Material & Methods
3.1 Preparation of slow ammonium releasing granules

Small round granules of brick soil were obtained from brick kiln situated at Sonepat town in Haryana state. Agro waste like cow dung, rice bran and neem leaves were collected locally. All the collected materials were dried separately in a oven for 3 days and powdered in a grinder and mixer. This supporting matrix were then mixed in different ratios to get different type of slow release super granules (SRFs) (Table 3.1). Ammonium sulphate \([\text{(NH}_4\text{)}_2\text{SO}_4]\) was mixed with these different combinations in a ratio of 200/500 mg of the supporting matrix g\(^{-1}\) and finally immobilized with the help of 10% and 25% of polyvinyl alcohol (PVA) (Table 3.1).

3.2 ESTIMATION OF RELEASED AMMONIUM FROM SLOW AMMONIUM RELEASING GRANULES IN WET SOIL AND WATER

Ammonium released was measured periodically from organo based slow release supergranules using method given by M.W. Weatherburn (1967) with some modifications as follows:

3.2.1 Preparation of Reagents

Reagent A:

5 gm. of phenol and 25 mg. of sodium nitroprusside were dissolved in 500 ml of distilled water and stored in amber bottle for 1 month.

Reagent B:

2.5 g of sodium hydroxide and 4.2 ml of sodium hypochlorite were dissolved in 500 ml of distilled water and stored in amber bottle for 1 month.
Table 3.1: Composition of different combinations of supporting and binding materials used to prepare SRFs

<table>
<thead>
<tr>
<th>Materials used as supporting matrix</th>
<th>Ratio of supporting matrix</th>
<th>Conc. of Polyvinyl alcohol</th>
<th>Symbol of SRFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS</td>
<td>10%</td>
<td>ESG1</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>3 : 1</td>
<td>10%</td>
<td>ESG2</td>
</tr>
<tr>
<td></td>
<td>1 : 3</td>
<td>10%</td>
<td>ESG2</td>
</tr>
<tr>
<td></td>
<td>1 : 1</td>
<td>10%</td>
<td>ESG2</td>
</tr>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS</td>
<td>25%</td>
<td>ESG4</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>3 : 1</td>
<td>25%</td>
<td>ESG5</td>
</tr>
<tr>
<td></td>
<td>1 : 3</td>
<td>25%</td>
<td>ESG6</td>
</tr>
<tr>
<td></td>
<td>1 : 1</td>
<td>25%</td>
<td>ESG6</td>
</tr>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS : RB</td>
<td>10%</td>
<td>ESG7</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>2 : 1 : 1</td>
<td>10%</td>
<td>ESG8</td>
</tr>
<tr>
<td>Rice bran (RB)</td>
<td>1 : 1 : 1</td>
<td>10%</td>
<td>ESG9</td>
</tr>
<tr>
<td></td>
<td>1 : 2 : 1</td>
<td>10%</td>
<td>ESG9</td>
</tr>
<tr>
<td></td>
<td>1 : 1 : 2</td>
<td>10%</td>
<td>ESG9</td>
</tr>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS : RB</td>
<td>25%</td>
<td>ESG11</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>2 : 1 : 1</td>
<td>25%</td>
<td>ESG12</td>
</tr>
<tr>
<td>Rice bran (RB)</td>
<td>1 : 1 : 1</td>
<td>25%</td>
<td>ESG13</td>
</tr>
<tr>
<td></td>
<td>1 : 2 : 1</td>
<td>25%</td>
<td>ESG13</td>
</tr>
<tr>
<td></td>
<td>1 : 1 : 2</td>
<td>25%</td>
<td>ESG13</td>
</tr>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS : RB : NL</td>
<td>10%</td>
<td>ESG15</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>2 : 1 : 1 : 1</td>
<td>10%</td>
<td>ESG16</td>
</tr>
<tr>
<td>Rice bran (RB)</td>
<td>1 : 1 : 2 : 1</td>
<td>10%</td>
<td>ESG16</td>
</tr>
<tr>
<td>Neem leaves (NL)</td>
<td>1 : 1 : 1 : 1</td>
<td>10%</td>
<td>ESG17</td>
</tr>
<tr>
<td>Cow dung (CD)</td>
<td>CD : BS : RB : NL</td>
<td>25%</td>
<td>ESG18</td>
</tr>
<tr>
<td>Brick soil (BS)</td>
<td>2 : 1 : 1 : 1</td>
<td>25%</td>
<td>ESG19</td>
</tr>
<tr>
<td>Rice bran (RB)</td>
<td>1 : 1 : 2 : 1</td>
<td>25%</td>
<td>ESG19</td>
</tr>
<tr>
<td>Neem leaves (NL)</td>
<td>1 : 1 : 1 : 1</td>
<td>25%</td>
<td>ESG20</td>
</tr>
</tbody>
</table>
Stock solution

100 mg of ammonium sulphate was dissolved in 100 ml of distilled water.

3.2.2 Standard curve for Ammonium Measurement

Stock solution of (NH₄)₂SO₄ was taken in different concentration which contain 10-50 µg of NH₄⁺ in various sets. Each test tube contain 0.01, 0.02, 0.03, 0.04 and 0.05 ml of stock solution and made up to 0.1 ml with distilled water. One ml of reagent A and same amount of reagent B were added in the reaction vessel and kept for 15 min. at room temperature. Absorbance were measured at room temperature at 625 nm by using spectrophotometer. Standard curve was plotted between concentration and optical density (Fig. 3.1).

3.2.3 Estimation of released ammonium from the granules

Newly developed of slow ammonium releasing supergranules were kept in a beaker of 50 ml capacity and 20 ml distilled water was added. Distilled water was extracted back and ammonium release was measured using the phenol hypochlorite method of Weatherburn (1967) at different time intervals.

To estimate release of ammonium from SRF granules in wet soil, a 100 ml glass beaker was taken and slow ammonium releasing supergranules were kept in it and allowed to release ammonium in wet soil as a function of
Fig. 3.1: Standard Curve for estimation of Ammonium
time. Each beaker contains 30 g of wet soil with 20 ml of distilled water. 5 ml of distilled water was added to each beaker to dissolved ammonium during the desired period and shaken. The distilled water was extracted back of setting soil mixed with granules and amount of ammonium released was estimated by phenol hypochlorite reaction at different intervals i.e. 1 day – 40 days. The experiments were done three times each with duplicate determination (n=6) and the values were calculated using standard curve made with ammonium sulphate (Fig. 3.1) The data were analyzed using statistical analysis based on ‘t’ test.

3.3 PREPARATION OF EXPERIMENTAL PLOT

To examine the performance of slow ammonium releasing supergranules in field, experimental fields were prepared during two subsequent years for each crop. The details of layout and allocation of treatments are shown (Fig. 3.2, 3.3, 3.4).

3.3.2 Experimental plot for wheat (*Triticum aestivum* L. cv. 306)

The treatments were studied in random block design with a single variety of wheat (*Triticum aestivum* L. cv. 306) in each plots. There were four treatments, which were replicated three times. Each plot area was 2'-2' feet.

Description of treatments under study is given below:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (- Nitrogen)</td>
<td>C</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>N</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-18</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-19</td>
</tr>
</tbody>
</table>
Experimental design = RBD  
Replications = 3
Treatments = 4;  Each plot size = 2' - 2' feet
Total no. of plots = 12;

Fig. 3.2 Layout plan of experimental plot of wheat (*Triticum aestivum* L. cv. 306)

3.3.3 Experimental plot for spinach (*Spinacea oleracea* L.)

The treatments were studied in random block design with a single variety of spinach (*Spinacea oleracea* L.) in each plots. There were four treatments which replicated for three times. Each plot area was 2'-2' feet.

Description of treatments under study is given below:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ( - Nitrogen)</td>
<td>C</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>N</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-18</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-19</td>
</tr>
</tbody>
</table>
### Experimental plot for garlic (Allium sativum L.)

The treatments were studied in random block design with a single variety of garlic (*Allium sativum* L.) in each plots. There were four treatments and replicated three times. Each plot area was 2'-2' feet. Description of treatments under study is given below:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (- Nitrogen)</td>
<td>C</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>N</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-18</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-19</td>
</tr>
</tbody>
</table>

**Experimental design** = Random block design (RBD)

- **Treatments** = 4; **Replications** = 3
- **Total no. of plots** = 12; **Each plot size** = 2'-2' feet

*(Fig.3.3)*: Layout plan of experimental plot of Spinach (*Spinacea oleracea* L.)

#### 3.3.4 Experimental plot for garlic (*Allium sativum* L.)

The treatments were studied in random block design with a single variety of garlic (*Allium sativum* L.) in each plots. There were four treatments and replicated three times. Each plot area was 2'-2' feet. Description of treatments under study is given below:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (- Nitrogen)</td>
<td>C</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>N</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-18</td>
</tr>
<tr>
<td>Slow releasing supergranule</td>
<td>ESG-19</td>
</tr>
</tbody>
</table>
Experimental design = Random block design (RBD)  
Treatments = 4;  
Replications = 3  
Total no. of plots = 12;  
Each plot size = 2'-2' feet

(Fig.3.4): Layout plan of experimental plot of garlic (*Allium sativum* L.)

### 3.4 STUDIES OF CLIMATIC CONDITIONS OF EXPERIMENTAL STATION

The climatic condition of the experimental site were studied by weather station (DL 2e Data logger, Delta T Device, Cambridge England) situated at Chaudhary Charan Singh Haryana Agricultural University, Regional Research Station Farm, Rohtak, Haryana (India).

### 3.5 STUDIES OF SOIL PROPERTIES OF EXPERIMENTAL STATION GROWN WITH DIFFERENT CROPS

#### 3.5.1 Collection of Soil Samples

For the collection of the soil, grass, weeds and other plants etc. were removed and cleaned. Top soil (100-200 gm) was collected from a depth of
about 0-20 cm. and mixed thoroughly before sampling. The soil samples were
dried in an oven until constant weight was obtained and then powdered
homogenously.

3.5.2 Analysis of physico-chemical properties of soil

3.5.2.1 Organic Carbon (OC)

Organic carbon in soil samples was estimated by wet digestion method
of Walkley and Black (1934) with slight modification. Organic carbon was
oxidized solely by the action of acid dichromate, heated with the exothermic
mixing of aqueous dichromate and concentrated sulphuric acid. Oxidation of
OC by this method is notoriously incomplete due to the low temperature
(~120°C) reached, but it can easily be improved by supplementary heating on
a hot plate or block digester (Schollenberger, 1945; Nelson and Sommers,
1975). Upon oxidation, OC is calculated from the titer of ferrous ammonium
sulphate obtained in the back titration of unused dichromate.

3.5.2.2 Estimation of available phosphorus in soil

Available phosphorus in soil was determined by the method of
Richards et al., 1968.

Reagents:

Sodium bicarbonate (N/2) pH 8.5

Activated carbon: Darco G-60.

Ammonium Molybedate

Working solution

10 g of SnCl₂·2H₂O was dissolved in 25 ml of conc. HCl and made upto
132 ml with distilled water.
**Procedure for detection of available phosphorus in soil**

2-3 g of black Darco G-60 and 50 ml of sodium bicarbonate (N/2) solution were added to 2 g dried powdered soil. It was shaken thoroughly for 30 minutes and then filtered through Whatmann No. 40 filter paper. To 5 ml of filtered soil extract 5 ml of ammonium molybedate was added and this was diluted to about 20 ml with distilled water. 1 ml of stannous chloride solution (working solution) was then added to it and the final volume was made upto 25 ml with distilled water and was shaken thoroughly. The colour intensity was read using the colorimeter at 660 nm after 10 minutes.

**3.5.2.3 Estimation of potassium**

Potassium was estimated by flame photometer (Perkin-Elmer model 52, flame photometer with acetylene or propane burner).

**Reagents**

A. **Ammonium acetate 1 N**: To 700 or 800 ml of water, 57 ml of concentration acetic acid and then 68 ml of concentration ammonium hydroxide was added. It was diluted to a volume of 1 liter and was adjusted to pH 7.0 by the addition of more ammonium hydroxide or acetic acid.

B. **Potassium chloride 0.02 N**: 1.491 g of dry potassium chloride in water was dissolve and diluted to a volume of exactly 1 liter.

C. **Potassium chloride: 0.02 N in 1 N ammonium acetate**: 1.49 g of dry potassium chloride was dissolved in reagent A and diluted to a volume of exactly 1 liter with additional A.
D. Lithium chloride, 0.05 N: 2.12 g of dry lithium chloride was dissolved in water and diluted to 1 liter.

Procedure

Reagents B and D were used to prepare a series of standard potassium chloride solutions, each containing the same concentration of lithium chloride. A similar series of standard potassium solutions using reagents C and D, and use A for dilution were prepared. The concentrations of potassium chloride were 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5 and 2 meq./l. The optimum concentration of lithium chloride varies with individual flame photometers but was usually 5 to 10 meq./l. Standard solutions were made in water employed for the analysis of waters and water extracts of soils; whereas, those made up in ammonium acetate solution were used for the analysis of ammonium acetate extracts of soils. The flame photometer was calibrated for operation over the concentration range 0 to 0.5 meq./l of potassium, using the first 6 standard solutions of the appropriate series. The first and the last 4 solutions of the appropriate series were used to calibrate the instrument for operation over the concentration range 0 to 2 meq./l of potassium.

An aliquot of the solution was pipet out to analyse containing less than 0.1 meq. of potassium into a 50 ml volumetric flask. An amount of reagent D was added, which when diluted to a volume of 50 ml, gave a concentration of lithium chloride exactly equal to that in the standard potassium chloride solutions. It was diluted to volume with water or with A, if ammonium acetate
extracts were being analyzed, mixed, and determined the potassium concentration by use of the flame photometer and the appropriate calibration curve.

3.6 SUPPLY OF SRFs IN EXPERIMENTAL FIELD

Plants grown in experimental field were applied with slow ammonium releasing supergranules of the ratio of cow dung, brick soil, rice-bran and neem leaf i.e. 2:1:1:1 (ESG-18) and 1:1:2:1 (ESG-19), which were proved to be best out of 20 newly developed supergranules.

3.7 COLLECTION OF SEEDS FOR SOWING IN EXPERIMENTAL FIELD

The certified seeds of wheat (Triticum aestivum L. cv. 306) were obtained from Agricultural Research Centre, Rohtak of CCSHAU, Hissar and seeds of other crops were procured from the local market.

3.8 MEASUREMENT OF GROWTH PARAMETERS FOR CROP PLANTS APPLIED WITH SRFs

3.8.1 Determination of fresh weight

The fresh weight of different plant parts was recorded at a regular interval using single pan electric balance. Distilled water was used for washing different plant parts and excessive water was removed after placing them in two layers of filter paper before weighing.

3.8.2 Determination of dry weight

Dry weight of the same tissue was recorded after drying it in a hot air electric oven 60°C for 48 h (till it became constant).
3.8.3 Measurement of Root length and shoot length

The length of root and shoot was determined regularly on a meter scale and were averaged. Six measurements was calculated.

3.8.4 Counting of number of leaves, primary branches and primary root hairs.

Physical growth parameters like number of leaves, number of primary branches and primary root hairs were counted at regular intervals of time and averaged. (n=6).

3.8.5 1000 – Seed Weight (g)

Random samples of seeds were collected from produce of each one thousand seeds were counted and then weighed with the help of digital electronic weighing balance to get 1000 - seed weight.

3.9 STUDY OF BIOCHEMICAL PARAMETERS OF PLANTS APPLIED WITH SRFs

3.9.1 Assay of *in vivo* nitrate reductase activity (NRA)

The activity of nitrate reductase in leaves was assayed by *in vivo* method (Srivastava; 1975) with slight modifications

Freshly harvested 0.5 g leaves were taken in black vials of 20 ml capacity containing 8.0 ml of 0.1M sodium phosphate buffer (pH 7.4), 1.0 ml of 0.2 M KNO₃ and 1.0 ml of 25% n-propanol. These vials were sealed and incubated in dark for 30 min at 30°C. Nitrite released in the incubation mixture due to the enzyme activity was measured by colour development. For this 2.0 ml of aliquot from the incubation mixture (drawn immediately after the termination of assay), 2.0 ml of 1.0% sulphanilamide in HCl (w/v) and 2 ml of 0.02% N (1-Naphthyl) ethylene diamine-dihydrochloride (NED) (w/v) were
added. After 20 min absorbance was read by Vis-spectrophotometer (Systronic 106) at 540 nm. A pink colour was developed due to the formation of diazo compound with sulphanilamide and nitrite which is coupled with NED. The amount of nitrite was calculated as μ moles $\text{NO}_2^-/\text{h/g}^{-1} \text{ fr.wt.}$ with the help of standard curve prepared from sodium nitrite (Fig. 3.5).

3.9.2 Determination of total organic nitrogen content

**Preparation of Nesseler's reagent**

In 400 ml of distilled water 100 g of NaOH was dissolved. In a separate flask 100 g of mercuric iodide (HgI$_2$) and 70 g of potassium iodide (KI) were also dissolved in 400 ml of distilled water and left for cooling. After some time, this iodide solution was mixed with cooled sodium hydroxide (NaOH) under the continuous stirring. The volume was made up to one litre with distilled H$_2$O and mixture was kept at room temperature for 24 h. The mixture was filtered off to make it free from the visible suspensions or precipitates, if required.

The clear solution was used as Nesseler's reagent, which is an alkaline solution of KI-HgI$_2$ complex. It reacts with ammonium to form a solution of dimercuric ammonium iodide; and brown to yellowish in colour.

$$2(\text{HgI}_2 + 2\text{KI}) + 2 \text{NH}_3 \rightarrow \text{NH}_2 - \text{Hg}_2 - \text{I}_3 + 4\text{KI} + \text{NH}_4 - 1$$

(Red brown or yellowish)

The following precautions were taken during preparation of Nesseler's reagent:

1. All solutions were cooled before Nesselerization.
2. Dilute solutions were used.
Fig. 3.5: Standard curve for estimation of Nitrate reductase activity
3. Nesseler's reagent was added rapidly and used with the solution at once.

4. Only clear preparation of Nesseler's reagent were used.

**Estimation of organic nitrogen**

The oven dried (at 60° C for 48 hrs) samples of the leaves obtained from the desired treatments were used for the estimation of organic nitrogen by the modified microkjeldahl method (Lang, 1958).

About 100 mg of dried and powdered plant samples were digested in microkjeldahl flasks containing 4.0 ml of concentrated H₂SO₄ on heating mental by constant heating of 70-80°C and 2-3 crystals of salicylic acid was added to make the solution clear. The colourless solution was then cooled and made upto 100 ml by addition of glass distilled water.

The aliquot of the above solution prepared by microkjeldahl method were used for colour development by adding Nesseler's reagent. The reagent mixture contained: 0.5 ml of the digested aliquot, 2.5 ml of distilled water, and 2.0 ml of Nesseler's reagent in a total volume of 5.0 ml. mixture was kept for 20 min. at room temperature. Absorbance was recorded at 440 nm for yellow colour developed using Vis-spectrophotometer (Systronic 106). Total organic nitrogen content was calculated using standard curve prepared from the acidic ammonium sulphate solutions (Fig. 3.6).

**3.9.3 Estimation of protein**

Protein was estimated by the method of Bradford (1976)
Fig 3.6: Standard curve for estimation of Total Organic Nitrogen
In a 1.0 litre volumetric flask, 100 mg of commassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol. 100 ml of 85% phosphoric acid was added and the volume was made up to 1.0 litre with distilled water. The solution was filtered through Whatmann No. 1 filter paper and stored at 4°C.

0.5 g of fresh leaf samples were extracted with 2 ml of phosphate buffer (pH 7.0). This solution was centrifuged at 12000 × g for 15 min. To 0.1 ml of the supernatant, 0.1 ml of NaCl and 1.0 ml of coomassie brilliant blue solution was added. The optical density was measured at 595 nm using Vis-spectrophotometer (Systronic-106). The content of total protein was calculated using a standard curve prepared for Bovine Serum Albumin (Fig. 3.7).

3.9.4 Polyacrylamide sodium dodecyl sulphate slab gel electrophoresis (SDS-PAGE) of proteins

Polyacrylamide gel electrophoresis (PAGE) was performed on vertical slab gel in a electrophoresis chamber (10 cm × 10 cm × 2 mm) as described by Lammeli (1970). The SDS-PAGE was performed on 15% gel concentration and staining of the proteins was done by coomassie brilliant blue R-250 solution.

Sample buffer (pH 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>20%</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.2%</td>
</tr>
<tr>
<td>SDS</td>
<td>4%</td>
</tr>
<tr>
<td>Tris base</td>
<td>100 mM</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>2%</td>
</tr>
</tbody>
</table>
Fig. 3.7: Standard curve for estimation of protein
**Prefixing solution:**
Methanol 5%
Acetic acid 7%

**Protein stain solution**
Coomassie brilliant blue R 250 0.02%
Methanol 45%
Acetic acid 10%

**Destaining solution**
Methanol 10%
Acetic acid 7%

As above without the dye coomassie brilliant blue R-250.

**Procedure**

Dried glass plates and spacers were cleaned and assembled properly. The assembly was held together with bulldog clips. White petroleum jelly or 2% agar (melted in a boiling water bath) was applied around the edges of the spacers to hold them in place and the chamber was sealed between the glass plates.

**Separating gel mixture was prepared as follows:**

*For 10% gel*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock acrylamide solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>Tris-HCl (pH 8.8)</td>
<td>8 ml</td>
</tr>
<tr>
<td>Water</td>
<td>18.1 ml</td>
</tr>
</tbody>
</table>

The mixture was degassed on a water pump for 3-5 min and then added with the following.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate solution</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20µl</td>
</tr>
</tbody>
</table>
The gel solution in the chamber was poured between the glass plates and left to set for 30-60 minutes to prepare a SDS-PAGE gel of 15% Stacking gel (4%) was prepared as follows:

- Stock acrylamide solution 1.35 ml
- Tris - HCl (pH 6.8) 1 ml
- Water 7.5 ml in a total volume of 10ml

Degassing of the above is done and SDS (10%) 0.1 ml and ammonium persulphate solution (5%) 5 µl were then added to it.

The stacking gel mixture was poured and the comb was placed in the stacking gel and allow the gel was allowed to set for 30-60 min. After the stacking gel had polymerized, the comb was removed without distorting the shapes of the well. The gel after removing the clips and agar etc. was carefully installed in the electrophoresis apparatus. Electrophoresis apparatus was filled with electrode buffer and air bubbles at the bottom of the gel was removed. The cathode is connected. The electrode buffer and the plates were kept cooled so that heat generated during the run did not affect the gel and resolution.

The samples prepared for electrophoresis following suitable extraction procedure was adjusted for the protein concentration in each sample using the 5-strength sample buffer was adjusted in such a way that the same amount of protein was present per unit volume.

The sample solutions were cooled and the required volume in a microsyringe was carefully injected into a sample well through the electrode buffer. The position of wells on the glass plate with a marker pen were
marked and the presence of bromophenol blue in the sample buffer facilitated easy loading of the samples. Similarly, few wells were loaded with standard marker proteins in the sample buffer.

The current of 10-15 mA was supplied and turned on 30mA until the samples traveled through the stacking gel. The same was continued to run at 30mA until the bromophenol blue reached the bottom of the gel (about 3h).

Once the run was complete, the gel between the plates was removed and immersed in staining solution for at least 3h or overnight with uniform shaking. The proteins absorbed the coomassie brilliant blue.

The gel was then shifted to a suitable container with at least 200-300 ml destaining solution was transferred and shaken gently continuously. Dye not bound to proteins was removed thus. Until the background of the gel is colourless destaining solution was changed frequently. The proteins fractionated into bands were seen coloured blue. As the proteins of minutes quantities were stained faintly, destaining process should be stopped at appropriate stage.

3.9.5 Estimation of chlorophyll

Chlorophyll was estimated in leaf by the method described by Strain and Svec (1966).

500 mg leaf were extracted with 80% acetone and centrifuged at 5000 rpm for 10 minutes. Amount of supernatant was made upto known volume with acetone absorbance of supernatant was recorded at 665 nm and 645 nm.
Chlorophyll was calculated as follows:

\[
\text{Chlorophyll a (\(\mu g/ml\)) = 11.63 \times A_{665} - 2.36 \times A_{645}}
\]

\[
\text{Chlorophyll b (\(\mu g/ml\)) = 20.11 \times A_{645} - 5.18 \times A_{665}}
\]

\[
\text{Total Chlorophyll (\(\mu g/ml\)) = 6.45 \times A_{665} + 17.72 \times A_{645}}
\]

3.9.6 Estimation of oxalate in leaves samples

3.9.6.1 Pre-treatment of Food Sample

50g of leaves sample were crushed and 2 g of this crushed leaves was homogenized in 5 ml distilled water in pestle mortar. The homogenate was centrifuge at 5000 rpm for 15 min and both pellet and supernatnat were collected. The supernatant was used for determination of soluble oxalate. The pellet was mixed with 2 ml of IN HCl, kept at 100°C in a boiling water bath for 1 hr, diluted to 4 ml with distilled water and used for determination of insoluble oxalate.

3.9.6.2 Determination of Oxalate Oxidase

Oxalate in leaves was determined by the method of Sharma et al., 2000 by using free barley oxalate purchased from Sigma oxidasepur instead of immobilized oxalate oxidase and based up on measurement of \(H_2O_2\) generated in test samples containing oxalate by barley oxalate oxidase with the colour reaction consisting of 4-microanimophenozene, phenol and peroxidase as chromogenic system. The reaction mixture containing 1.7 ml (0.05 sod. succinate buffer) pH 3.2, 0.1 ml CuSO\(_4\) (0.01M) and 0.1 ml of oxalate oxidase was pre-incubated for 5 minutes at 35°C. 0.1 ml of test sample was added and incubated at 35°C for 5 minutes under continuous
stirring. 1.0 ml colour reagent was added and kept for 15 minutes at room temperature. $\lambda_{520}$ was read and amount of oxalate was calculated from standard curve between oxalate concentration $\lambda_{520}$ (Fig. 3.8).

3.10.7 Estimation of sedimentation value of grains of wheat

This method was determined by the method followed by Dr. Shashi Madan, Scientist (Bio-Chemistry) CCSHAU, Hissar.

Reagent

SDS/Lactic acid reagent: This reagent was prepared by mixing 20% SDS (w/v), 80% lactic acid (v/v) and distilled water in ratio of 20:1:8.

Procedure

Wheat meal (6 g) was added to 50 ml water in a 100 ml cylinder, a stopcock set going and the material dispersed by rapid shaking horizontally for 15 sec. The contents of the material were again shaken for 15 sec at 2 min and 4 min. Immediately after the last shake, 50 ml of SDS lactic acid reagent was added and mixed in by inverting the cylinder 4 times. Inversion (four times) was repeated at 6, 8 and 10 min, the contents of the cylinder were allowed to settle for 20 min (whole meal) before the sedimentation volumes were read.

The data is expressed in ml.

3.10.8 Estimation of grain hardness in wheat

Grain hardness was determined using Hardness Tester of Tokyo, Japan. The data is expressed in Kg./grain.
Fig. 3.8: Standard curve for oxalate
STATISTICAL CALCULATIONS

The formula given by Snedecore (1961) was used for calculating standard error.

\[
S_x = \frac{S}{\sqrt{N}}
\]

Where

- \( S_x \) = Standard error
- \( N \) = Number of observations
- \( S \) = \( \frac{x^2}{\bar{x}} \)
- \( x^2 \) = Sum of the deviation square from the mean

Calculation of significant differences

Significant differences was calculated by 't' test (Steel and Torrie, 1960) with a formula appropriate for unpaired observations and unequal variance.

The equation is as follows:

\[
t = \frac{d}{s \sqrt{d}}
\]

\( d \) = Difference between the means

\[
S/d = \frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}
\]

Where

- \( n_1 \) = sample size for control
- \( n_2 \) = sample size for experimental

\( S_1^2 \) and \( S_2^2 \) values are obtained by the following method

The standard error was multiplied \( \sqrt{n} - 1 \) for experiment and result was squared.
\[ S_1^2 = \text{Standard error} \times \sqrt{\text{sample size}_1}^{-1.2} \]
\[ S_2^2 = \text{Standard error} \times \sqrt{\text{sample size}_1}^{-1.2} \]

By calculating from the table of 't' values at 0.05% or 0.01% it was observed whether the obtained values in the table for the degrees of freedom indicated were larger or smaller. If the calculated value was larger than the tabular value, it was taken significant, otherwise insignificant. The term insignificant means that the two means compared are not statistically different. Significant refers to difference, which can be demonstrated statistically.