetric flask, dissolved and diluted upto the mark with distilled water.

4. Working standard solution: From the above solution 1 ml was taken out and the volume was make up to 100 ml in volumetric flask. The concentration of the solution was made 0.01 mg/ml.

5. 80% ethanol solution:

6. Sample : plant material(leaf and seed)

7. Glass wares: test tubes, beakers, macro and micro pipettes, reagent bottles, measuring cylinder, cubettes, centrifuge tube.

8. Equipments: Spectrophotometer (Systronics-118), incubator (Remi-CI-6S), electrical balance (Sartorius-BL 60S), centrifuge (Rmi-R 24), waterbath.

9. Miscellaneous materials: Test tube rack, mortar and pestle.

Procedure

1. Preparation of different standard catechol solution: A set of standard solutions including the blank were prepared in 9 test tubes with different aliquots of stocked catechol solutions (0.01 mg/ml). For this purpose 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 ml for standard catechol solution using micro pipettes into all the test tubes diluted each solution up to 3.0 ml with distilled water using micro pipettes.

A blank solution was prepared taking 3.0ml distilled water in another test tube. Now 0.5 ml Folin-phenol was added to each of the above nine test tubes and incubated for 5 minutes at room temperature. Then
1.0 ml 20% Na$_2$CO$_3$ solution was added to each of them and mixed well.

2. Measurement of OD: OD of the standard solutions as well as the unknown sample solution was measured at 650 nm.

3. Calibration curve: A plot of the absorbance against the concentration is drawn using micro-soft excel.

4. Calculation: The concentration of the unknown sample solution was found from the calibration curve.

3.17. Estimation of RNA and DNA from biological materials (Choudhuri and Chatterjee, 1970)

Materials required

1. 10% cold TCA
2. Ethanol: Ether (1:1)
3. 0.5(N) NaOH
4. HClO$_4$
5. Diphenylamine Reagent: Dissolved 1g of DPA in 100 ml of glacial acetic acid and add 2.5 ml of concentrated H$_2$SO$_4$. This must be prepared fresh.
6. Orcinol Reagent : Dissolved 300 mg of orcinol in 5 ml of ethanol. Add 3.5 ml of this just before use to 100 ml of 0.1% solution of FeCl$_3$ in concentrated HCl.
7. Preparation of standard curve: Authentic sample of RNA and DNA were taken. took solutions of RNA and DNA as 100 µg/ml in Saline
citrate buffer (SCB) [0.15 M NaCl and 0.015 M Sodium citrate, pH 7.0] were prepared.

8. From the stock solution of both RNA and DNA the following gradients were made (separately form RNA and DNA).

Procedure

1. Homogenize about 1g plant tissue with 10 ml of (if needed more amount can be added) cold 10% TCA and centrifuged for 10' at 4000 rpm (use neutral sand for crushing).

2. The pellet were taken and suspended in 5 ml (if needed more can be added) of ethanol: ether mixture (1:1). Centrifuged for 10' for 4000 rpm.

3. Take the pellet and suspend in 5 ml of 0.5 N NaOH. Mix well and leave at 37°C for 18hrs.

4. Centrifuged for 10 minute at 4000 rpm. Keep the supernatant and pellet separately. Pellet contained some protein and most DNA. Supernatant contained RNA in hydrolyzed form as well as proteins.

For DNA

1. The pellets were taken and to it add 1 ml of perchloric acid heated in boiling water bath for 1 hr cool to room temperature.

2. Centrifuged for 10' at 4000 rpm and take the supernatant (sup) which contained nucleotides released from DNA. Make upto known volume with HClO₄ and estimate DNA by diphenylamine reagent.
For RNA

The supernatant from step 4 and add equal vol. of 10% TCA and mixed well. Centrifuged for 10' at 4000 rpm.

1. The supernatant which contains nucleotides released from RNA was taken and up to known volume with 10% TCA and RNA was estimated by adding orcinol reagent.

Development of colour

A. For DNA: Take 3 ml of sample and add 5 ml of diphenylamine reagent. Heated in a boiling water bath for 10-15 minutes and cooled to room temperature read at 595 nm.

B. For RNA: 2 ml of sample was taken and to it 3 ml of orcinol reagent. Heated in boiling water bath for 10-15 minutes and cooled to room temperature read at 665 nm.

3.18. Estimation of pH from soil (Jackson, 1972)

Materials required

1. pH meter (Sartorius-CP64) with glass electrodes.

2. Thermometer

3. Buffer solutions, preferably of pH 4.0 and 9.2 buffers of different pH was prepared by dissolving standard pH tablets in 100 ml of distilled water.

Procedure

Put the switch on and recorded pH 7.0. Washed the electrode with distilled water and connected it with the pH meter. Dip the electrode in buffer of pH 4.0 and move the temperature compensation knob to the
temperature of buffer. Turn selector switch to pH range of 0-7 and adjust the set buffer knob until the meter reads pH 4.0. Move selector switch to zero. Remove the electrode from buffer, and washed it with distilled water. Dipped in a buffer of pH 9.2. Turned the selector switch to pH range of 7-14 and adjusted the set buffer knob until the meter reads pH 9.2. In doing so, the meter was calibrated both for pH ranges from 0 to 7 and from 7 to 14. Put selector switch to zero. Washed the electrode with distilled water and dipped in the sample. Adjusted temperature compensation knob to the temperature of sample. Put selector switch to pH range of 0 to 7 and read the meter for the pH of sample. If pH exceeds 7, move selector switch to pH range of 7 to 14 and read the meter. Turned the selector switch to zero, switched off the instrument and remove the electrode. Keep the electrode dipped in distilled water when not in use.


Materials required

1. Conductivity meter
2. Conductivity cell
3. Thermometer

Procedure

Studied carefully the operation manual of the conductivity meter (-306). Note the temperature of sample and adjusted the temperature compensation knob of the conductivity meter to the temperature of sample. Keep the selector switch to ×1000 and calibrate to CAL mark. Dipped the conductivity cell in the sample contained in a beaker and connected the cell
terminals to the sockets provided in the instrument. If meter shows negligible deflection, disconnect the cell terminals. Move the selector switch to x100 and calibrate to CAL mark. Reconnected the cell terminals and noted the deflection. If it is still negligible, disconnected the cell, moved the selector switch to x10 and calibrated to CAL mark. Reconnected the cell and noted the deflection (dial reading). Switched off the meter, disconnected the cell and washed it with distilled water.

**Calculation**

\[
EC (S) = DR \times SS
\]

where, \( EC = \) electrical conductivity; \( DR = \) dial reading; and \( SS = \) value of selector switch.

In meters not provided with selector switch and temperature compensation knob, EC is computed as follows:

\[
EC (S) = OEC \times CC \times TF \text{ at } 25^\circ C
\]

where, \( OEC = \) observed conductance; \( CC \) cell constant (supplied by the manufacturer); and \( TF = \) temperature factor.

### 3.20. Estimation of organic carbon from soil (Wakely & Black, 1947)

**Materials required**

1. Standard 1(N) \( K_2Cr_2O_7 \) solution: Accurately weighed out 12.258 g air dried \( K_2Cr_2O_7 \) in a 250 ml volumetric flask dissolved and diluted up to mark with distilled water

2. Conc. \( H_2SO_4 \) (sp. gr. 1.84)
3. Syrupy H₃PO₄ (35 %)

4. 0.5 (N) Mohr salt solution: 49.0 g (NH₄)₂SO₄, FeSO₄, 6H₂O was dissolved in 200 ml distilled water and 5 ml conc. And diluted to 250 ml.

5. Diphenyl amine indicator: 0.5 g of diphenyl amine was dissolved in 20 ml water and 100 ml of conc. H₂SO₄.

Procedure

1. Two 500 ml conical flask was taken.

2. 1.0 g of soil sample was added to one of the flask.

3. 10 ml of K₂Cr₂O₇ solution was added to each of the flask.

4. 20 ml of conc. H₂SO₄ was added to each of the flask.

5. The flask were covered with a watch glass and allowed to stand for about half an hour.

6. 200 ml of distilled water was added to each of the flask.

7. 10 ml of orthophosphoric acid was added to each of the flask.

8. The solution turned blue in colour after addition of 1 ml diphenyl amine indicator. (Hest prior to titration 10 drops D.P.A was added into the flask).

9. Titration was done with 0.5 (N) Mhor's salt or FAS.

10. The solution turned blue to green in colour at the end of the titration.

Calculation

Organic carbon % in the soil sample

\[ Q = 0.5 \times (B - S) \times 0.003 \times (100/W) \] .......................... (1)
where \( W \) = weight of the soil sample taken.

\[ B = \text{volume of FAS solution used for blank titration (in ml).} \]

\[ S = \text{volume of FAS solution used in sample titration in ml.} \]

So, putting the values of \( W, B, S \) in the equation (1) we get the value of \( Q \).

3.21. Estimation of soil total nitrogen (Jackson, 1972)

Materials required

1. Conc. \( \text{H}_2\text{SO}_4 \)
2. \( \text{CuSO}_4 \)
3. \( \text{Na}_2\text{SO}_4 \)
4. 40\% \( \text{NaOH} \)
5. 4\% Boric acid
6. Mixed indicator (Bromocresol green)
7. 0.02 (N) \( \text{H}_2\text{SO}_4 \)

Procedure

1. 0.5 g of the sample was taken in a Kjeldahl flask and 0.5 g of \( \text{CuSO}_4 \) and 2 g of \( \text{Na}_2\text{SO}_4 \) is added to it.

2. To it 7 ml of conc. \( \text{H}_2\text{SO}_4 \) was added and the flask is heated to avoid loss through foaming.

3. Gradually the sample turns dark. As the digestion proceeds the colour of the sample turns pale green. The heating is continued for additional 30 minutes.
4. The flask is cooled and 200 ml of distilled water was added to it. The solution is made alkaline with 40 % NaOH until flocculation starts.

Distillation

1. The solution is distilled and the distillate was collected in 50 ml of Boric acid.

2. The ammonia absorbed in boric acid is titrated with 0.02 N H₂SO₄ using a mixed indicator of methyl red and bromocresol green. The end point of the titration is red colour.

Calculation

Therefore the amount of total nitrogen in the sample in percentage:

\[
= \frac{\text{Burette reading} \times 0.28}{\text{Sample taken} \times 1000} \times 100
\]

3.22. Estimation of PO₄³⁻ (Saxena, 1998)

Materials required

1. Potassium dihydrogen phosphate (KH₂PO₄)

2. Ammonium molybdate

3. Stanous chloride (SnCl₂)

Procedure

KH₂PO₄ was used to prepare standard calibration graph in the range of 0.2mg/L – 1mg/L. During analysis 4 ml of ammonium molybdate and 0.5 ml of SnCl₂ and 5 ml of sample generally used in the 100 ml volumetric flask and make up the volume up to 100 ml. After 10-12 minutes, OD was measured through spectrophotometer. Then the phosphate concentration was
determined with the help of the previously done calibration graph with measured OD of the sample. OD was measured at 690 nm.


Materials required

1. Potassium chloride
2. Distilled water

Procedure

KCl is used for the preparation of calibration curve in the range of 2 mg/L - 10 mg/L. Again the digested sample was taken for analysis of ‘K’ of the soil samples, with the help of flame photometer reading with the help of the calibration curve through calculation.


For analysis of soil biological parameter the book of Experiment in Microbiology and Plant Pathology, Tissue Culture and Mushroom Production Technology was consulted.

Plate counting

1. Material required For nutrient medium, Peptone, Beef extract, Agar, Distilled water.

2. For dilution plating: Distilled water test tubes, 1 ml micro-pipettes, petridishes, test tube stand, incubator, laminar air flow.

Composition of medium

1. Thronton Medium
i) Dipotassium phosphate 1.0 g
ii) Potassium Nitrate 0.5 g
iii) Magnesium sulphate 0.2 g
iv) Calcium chloride 0.1 g
v) Sodium chloride 0.1 g
vi) Ferric chloride trace
vii) Aspargin 0.5 g
viii) Agar Agar 15 g
ix) Mannitol 1.0 g
x) Distilled water 1.0 L

pH 7.2 ± 0.1

**Procedure**

1 g of soil was dissolved in 100 ml distilled water. 9 ml of sterile distilled water was taken in each test tube kept in a test tube stand. 1 ml of soil solution was added to 9 ml of sterile distilled water to give a dilution of $10^{-1}$. Similarly, 1 ml of solution from this test tube was transferred to 9 ml sterile distilled water in the next tube to prepare a dilution of $10^{-2}$. Thus, dilution of $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ were prepared. About 25 ml of molten Agar was plated in petridishes and 1 ml of soil mixed with water from each dilutions was added to the agar and kept at 37°C in the incubator after solidification. The mixture was spread uniformly. The bacterial colony forming units were counted at a mean dilution after 48 hours.
Calculation

No. of cells/ml = No. of colonies/amount plated \times \text{dilution}

Since 1 ml contain $X$ cells.

100 ml contain $100 \times X$ cells.

Since 1 g soil was dissolved in 100 ml of solution.

So, 1 g of soil contains $100 \times X$ cells.

3.25. Methods of statistical analysis

To analyze the tabulated data as observed in the field experiment and laboratory analysis in different aspects, Cochran and Cox (1959). Fisher (1960), Panse and Sukhatme (1967), Gomez and Gomez (1984) were consulted. For interpreting the effect of different treatments under different cases, for comparison of F-values and for determination of Least Significant Difference (L.S.D) at 5% level of significance, Fisher & Yates table (1953) was followed.