Chapter 4

MATERIALS AND METHODS
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MATERIALS & METHODS

4.1 Description of Survey Area

The present study has been done in and around Nadia district, West Bengal. The district comprises four subdivisions: Krishnanagar Sadar, Kalyani, Ranaghat and Tehatta. Krishnanagar Sadar subdivision consists of Krishnanagar municipality, Nabadwip municipality and seven community development blocks: Kaliganj, Nakashipara, Chapra, Krishnanagar–I, Krishnanagar–II, Nabadwip and Krishnaganj. Kalyani subdivision consists of Chakdaha municipality, Gayespur municipality, Kalyani municipality and two community development blocks: Chakdaha and Haringhata. Ranaghat subdivision consists of Shantipur municipality, Ranaghat municipality, Birnagar municipality and four community development blocks: Hanskhali, Shantipur, Ranaghat–I and Ranaghat–II. Tehatta subdivision consists of four community development blocks: Karimpur–I, Karimpur–II, Tehatta–I and Tehatta–II. Krishnanagar is the district headquarters. According to the 2011 census Nadia district has a population of 5,168,488, roughly equal to the United Arab Emirates or the US state of Colorado. This gives it a ranking of 18th in India (out of a total of 640). The district has a population density of 1,316 inhabitants per square kilometre (3,410/sq mi). Its population growth rate over the decade 2001-2011 was 12.24%. Nadia has a sex ratio of 947 females for every 1000 males, and a literacy rate of 75.58%.
Figure 4.1 Detailed political map of Nadia district, West Bengal, India. Waste samples were collected from different places of Nadia district.
4.2 Selection of Sampling Sites

Depending on the objective of the present study, a random survey was done in and around Nadia district, West Bengal to screen out the suitable sampling sites.

4.3 Collection of Sample from Sources

The present study has been done in and around Nadia district, West Bengal. The district comprises four subdivisions: Krishnanagar Sadar, Kalyani, Ranaghat and Tehatta. Krishnanagar Sadar subdivision consists of Krishnanagar municipality, Nabadwip municipality and seven community development blocks: Kaliganj, Nakashipara, Chapra, Krishnanagar–I, Krishnanagar–II, Nabadwip and Krishnaganj. Kalyani subdivision consists of Chakdaha municipality, Gayespur municipality, Kalyani municipality and two community development blocks. Depending upon the above survey, total of eight sites were selected for this present study (Table 4.1). The sites were catalogued as – S1 to S8. The detailed information regarding the sites, soil types, date of collection and other necessary details are provided in the table below. Samples were collected randomly from these mentioned locations in and around Nadia district, West Bengal, at specific intervals. At each sites, the soil dug to a 0 - 20cm was scooped into sterilize polythene bags, labelled and brought to laboratory for analysis.
Table 4.1 Characteristic features of sampling sites, and sample types. All the sampling sites are located within Nadia district, West Bengal, India.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Sample Type</th>
<th>Date of Collection</th>
<th>Place of Collection</th>
<th>Altitude (Above Sea Level)</th>
<th>Soil Type</th>
<th>Colour</th>
<th>Smell</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Cooking debris</td>
<td>11.01.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Clay</td>
<td>Blakish</td>
<td>Pungent</td>
</tr>
<tr>
<td>S2</td>
<td>Rotten Tea</td>
<td>12.01.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Humus</td>
<td>Blakish</td>
<td>Pungent</td>
</tr>
<tr>
<td>S3</td>
<td>Jute rotting pond</td>
<td>21.01.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Clay</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>S4</td>
<td>Normal pond</td>
<td>02.02.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Clay</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>S5</td>
<td>Garbage of rotten flower</td>
<td>21.03.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Sandy</td>
<td>Slightly Blakish</td>
<td>Pungent</td>
</tr>
<tr>
<td>S6</td>
<td>Garden litter</td>
<td>03.04.13</td>
<td>Aranghata</td>
<td>17 mt</td>
<td>Loamy</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>S7</td>
<td>Paper mill water</td>
<td>05.04.13</td>
<td>Chakdaha</td>
<td>11 mt</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>S8</td>
<td>Fertile soil</td>
<td>08.04.13</td>
<td>Kalyani</td>
<td>11 mt</td>
<td>Loamy</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>
4.4 Characterization of collected samples

The collected samples were initially analyzed for three different parameters – as Moisture content, pH and Temperature (Table 5.1).

4.4.1 Moisture content of soil sample

The moisture content was calculated as percent moisture on a weight basis. Ten grams of wastes mixed soil sample was taken and dried in an oven at 60 °C for 24 - 48 hours. The weight of collected samples after drying was noted. The moisture content, of the solid wastes was determined by using the following equation (Rowel, 1995)

\[
\text{Moisture content (M %)} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where,

\( W_1 = \) initial weight of sludge mixed soil sample, \( W_2 = \) final weight of sample after drying.

4.4.2 pH of soil sample

The pH of the collected samples was determined using pH mater (Systronics, model no.361). In a beaker, 10g of collected samples was mixed with 50 ml double distilled water and kept on a magnetic stirrer for 30 min, it was then allowed to settle and pH was measured.

4.4.3 Temperature of soil sample

The temperatures of the collected samples were determined using Soil Probe Thermometer (Model: Luster Leaf 1618 Rapitest Soil thermometer). The
thermometer was bored down to soil samples for about 10 – 20 cm in depth, and the data was recorded when the readings got stabilized.

4.5 Isolation of Microbes from collected Samples

The microbial community were isolated from the collected samples by serial dilution method employing pouring as well as spread plating technique on isolation media (Aneja, 2005). For enumeration of total bacterial and fungal load in the samples, 100μl of each dilution was spread on pre-sterilized agar plates, incubated and observed for the appearance of the colony. For bacterial isolation, the aliquots were plated on nutrient agar (NA), incubated at 30 °C for 24 - 48 hours and incubated at 28°C for 3-5 days.

4.6 Maintenance of the culture:

Glycerol stocks were prepared and stored at – 80 °C for long term preservation. Pure cultures of the bacterial isolates were incubated at 30 °C for 24 hours in LB broth. Then 0.5 ml of each of the cultures was transferred into cryotubes and 0.5 ml broth containing 40% glycerol was added. The samples were mixed gently and stored at 80 °C.

4.7 Evaluation of Cellulolytic Activity

The samples were placed in sealed plastic bags surrounded with ice and brought to the laboratory within 2 h. One gram of bacterial contents was diluted with 100 mL distilled water and homogenized in a constant-temperature
oscillation water bath at 80°C, for 30 min. After gradient dilution, the samples were poured in CMC-Congo red- Agar (Hendrick et.al.,1995) plate and incubated at 37°C for 24 h. After incubation, cellulolytic strains was isolated by showing distinct clear zone around the colony. Strains showing high cellulase activity were used in subsequent experiments.

4.8 Selection of Most Effective Bacterial Strain

Depending on the cellulolytic activity of the isolated bacterial strains, total two bacterial strains, i.e. – S2 and S7, were selected for further studies. These two strains were further analyzed for their growth and PGPR characters.

4.9 Growth Curve analysis of Bacterial Strain

4.9.1 Bacterial growth at different temperatures:

The ability of the isolated bacterial strains to grow at different incubation temperature (20°C, 25°C, 30°C, 40°C, 50°C and 60°C) was assessed by inoculating the bacterial species in to LB broth and incubated at different temperature. The growth of bacteria was measured spectrophotometrically at 600 nm in a UV-Vis spectrophotometer (Aneja, 2005).

4.9.2 Bacterial growth of different pH:

The ability of bacterial isolates to at different pH (pH 4 - 9) was tested in LB broth (Appendix B) by adjusting the pH of culture medium using 1N HCl and
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1N NaOH. The growth of bacteria was measured spectrophotometrically at 600nm (Aneja, 2005).

4.10 Characterization of isolated Bacterial Strain –

The isolated bacterial strains were characterized by their morphological, cultural and staining properties. Morphological characteristics of the colony of each isolate were examined on Nutrient agar plates.

4.10.1 Colony Morphology

All the isolates were streaked on Nutrient agar plates. After 3 days of incubation, different characteristics of colonies such as shape, size, elevation, surface, margin, colour, etc were recorded.

4.10.2 Gram nature of each isolates

Gram nature of each isolates was initially determined by using Crystal violet and Safranin staining according to standard microbiological protocol. A loopful of the freshly bacterial culture was air dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water. Grams iodine (0.4% w/v) was added and allowed to stand for 30 seconds before being rinsed off. The stain was washed with ethanol (95.0% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one minute. This was then washed with water for 5 seconds. It was then further confirmed by Hagedorn and Halt
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(1975) method using methyl red agar medium (beef extract 3g; poptone 5g; methyl red 150mg; agar 15g; distilled water 1 lit; pH 7.0) for detection of Gram positive bacteria and Gould et al (1985) method using sterile crystal violet agar medium (beef extract 3g; poptone 5g; crystal violet [0.05/w/v] 4ml; agar 15g; distilled water 1 lit; pH 7.0) for detection of Gram negative bacteria

4.11 Maintenance of isolates

All the isolates were maintained at 4°C in equal volumes of nutrient broth and 30% glycerol.

4.12 In vitro screening of isolates for different plant growth promoting activities

Different direct plant growth promoting activities of the bacterial isolates viz. phosphate solubilisation, IAA production, ammonia production, siderophore production and indirect activities such as hydrocyanic acid (HCN) production were studied.

4.12.1 Detection of Phosphate solubilisation ability

Phosphate solubilizing ability of the bacterial isolates were measured by following standard microbiological technique. Inoculated the bacteria into the sterile Petri plates on Pikovskaya’s medium containing 5 g of tri-calcium phosphate \([\text{Ca}_3(\text{PO}_4)_2]\) as sole phosphorus source for selectively screening of bacteria which have the ability to release inorganic phosphate from tri-calcium
phosphate. After 3-days of incubation at 30°C, phosphate solubilizing bacteria developed clear zones around colonies. All the isolates having phosphate solubilizing activity were preserved in the laboratory as a pure culture for their further investigation.

4.12.2 Detection of IAA production by the selected isolates

The P- solubilising rhizobacteria were then selected for their phytohormone (Auxin) production ability. It was ascertained by the method of Gordon and Weber (1951). 1 ml of each freshly cultured test bacterial suspension was inoculated separately in Nutrient Broth medium containing L-tryptophan (5gm/Lit), and incubated for 96 hour. Cultures were centrifuged at 10,000 rpm for 10 minutes. 2ml of supernatant was taken. 2 drops of Orthophosphoric acid and 4ml of Solawaski’s reagent were added to it. (Solawaski’s regent – 50ml of 35% Perchloric acid and 1ml of freshly prepared 0.5 (M) FeCl₃). Then it was kept in dark for 20 min at room temperature. Salkowski reagent was used for detecting the presence of IAA. The reagent gives reaction with IAA and does not interact with L-tryptophan This reagent is an important option for qualitative and semiquantitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants based on the change of colour from yellow to pink, the presence of Indole Acetic Acid was confirmed. The values of the various strains along with Salkowski reagent was checked using UV-VIS spectrophotometer and their values were noted down in terms of Optical
density. O.D. was taken at 540 nm. The produced IAA level in each culture medium was estimated by comparison with a standard IAA graph. The measurements were taken in triplicate. The IAA producing bacterial strain was screened for further study.

4.12.3 Detection of Siderophore production by selected isolates

Siderophore production was checked on solid CAS (Chromazurol S) universal blue agar plates (Schwyn and Neilands 1987) which was prepared by adding two solutions (solution A and B) as follows:

Solution A: 60.5mg CAS was dissolved in 50ml water and mixed with 10ml iron (III) solution [1mM FeCl3,6H2O, 10mM HCl] and slowly added to 72.9mg HDTMA [hexadecyl trimethyl ammonium bromide] dissolved in 40ml water. The resultant dark blue liquid was autoclaved.

Solution B: A basal medium containing deferrated 1M sucrose 3ml; deferrated 1M CaCl2 0.4ml; deferrated 1M MgSO4,7H2O 0.8ml; deferrated 2% K2HPO4 10ml; NaCl 0.2g; NaMoO4 0.0005g; Pipes [free acid] 30.24g and agar 15g; in800ml water was prepared. The pH of the medium was adjusted to 6.8 by the addition of 50% [w/w] NaOH and autoclaved. After cooling to 50°C; 30ml of deferrated casamino acids [10%] was added as sterile solutions. Finally solution A was added to solution B along the glass wall with enough agitation to achieve maximum mixing without formation of foam.
Actively growing cultures were spot inoculated on the CAS blue agar plate and incubated at 30°C for 48 h. Formation of yellow-orange halo around the colony indicated production and release of the siderophores on the agar plate.

### 4.12.4 Detection of Ammonia production by selected isolates

Each strain was tested for the production of ammonia in peptone water broth. (Peptone 10.0 g; NaCl 5.0 g; Dist. Water 1 lit pH 7). Overnight broth cultures were inoculated in 10 ml peptone water and incubated at 30°C for 4 days. After incubation period, Nessler’s reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was recorded as a positive test for ammonia production (Cappucino and Sherman 1992).

### 4.12.5 Detection of HCN production by selected isolates

Screening of bacterial isolates for hydrogen cyanide (HCN) production was determined as per methodology described by Bakker and Schipper (1987). Bacterial cultures were streaked on King’s B (proteose peptone 20g; glycerol 10g; K₂HPO₄ 1.50g; MgSO₄,7H₂O 1.50g; agar 15 g; distilled water 1 lit; pH 7.2) medium containing 4.4 g /liter of glycine. Then Whatman filter paper No. 1 was soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. The Petri plates were sealed with paraffin and incubated at 30±0.1°C for 4 days. Development of light brown to dark brown colour in filter paper indicated HCN production.
4.12.6 Detection of Potassium solubilisation ability

Potassium solubilizing ability of the bacterial isolates were measured by following standard microbiological technique. Inoculated the bacteria into the sterile Petri plates on Aleksandrov medium. After 3-days of incubation at 30°C, potassium solubilizing bacteria developed clear zones around colonies. All the isolates having potassium solubilizing activity were preserved in the laboratory as a pure culture for their further investigation.

4.12.7 Detection of Nitrogen fixing ability

Nitrogen fixing ability of the bacterial isolates were measured by inoculation of the bacteria into nitrogen free Jansen media. After 3 days of incubation at 30°C. Only growing colony represent nitrogen fixing ability as the medium devoid of nitrogen so only nitrogen fixing organisms can survive in that medium.

4.13 Selection of the best PGPR

The performances of all the selected bacterial isolates were critically considered in the light of Plant Growth Promoting activities and the best two Plant Growth Promoting Rhizobacterial strains were selected according to their multiple positive PGPR activities.
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4.14 Biochemical characterization of the best 2 PGPR strains

These two best isolates which showed PGPR activity were further characterized by following standard biochemical tests as depicted bellow -

4.14.1 Indole production

This test is done to determine if bacteria can breakdown the amino acid tryptophan into indole. In this study we use KB003 Hi25™ identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.

4.14.2 Methyl Red Test- Voges –Proskaur (MRVP)

This test is used to determine two things. The MR portion is used to determine conversion of glucose to acidic products like lactate, acetate and formate. The VP portion is used to determine conversion of glucose to acetone. In this study we use KB003 Hi25TM identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.
4.14.3 Citrate Utilization

This defines the ability of bacteria to convert citrate into oxaloacetate. In this study we use KB003 Hi25TM identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.

4.14.4 Catalase Test

In this study we use KB003 Hi25TM identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.

4.14.5 Gelatin Hydrolysis Test

This test was performed to determine the ability of the bacterial sample was inoculated into motility media using a needle and incubated at 30°C for 24 hours and checked for the bacterial migration.

4.14.6 H₂S Production Test

In this study we use KB003 Hi25TM identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.
4.14.7 Urease Test

In this study we use KB003 Hi25TM identification test kit of Hi Media Laboratories Pvt Ltd, was inoculated and incubated at 37°C for 48 hrs and proper colour change as directed in kit manual was noticed for result.

4.15 Molecular Characterization

4.15.1 Genomic DNA isolation, PCR amplification and Sequence Determination

To indentify the isolates, genomic DNA extraction and purification from each sample were done as described by Soerson et al. (1978). Each genomic DNA used as template for 16S rRNA gene amplification and PCR amplification was done with aid of universal primer 16S F (5' AGA GTT TGA TCC TGG CTC 3') and 16S R (5' GGT TAC CTT GTT ACG ACT T 3').

The amplification was done using a Themocycler (Model: Eppendroff Master Cycler Pro) with initial denaturing at 95°C for 6 min followed by 35 cycles of 95°C for 30 second, 52°C for 30second, 72°C for 30 second, and final extension at 72°C for 10 min. The presence of PCR amplified products were determined by agarose gel electrophoresis of 10 µl of the reaction product in 1.5 % agarose gel. The polymerase chain reaction (PCR) product was sequenced by Chromas Biotech Pvt. Ltd (India). Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was done using the ABI 3500 XL genetic
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analyzer and Big Dye Terminator version 3.0 cycle sequencing kit. The 16S rRNA gene sequences were aligned and compared with the closely related neighbour sequences retrieved from the GenBank database of the National Centre for Biotechnology Information, via BLAST search program. Phylogenetic analysis of all the isolate was performed using the software package MEGA version 5.0 after obtaining multiple alignments of data by CLUSTAL W. Pair wise evolutionary distances were computed using the correction method and clustering was performed using the neighbour-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 replicates. The 16S rRNA gene sequences have been deposited to GenBank and have been assigned with NCBI accession numbers.

4.16 Impact of Plant Growth

4.16.1 Rice seed germination:

Germination tests were carried out to determine the effect of inoculation of PGPR strains on the rates of seed germination. For this, Rice (*Oryza sativa* L.) seeds were used as plant materials. Healthy seeds were surface sterilized with 0.1% HgCl₂ for 2 min and rinsed six times with sterile distilled water. Two PGPR strains were grown in respective broth on shaking incubator (180 rpm) at 28 ± 2°C for 24 h. The surface sterilized seeds of rice were inoculated in broth culture of the PGPR strain cultures for 30 min including normal water (C) as control. Ten inoculated seeds of each treatment were placed in separate petri-
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plate containing soaked (with distilled water) filter papers the petri-plates were incubated at 25 ± 2°C for 6 days. Seed germination was recorded regularly starting from the 2\textsuperscript{nd} day on the basis of number of the germinated seed out of total germination. Each treatment was replicated three times. Percentages of germination were calculated.

4.16.2 Spinach Plants

4.16.2.1 Effect of single and co-inoculation on growth and yield Spinach plants

Only pot experiments were conducted to test the potential of selected PGPR isolates alone as well as in combination for promoting growth and yield of Spinach plants under natural conditions.

4.16.2.2 Layout, Design and Treatments:

In these experiments, three PGPR bacteria, either alone or in combination, were applied along with one controls (water) and the total four treatment combinations were laid out with three replications.

4.16.2.3 Pot experiments

Pot trials were conducted in the green house of the Department of Botany, University of Kalyani, West Bengal. Palak seeds were sawn into sterilized pots. Five seeds were sown in each pot containing 12 kg soil per pot which was
thinned to one plant after 15 days of germination. Pots were placed in the net
house under ambient light and temperature. The inocula for the pot trials were
prepared by culturing the selected bacterial strains on nutrient agar. A single
colony was transferred to 250 ml flasks containing nutrient broth, and grown
aerobically in flasks on a rotating shaker (95 rpm) for 24 h at 27°C. The
bacterial suspension was then diluted in sterile distilled water to a final
concentration of 108 CFU ml-1. 1ml of log culture (108 cells) of each bacterial
isolates was transferred as inoculum in the corresponding treatments. Treated
and non treated pots were irrigated with sterilized water daily. After every 7
days interval 2ml of microorganism inoculum was inoculated in the
(corresponding pot as booster dose. At maturity stage and data regarding yield
and yield contributing parameters were collected and analyzed statistically.

4.16.2.4 Harvesting

At maturity, the Spinach plants were harvested. It was started from20 days after
transplanting.

4.16.2.5 Observations recorded

Ten plants of each plot were randomly selected for recording observations in
all replications. The mean value of each treatment was used for statistical
analysis. Observed data about growth and yield parameters were recorded. The
parameters were as follows:
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a. **Plant Height (cm):**

Plant height has been recorded as average height of plant at 20 days after transplanting. The measurement was made by the help of meter scale from the base of the plant to the top at the opening of the first flower if any.

b. **Number of leaves per plant:**

Green leaves were randomly selected at mature vegetative stage and the numbers of leaves were counted. The average values were taken for statistical analysis.

c. **Area of leaves per plant:**

Green leaves were randomly selected at mature vegetative stage, and the areas of leaves were measured. The average values were taken for statistical analysis.

d. **Yield:**

Finally the plants were harvested for final yield. Both fresh and dry weight of total plants were counted and recorded for further calculation.

4.17 **Impact on Soil Fertility**

Soil samples were collected in triplicate from different treatments to analyze the properties of soil after fifteen days of initial mixing (0 day). Soil samples were collected by taking the monoliths of 10 x 10 x 20 cm from the replicate plots/pots of each treatment at different intervals.
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4.17.1 pH

The pH of soil and sewage sludge was measured in the suspension of 1: 5 (w / v) with the help of pH meter (Model EA940, Orion, USA) standardized with pH 4, 7 and 9.2 reference buffers.

4.17.2 EC

Soil Electrical Conductivity was measured by conductivity meter (Model 303, Systronics, India).

4.17.3 Organic Carbon

For organic carbon estimation, 1 g sieved soil sample was taken in a conical flask and 10 ml of 1 N K₂Cr₂O₇ and 20 ml of concentrated H₂SO₄ were added with constant shaking. The solution was allowed to stand for 30 min for the reaction to complete and then 200 ml of distilled water was added to the flask to dilute the suspension. Thereafter 10 ml of orthophosphoric acid and 1 ml of o-phenanthroline indicator was added. A deep blue colour appeared. It was titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the colour changed from violet to blue and finally to bright green. Organic matter in the sample is oxidized with the mixture of potassium dichromate and concentrated sulphuric acid. The excess of potassium dichromate, which is not reduced by the organic matter of the sample, is determined by titration using standard ferrous ammonium sulphate solution in the presence of orthophosphoric acid using o-phenanthroline indicator. A blank (without soil) was also run following
the same steps. The percent of organic carbon in soil was calculated using the following formula:

\[
\text{Organic carbon (\%) = } \frac{N \times (X-Y) \times 0.003 \times 100}{W}
\]

Where, \(N\) is the normality of ferrous ammonium sulphate, \(W\) is g of sample taken, \(X\) is the volume (ml) of 0.5 N ferrous ammonium sulphate required for reducing 10 ml \(K_2Cr_2O_7\) solution (blank reading) and \(Y\) is the volume (ml) of 0.5 N ferrous ammonium sulphate required for reducing the excess of chromate (sample reading).

### 4.17.4 Nitrogen

Total nitrogen (N) content was determined by using micro-kjeldahl technique through the Gerhardt Automatic N analyzer (Germany). The method for nitrogen analysis is composed of three distinct steps (a) Digestion step (b) Distillation step, and (c) Titration step. 1 g sieved soil sample was taken in a tube and 10g \(K_2SO_4\) and 0.5 g \(CuSO_4\) were added. 10 ml of concentrated \(H_2SO_4\) was then added and after mixing the solution was kept for 10 minutes. All the tubes were then loaded on to the digestion rack. Samples were digested by heating at 90 °C and then increasing the temperature step wise from 90 to 150 °C, from 150 to 250 °C and 250 to 350 °C and then 350 to 390 °C. After digestion the sample was cooled. The distillation step involves separation and isolation of nitrogen from digestion tube. The digestion tube was connected to
distillation unit containing 2 % of 40 ml boric acid and 2 to 3 drops of indicator. This was accomplished by raising the pH with NaOH. During the distillation process the ammonium is converted to ammonia and the distillate is collected in trapping medium (2 % boric acid). The ammonium is bound to boric acid in form of ammonium borate. For titration 0.1 N HCl taken in burette was added slowly to the flask containing the distillate and the end point was noted as the change in colour. A blank without any sample, but following the same steps was also digested, distilled and titrated. Percent nitrogen was calculated as follows:

\[
\text{Percent Nitrogen} = \frac{\text{(Blank reading – Sample reading) x Normality of titrant x 1.4007}}{\text{Weight of sample (g)}}
\]

4.17.5 Potassium

Exchangeable Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) in soil and sewage sludge were extracted in ammonium acetate by repeated leaching methodology and then concentrations were determined with the help of Atomic Absorption Spectrophotometer (Model 2380, Perkin Elmer, USA).

4.17.6 Phosphorus

The available phosphorous in the soil or sewage sludge sample was quantified by NaHCO\(_3\) extraction method given by Olsen et al. (1982). Available P in
sieved sample was extracted by taking 5 g sieved soil and 100 ml 0.5 N NaHCO$_3$ having pH 8.5 after adding one teaspoon of carbon black. The solution was shaked for 45 min and then allowed to stand for overnight. It was filtered and filtrate was used for estimation of available P. To 5 ml of filterate in 25 ml volumetric flask, 5 ml ammonium molybdate and 1 ml SnCl$_2$ was added. The volume of the reaction mixture was maintained upto 25 ml with the help of distilled water. The optical density (O.D.) of the resultant light blue colour was taken at 660 nm using s UV-VIS spectrophotometer (Model- 119, Systronics, India). A blank was also prepared following the above protocol but without extract of sample and O. D. was taken at the same wavelength. The content of available P was calculated using the following formula:

\[
\text{Available P (mg kg}^{-1}) = \frac{C \times V}{v \times w}
\]

Where C is the concentration of P as read from the standard curve, V is final volume of extractant (ml), v is volume of aliquot (ml) and w is the weight (g) of sample analyzed.

4.18 Statistical analyses

Data of the physiological and growth characteristics were subjected to four-way analysis of variance (ANOVA) to examine the individual and combined effects of age, variety, NPK level and treatment. Data of yield parameters were analyzed through three-way ANOVA for assessing the significance of changes
due to varietal, treatment and NPK level. Duncan’s multiple range test was performed as post hoc for various measurements after subjecting to various ANOVA tests. All the statistical tests were performed using SPSS software (SPSS Inc., version 14.0).