CHAPTER III

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MATERIALS AND METHODS

In order to study the objectives of the research programme as indicated in the introduction chapter, three types of studies, viz: field survey studies, laboratory studies and pot culture experiments were undertaken. Details of each study are given below.

3.1.0 Field survey and enumeration of Frankia population from major Casuarina growing areas of Kerala

A survey was conducted to collect the root nodules and rhizosphere soil samples for the analysis of Frankia population from the existing three major Casuarina plantations of Kerala.

The samples containing root nodules and rhizosphere soil were collected from the following places in Kerala.

<table>
<thead>
<tr>
<th>District</th>
<th>Location</th>
<th>Date of Collection</th>
<th>Field name</th>
<th>Variety of Casuarina</th>
<th>Age of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kollam</td>
<td>Kerala Forest Development Corporation, Punaloor</td>
<td>4-6-2004</td>
<td>Kuravoor-3</td>
<td>Casuarina equisetifolia</td>
<td>4 years.</td>
</tr>
<tr>
<td>Thrissur</td>
<td>Kerala Forest Development Corporation, Kondazhy</td>
<td>13-6-2004</td>
<td>Kayampoovam puthirithara</td>
<td>Casuarina equisetifolia</td>
<td>6 years</td>
</tr>
<tr>
<td>Alapuzha</td>
<td>Kerala Social Forestry Department, Kommady.</td>
<td>2.7-2004</td>
<td>Payalkulangara estate</td>
<td>Casuarina equisetifolia</td>
<td>20 years</td>
</tr>
</tbody>
</table>
The roots with nodules were washed and nodules were excised from the roots. (Plate 1) Rhizosphere soils were also collected and the collected samples were stored in sterile plastic bags at 5°C in the refrigerator.

3.2.0 Laboratory studies

The samples collected during the field survey were brought to the laboratory for undertaking further studies. The samples were processed as per the requirement of each analysis and kept in the containers with proper labelling and without any contamination.

3.2.1 Analysis of rhizosphere soil samples

Analysis of physical and chemical properties of rhizosphere soils was conducted as given below. Isolation, identification and enumeration of *Frankia* population in rhizosphere soil were also carried out.

3.2.2 Analysis of physico-chemical properties of soil

The analysis was carried out at the Agroclinic and Research Centre, Kottayam using appropriate method as given below.

3.2.2.1 Soil pH

Soil pH was determined as per the procedure given by Jackson, (1973). Soil: water in 1:2 ratio was taken and this soil suspension was used to analyse soil pH using a pHmeter by potentiometric method.
20g soil was transferred to a 100 ml beaker. 40ml distilled water was added. Stirred well with a glass rod and allowed to stand for half an hour with intermittent stirring. Adjusted the pH meter with the standard buffer solutions. Washed the electrodes with water and carefully wiped. The electrodes were then immersed in the beaker containing soil water suspension and pH meter was switched on. The reading was recorded which was automatically displayed in the meter.

3.2.2.2 Soil Electrical Conductivity (EC)

Electrical conductivity of soil was measured by using the conductivity meter using the method adopted by Clarson (2002). Soil suspension in 1:2 ratio was prepared with distilled water and stirred well. The instrument was switched on and was checked with 0.01N KCl solution before sample analysis. The electrodes were washed with distilled water. Sample was taken in a clean beaker. The range switch of the meter was turned to millimhos range. The temperature was set to 25°C. The electrodes were fully immersed in the sample solution. The readings displayed were recorded. The reading was expressed in millimhos/cm by multiplying with the cell constant.

3.2.2.3 Soil colour and soil texture

Soil colour was determined by the Quick method and soil texture by the Feel method (Clarson, 2002). Quick method is a visible method, determined by seeing the colour of the soil. Texture
refers to the relative percentage of sand, silt and clay. In Feel method, to determine the soil texture, handful of soil was taken in the hand and moistened with water. The soil was rubbed between the thumb and fingers and recorded the texture.

3.2.2.4 Estimation of Available Nitrogen in the soil

Available nitrogen in the soil was estimated by Alkaline permanganate method, suggested by Subbiah and Asija (1956).

**Principle**

The amount of soil nitrogen released by alkaline permanganate solution is estimated by distillation with sodium hydroxide. The distillate is collected in boric acid containing mixed indicator and titrated against standard sulphuric acid. The nitrogen so estimated is designated as available nitrogen which is correlated with crop response to nitrogen fertilizers.

**Reagents**

1. Potassium permanganate (0.32%)
2. Sodium hydroxide (2.5%)
3. Boric acid (2%)
4. Mixed indicator – 0.5g of bromocresol green and 0.1g of methyl red in 100 ml ethylalcohol.
5. Sulphuric acid. (N/50)
6. Distilled water.
7. Liquid paraffin.
**Procedure**

20g of soil was taken in a distillation flask. 20ml of distilled water was added to it, followed by 1ml of liquid paraffin to control frothing. Then added 100ml of 0.32% potassium permanganate solution and 100ml of 2.5% sodium hydroxide solution. Distilled the content in a steady rate collecting the liberated ammonia in a 500ml ice tumbler containing 20ml of 2% boric acid with mixed indicator. Continued the distillation until 10ml of distillate was collected in the beaker. The ammonia so collected was titrated against N/50 sulphuric acid. From the titre value, the available nitrogen content of the soil was calculated and expressed in mg/100g.

**Calculation**

1ml of N/50 $\text{H}_2\text{SO}_4 = 0.00028\text{g of nitrogen}$

\[
\text{Available nitrogen (kg/ha)} = \frac{TV \times 0.00028}{1000} \times \frac{1000}{20} \times 2 \times 10^6
\]

\[= TV \times 28 \text{ (kg/ha)}\]

**3.2.2.5 Estimation of Available Phosphorus in the soil**

Available phosphorus content in the soil was estimated by Olsen’s method (Olsen et al., 1954) for neutral or alkaline soil and by Bray method for acidic soil.

**Principle (Olsen’s Method)**

A solution of 0.5M sodium bicarbonate buffered at pH 8.5 is used for the extraction of available phosphorus in neutral or alkaline
soils. The extracted phosphorus in the solution is estimated colorimetrically by developing a blue colour of the chlorostannous indicator reduced to molybdic phosphoric acid.

**Reagents**

1. Olsen’s reagent – 0.5M sodium bicarbonate adjusted to pH 8.5

2. Dargo –G60 (activated carbon powder.)

3. 1.5% ammonium molybdate reagent – 15g ammonium molybdate was dissolved in 300ml distilled Water, warmed to 50°C and added 450ml of 10NHCl with constant stirring and made up to one litre with distilled water

4. Stannous chloride solution.
   a) Stock solution - Prepared by dissolving 10g stannous chloride in 25 ml conc. HCl.
   b) Working solution - Diluted 1ml of stock solution to 66ml with distilled water

5. Standard potassium hydrogen phosphate

**Procedure**

To 5g of soil, a pinch of Dargo- G60 was added. Shaken for 30 minutes with 50ml of Olsen’s extractant and filtered. To the filtrate 5ml 1.5% ammonium molybdate reagent and 1ml stannous chloride solution was added. Mixed well and kept for 10 minutes. The intensity of the blue colour developed was read in a photoelectric
colorimeter at 660nm. Standards of varying concentration was prepared using KHPO$_4$. The concentration of phosphorus in ppm was found out from the graph. Then the values were calculated for mg/100g.

**Bray Method**

**Principle**

This method is used as an index of available P in acid soil. The combination of HCl and ammonium fluoride is designed to remove easily acid soluble forms of P. This ammonium fluoride dissolves aluminium and iron phosphates in acid solution. The amount of phosphorus extracted is determined colorimetrically.

**Preparation of Bray extractant**

Dissolved 11.1 gm of ammonium fluoride(0.03N) in 200ml water. Diluted 88ml conc. HCl to 1.5 litre (1N). Added the fluoride solution to acid solution and was made up to 2 litres. Diluted the stock solution 5 times to get the working solution.

**Procedure**

To 5gm of soil sample, 50ml Bray working solution was added. Shaken well for 5 minutes and filtered. Pipetted 5ml of the filtrate into a 50 ml conical flask and added 10ml of 0.6M boric acid followed by 1ml of chlorostannous acid working solution. Mixed well and colour was read after 5 minutes at 660 nm. The blank was prepared with 5ml Bray working solution instead of soil extract. A graph was
plotted and available phosphorus in soil was calculated from the graph and was expressed in mg/100g.

**Calculation**

Weight of soil = 5gm

Volume of extractant = 50ml

Volume of aliquot = 5ml

Available phosphorus (kg/ha) = \( \frac{ppm \times 50}{5} \times 2 \times 10^6 \)

= ppm X 20 (kg/ha)

**3.2.2.6 Estimation of Available Potassium in the soil**

The available potassium in the soil was determined by flame photometric method suggested by Stanford and English, (1949)

**Principle**

Available potassium was estimated by extraction with neutral normal ammonium acetate. The potassium ions in the exchangeable sites are replaced with ammonium ions leached from soils. The potassium ions in the solution is then determined by Flame photometer. The principle underlying the flame photometry is that the elements when excited in a flame emit radiation at characteristic wavelengths. It measures the emission intensity in proportion to the concentration of potassium in the solution.
Reagents

1. Neutral ammonium acetate solution

2. Potassium chloride stock standard – 1.9068g pure dry potassium chloride was dissolved in distilled water and made upto 1 litre. This gives 1000 ppm of potassium.

3. Working standard – 10ml of stock solution is diluted to 100ml. This gives 100ppm of potassium.

Procedure

5g soil was taken in a 100ml shaking bottle. 25ml neutral normal ammonium acetate solution was added and shaken for 5 minutes in a mechanical reciprocating shaker and filtered. The amount of potassium in the filtrate was measured in a flame photometer. A standard graph was prepared using potassium chloride in varying proportion of working standard and the available potassium in the soil was calculated from graph and expressed in mg/100g.

Calculation

\[
\text{Available K (kg/ha)} = \frac{\text{graph reading (ppm)} \times 25 \times 2 \times 10^6}{5 \times 10^6}
\]

\[
= \text{ppm} \times 10 \text{ (kg/ha)}
\]
3.2.2.7 Estimation of Available Calcium in the soil

Available calcium in the soil was measured by Versanate method (Clarson, 2002).

**Principle**

Calcium form stable complex with EDTA at pH 12. Calcium in solution can be titrated with 0.01 N Versanate using murexide indicator in presence of sodium hydroxide solution. The end point is a change of colour from orange red to purple at pH 12.

**Reagents**

1. Sodium hydroxide –16% solution
2. Murexide indicator
3. EDTA 0.01 N (Versanate).

**Procedure**

To an aliquot of the sample solution, 2-3 crystals of carbamate was added to prevent the interference of other elements if present. To this 45 mg murexide indicator powder was added. Titrated it with 0.01N EDTA solution till the colour changed from orange red to purple colour.

**Calculation**

Milliequivalents of calcium present =
\[
\frac{\text{Volume of versanate used} \times \text{Normality of versanate}}{\text{Volume of aliquot taken}} \times 500
\]

From the values obtained, the amount of available calcium was calculated in mg/100g of soil and was recorded.

3.2.2.8 **Estimation of Available Magnesium in the soil by Versanate method.**

Available magnesium in the soil was measured by Versanate method given by Clarson (2002).

**Principle**

Magnesium in solution can be titrated with 0.01N versanate (EDTA) using Erichrome black T-dye as indicator at pH 10 in the presence of ammonium chloride and ammonium hydroxide buffer. The end point is the change of colour from wine red to green.

**Reagents**

1. EDTA – 0.01N
2. Erichrome black T dye indicator

**Procedure**

To an aliquot of soil sample, 2-5 crystals of carbamate were added followed by 5ml of ammonium chloride ammonium hydroxide buffer 3-4 drops of Erichrome black T dye indicator was added to it. This solution was then titrated with 0.01N versanate till the colour changed to green.
Calculation

Milliequivalents of magnesium per litre =

\[
\frac{\text{Volume of EDTA} \times \text{Normality of EDTA}}{\text{Volume of aliquot taken}} \times 1000
\]

From this, the amount of magnesium was calculated and expressed in mg/100g.

3.2.3 Isolation and identification of *Frankia* from rhizosphere soil

Isolation of *Frankia* from rhizosphere soil samples was carried out using DPM medium (appendix) by modified sucrose fractionation procedure. The morphological characteristics were observed and recorded. Gram staining and catalase test were undertaken. Number of *Frankia* colonies from each soil sample was counted and recorded.

3.2.3.1 Isolation of *Frankia* by modified sucrose fractionation procedure

Isolation of *Frankia* and its enumeration was carried out by the method suggested by Baker and O’Keefe (1984).

Soil suspension was prepared and incubated in 0.7% (v/v) liquefied Phenol for 10 minutes. The suspension was then layered on to a sucrose gradient and centrifuged in a swinging bucket rotor at 100,000 g for 3 hours. After centrifugation the fractions obtained was inoculated to the DPM medium supplemented with
cycloheximide (30 $\mu$g/ml) and incubated until visible colonies were observed.

### 3.2.3.2 Gram staining technique

Gram staining technique of *Frankia* was carried out by the procedure adopted by Ananthanarayanan and Panicker (1996).

**Principle**

Gram staining is used for identification of unknown bacteria. The procedure is based on the ability of bacteria to retain the purple colour of crystal violet during decolorization with alcohol. Gram negative bacteria are decolorized by alcohol. Gram positive bacteria are not decolorized. After decolorization, safranin, a counter stain, impart a pink colour to the decolorized gram negative organisms.

**Reagents and stains**

1. Crystal violet
2. Gram’s iodine
3. 95% ethanol
4. Safranin

**Procedure**

The smear was prepared and fixed by heating it over a flame very briefly. It was then covered with crystal violet for 30 to 60 seconds. The stain was washed off rapidly with distilled water and excess water was drained off. The smear was then covered with
grams iodine for one minute. The iodine was washed off and decolourised rapidly with 95% ethanol. Washed immediately and then covered the smear with safranin for 30 seconds and excess stain was washed out. The smear was air dried and examined under oil immersion microscope.

3.2.3.3 Catalase test

Principle

Catalase break down hydrogen peroxide to oxygen and water. Organism to be tested is kept in contact with 30% \( \text{H}_2\text{O}_2 \). Bubbles of oxygen are released if the organism is catalase producer. (Ananthanarayanan and Panicker, 1996).

Procedure

The colony was immersed in 30% \( \text{H}_2\text{O}_2 \) using a sterile wooden stick and observed for immediate bubbling, confirming that it is catalase positive.

3.2.4. Root nodule analysis

Isolation and enumeration studies of \textit{Frankia} colonies in root nodules were conducted. Screening of suitable medium for growth and multiplication of \textit{Frankia} was also carried out. Staining of root nodules with trypan blue in lactophenol (0.05% W/V) for microscopic observation were done and the observations were recorded.
3.2.4.1 Isolation of *Frankia* from root nodules of *Casuarina equisetifolia*

Differential filtration technique was adopted for the isolation of *Frankia* from root nodules. Three different media viz: BuCT medium, Qmod medium and DPM medium (appendix) were used for the isolation in order to select the most suitable medium for the growth of *Frankia*.

**Procedure**

**Differential filtration technique**

Isolation of *Frankia* from the root nodules of *Casuarina equisetifolia* was carried out by the procedure suggested by Benson (1982).

Nodules were cleaned thoroughly with a stream of water and dissected into individual lobes. Lobes were surface sterilized with 30% H₂O₂ for 30 minutes and rinsed with distilled water. 1g of nodules from each location were homogenized in a mortar and pestle in 5 ml Frankia medium (appendix). Homogenized nodule suspension was filtered through nylon net of pore size 100µm and 20µm. the crushed nodule debris was collected into the 100µm filter and washed twice with 30ml Frankia medium. Agar plates containing respective medium supplemented with antibiotics nalidixic acid (30µg/ml) and amphotericin B (10 µg/ml) were taken and inoculated with homogenized root nodule suspension. The plates were incubated
at 30°C for 3 weeks. When the colonies were formed, they were removed aseptically and homogenized the mass and inoculated 1 ml of Frankia medium containing 0.3% (W/V) filter sterilized sodium pyruvate with a single homogenized colony.

3.2.4.2 Staining of root nodules of Casuarina

Staining was carried out by the procedure suggested by Phillips and Hayman (1970).

**Principle**

When stained with trypanblue in lactophenol, the peripheral hyphae take up stain and was blue coloured.

**Reagent**

1. Potassium hydroxide, 10%
2. Hydrochloric acid, 2%
3. Trypan blue, 0.05%
4. Lactophenol

**Procedure**

The root nodules were washed and cut into small bits. They were immersed in 10% KOH for stain penetration and autoclaved at 15 lb/sq pressure for 20 minutes. KOH solution was then poured out and the root bits were rinsed in water until no brown colour appeared in the rinsed water. Nodule bits were then acidified with 2% HCl for 3-4 minutes for proper staining. Acid was poured out without
rinsing with water and root nodule bits were stained with 0.05\% trypan blue in lacto phenol and boiled for about 10 minutes and cooled. They were then examined under the microscope and characters were observed.

3.3.0 Pot culture experiment

A pot culture experiment was conducted at ACRC, Kottayam in order to determine the efficiency of *Frankia* isolates on the test crop of different *Casuarina* species and to study the interaction effect of *Frankia* with nitrogen fixing soil bacterium *Azotobacter*, on the growth, yield and effectiveness of *Casuarina* species. The details of the experiment are as follows.

3.3.1 Collection of seeds

Seeds of two species of *Casuarina* viz *Casuarina equisetifolia* and *Casuarina junghuhniana* were obtained from the Institute of Forest Genetics and Tree breeding (IFGTB), Coimbatore. The details of seed collection are given below.

<table>
<thead>
<tr>
<th>Variety of Casuarina</th>
<th>Date of collection</th>
<th>Place of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Casuarina equisetifolia</em></td>
<td>9.8. 2004</td>
<td>Panampally, Tamilnadu</td>
</tr>
<tr>
<td><em>Casuarina junghuhniana</em></td>
<td>16.8.2004</td>
<td>Karunya, Tamilnadu</td>
</tr>
</tbody>
</table>

3.3.1.1 Seed treatment and Germination

Seed treatment and germination were carried out as per the procedure outlined by Turnbull and Martensz, (1981). Seeds of the
above varieties of *Casuarina* were washed and surface sterilized with 30% H$_2$O$_2$ for 15 minutes. (Plates 2 and 3) They were then rinsed three times with sterile water for 15 minutes. Vermiculite and sand in the ratio 2:1 was taken and the mixture was sterilized at 121°C in an autoclave. The seeds were sown in to trays containing sterile vermiculite: sand in 2:1 ratio. A thin layer of sand was used to cover the seeds. They were watered daily until the seeds started to sprout. They were kept in a shade house at a temperature of 28°C. Seeds germinated within one week. After 10 days, thinning of the seedlings was done. Zinc sulphate 0.5% solution was sprayed on four alternative days and they were watered daily. They were treated with Hoagland’s nutrient solution (appendix) once, on the 16th day of sowing. (Plates 4 and 5)

### 3.3.1.2 Transplanting of seedlings

When the seedlings were 49 days old, they were transplanted in to free draining pots of pot size 29x27cm (Height x width). The pots were filled with a mixture of vermiculite, sand and soil in the ratio 1:1:3 to contain 10 kg/pot. The initial pH of soil was 6.85. Before transplanting, the shoot length, root length and whole length of seedlings and other morphological features of seedlings were recorded. Physico-chemical analysis of soil was also done before transplanting.

24 pots were taken and the seedlings of two species were transplanted to the respective pots as per the treatments. Five
seedlings were planted on each pot (5 plants /pot). All the plants were given water daily.

### 3.3.2 Treatments and structure of pot culture experiment

- **Treatment –1**: Soil + *Casuarina equisetifolia*
- **Treatment –2**: Soil + *Casuarina junghuhniana*
- **Treatment-3**: Treatment-1 + *Frankia*
- **Treatment –4**: Treatment –2 + *Frankia*
- **Treatment –5**: Treatment-3 + *Azotobacter*
- **Treatment –6**: Treatment –4+ *Azotobacter*

#### 3.3.3 Number of treatments
- 6

#### 3.3.4 Number of replications
- 4

#### 3.3.5 Design
- CRD (Complete Randomized Design)

#### 3.3.6 Number of pots
- 24

#### 3.3.7 Period of pot culture study
- 10 months.

### 3.4.0 Preparation of nodule inoculum

Crushed nodule inoculum was prepared by the procedure suggested by Subba Rao and Rodriguez Burreuco, (1995). Root nodules collected during survey from different locations were cleaned thoroughly with water and surface sterilized with 30% H$_2$O$_2$ for 30 minutes. Rinsed with distilled water several times. 20g of nodules were weighed and ground with a sterile mortar and pestle. Nodules were homogenized in presence of 1% polyvinyl pyrrolidone (PVP) and 10% activated charcoal to decrease the blackening of solution due to the release of toxic phenolic compounds that is deleterious to the
seedlings. It was made up to 200ml with sterile distilled water and filtered using a muslin cloth.

3.5.0 Inoculation of the seedlings with root nodules suspension.

15 days after transplanting. Thinning of the plants after leaving one plant/pot was done. 10ml of the homogenized root nodules suspension was injected in to the base of each seedlings by using a sterile syringe. Thus the inoculum was introduced around the root of respective treatments.

3.6.0 Inoculation of seedlings with *Azotobacter chroococcum* culture

The treatments pertained to *Azotobacter* culture were inoculated with *Azotobacter chroococcum*. 20g of *Azotobacter* culture was applied to the base of respective treatments.

3.7.0 Nutrient application

A starter dose of nitrogen, at the rate of 2mg ammonium nitrate per pot were applied to all pots 3 weeks after inoculation.

Hoagland’s nutrient solution was given to all plants at 2 weeks intervals for 6 months in the following doses.

<table>
<thead>
<tr>
<th>Period</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 days</td>
<td>30ml</td>
</tr>
<tr>
<td>30 days</td>
<td>40ml</td>
</tr>
<tr>
<td>45 days</td>
<td>50ml</td>
</tr>
</tbody>
</table>
Upto 4 months Hoagland’s solution was added without nitrogen. After that, nitrogen containing Hoagland’s solution with diammonium phosphate (2g/l) was added.

Neem –garlic extract (1:1 ratio) was also sprayed two times in order to prevent pests, if any. The plants were watered daily. (plates 10 and 11)

Harvesting was done on the 290\textsuperscript{th} day of planting (9 months and 20 days old).

\textbf{3.8.0 Collection of data}

Height of the plants were taken, periodically and recorded. Shoot length, root length, no: of nodules per plant, number of connates, basal girth, wet weight of shoots and roots were taken at the time of harvesting. Shoots, roots with root nodules and rhizosphere soil from each treatments were collected and placed in
sterile polythene bags and kept for further studies. The physical and chemical properties of post harvest rhizosphere soil samples were analyzed by the methods previously described.

3.9.0 Isolation of Frankia from rhizosphere soils and root nodules at post harvest stage

Isolation, enumeration and identification of Frankia were evaluated as per the methods described previously and the observations were recorded. Frankia from nodules were cultivated in BuCT medium which was selected as the most suitable medium for the isolation of this strain of Frankia. Staining of root nodules with trypan blue and lactophenol were done.

3.10.0 Isolation of Azotobacter sp from rhizosphere soil samples at post harvest stage.

Dilution pour plate technique described by Dubey and Maheswari (2002) was adopted for the isolation of Azotobacter. One gram soil was mixed with 9ml sterile water. Shaken well. This corresponds to 1x10^{-1} dilution. From this, serial dilutions up to 1X10^7 was prepared. One ml of appropriate dilutions were transferred on to the agar plates. Jensen’s medium was used for the isolation (appendix). The plates were incubated at 30^0C for 4-6 days and the colony forming units developed were recorded.

Enumeration of Azotobacter colonies were done. Identification were carried out by observing the morphological colony characters,
microscopic examination by Gram staining and by biochemical tests and the observations were recorded.

**3.11.0 Plant analysis**

**3.11.1 Estimation of Total Biomass**

Shoot length, root length, number of nodules, basal girth, no: of connates were recorded at the time of harvest. The roots were washed gently with water to remove the soil. The plants were separated to stem, side branches, roots and nodules. They were then dried in hot air oven at 65°C for three days and weighed separately after cooling and shoot dry mass; root dry weight and total biomass content were determined and were expressed in g/plant.

**3.11.2 Assessment of Root: Shoot ratio and Quality index**

The root to shoot dry weight ratio was worked out by dividing the weight of dry root by the weight of dry shoots of each plant separately. Quality index assessment was made using the formula of Dickson et al., (1960).

Quality index =

\[
\frac{\text{Seedling dry weight (g)}}{\text{Height (cm)/ diameter (mm) + shoot dryweight (g) / root dry weight (g)}}
\]

**3.11.3 Analysis of Total Nutrient contents of plant material**

Plant shoots dried in hot air oven were ground well and they were passed through 0.4 – 0.5 mm sieve and stored in suitable
bottles and tightly corked. They were then analysed for nutrient concentration.

3.11.3.1 Analysis of Total Nitrogen content

Total nitrogen content in *Casuarina* shoot samples were analyzed by the Microkjeldhal’s procedure suggested by Humphries (1956).

**Principle**

The method involves the digestion of plant material to convert nitrogen into NH$_3$. During digestion it remains in the form of ammonium sulphate because of excess H$_2$SO$_4$. Ammonia is determined by distillation, following a strong alkalinization. The NH$_3$ gas so liberated, is absorbed in a known volume of boric acid, the quantity of which is determined by back titrating it with a standard acid.

**Reagents**

1. Diacid mixture - Sulphuric acid and perchloric acid in 5:2 ratio
2. Boric acid - 2%
3. Double indicator - 0.003g of methyl red and 0.006g bromocresol green in 100ml methanol.
4. Sulphuric acid. - N/50
5. Sodium hydroxide 40%
**Procedure**

0.5g of powdered plant material was digested using 10 ml of diacid mixture. The digest was made up to 50ml with distilled water. About 10ml of the acid digest was taken and transferred to microkjeldhal’s flask and added 10ml 40% NaOH to distill the ammonium evolved. 2% boric acid with 2-3 drops of double indicator was used as the indicator solution. Blanks were prepared without adding sample. Completion of distillation was tested using a moistened red litmus paper. After the completion of distillation, the contents were titrated against N/50 sulphuric acid. From the titre value the nitrogen content was calculated and expressed in percentage.

**Calculation**

\[
\text{Percentage of nitrogen} = \frac{x \times 0.00114 \times v \times 100}{10 \times w}
\]

- x = Volume of N/50 H₂SO₄ used
- v = Volume of diacid prepared

**3.11.3.2 Analysis of Total Phosphorus content**

The total phosphorus content in plant material was analyzed by the vanadomolybdate phosphoric yellow colour method proposed by Jackson, (1973).
Principle

Digested plant material is allowed to react with vanadomolybdate. A characteristic yellow chromogen of the vanadomolybdophosphoric system is formed, the intensity of colour is measured colorimetrically at 470nm.

Reagents

1. Triple acid mixture - Nitric acid: sulphuric acid: perchloric acid in 5:2:1 ratio.

2. Vanado molybdate reagent - Solution A
   25g ammonium molybdate was dissolved in 400ml warm distilled water and was cooled.

   Solution B
   Slowly added 1.25g of ammonium meta vanadate to 300ml of boiling water and was cooled. To it 250ml concentrated HNO₃ was added and cooled.

3. Phosphorus standard

Procedure

0.5g of powdered plant material was digested with 10ml triple acid mixture. After cold digestion, the digestion mixture was heated
at 180°C to 200°C till the digestion mixture becomes a clear solution. Then the digest was made up to 50ml with distilled water. An aliquot of 20ml of acid digest was taken in a 50 ml volumetric flask and 10 ml of vanado molybdate reagent was added and allowed to stand for 30 minutes to develop yellow colour. The volume was made up to 50 ml and the colour developed was read at 470nm in a photoelectric colorimeter. Phosphorus content was determined by comparing the reading with the standard graph and expressed in percentage.

3.11.3.3 Analysis of Total Potassium content and Total Calcium content

Total potassium and total calcium content of plant material was estimated by the procedure outlined by Jackson, (1973).

Principle

Potassium and calcium when excited in flame emits radiations of characteristic wavelength. This emission intensity is proportional to concentration of respective element in the solution which is measured through the flame photometer.

Reagents

Triple acid mixture - nitric acid: sulfuric acid: perchloric acid 5:2:1 ratio.

Procedure

0.5g of powered plant material was digested with 10ml triple acid mixture and the digest was made up to 100ml with distilled
water in a volumetric flask. This triple acid digest was used to estimate the potassium and calcium content using flame photometer and the results were expressed in percentage.

**Calculation**

Potassium (%) = \( \frac{OD\ value \times 100 \times \text{dilution}}{10 \times 0.5 \times \text{aliquot} \times 10000} \)

Calcium (%) = \( \frac{OD\ value \times 100}{5 \times 0.5 \times 10000} \)

**3.11.3.4 Analysis of Total Magnesium content**

Magnesium content was estimated using the method proposed by Ure (1983) in Atomic absorption spectrophotometer. The percentage was calculated by using the formula.

**Calculation**

Magnesium (%) = \( \frac{100 \times \text{Dilution} \times \text{OD Value}}{0.5 \times \text{aliquot} \times 10000} \)

**3.11.3.5 Nutrient uptake studies**

Nitrogen and phosphorus uptake by the *Casuarina* plants under study were calculated for each treatment by multiplying the contents of nutrients (expressed in percentage) with respective yields and the results were expressed in mg/plant.
3.11.4 Biochemical analysis

3.11.4.1 Estimation of Nitrogenase activity in root nodules of *Casuarina*

Nitrogenase enzyme activity in root nodules was estimated by acetylene reduction assay according to the procedure of Larue and Kurz (1973).

**Principle**

Acetylene is reduced to ethylene by the nitrogenase. The ethylene is measured by oxidizing it to formaldehyde and determining the formaldehyde calorimetrically.

**Reagents**

1. Acetylene gas
2. Ethylene gas.
3. Sodium metaperiodate 0.05M – 16.5 g of sodium metaperiodate was dissolved and made up to 1 litre with distilled water.
4. Potassium permanganate 0.005M – 0.79g of KMnO₄ was dissolved and made up to 1 litre with distilled water.
5. Sodium arsenite 4M – 52g of sodium arsenite was dissolved and made up to 1 litre with distilled water.
6. Sulphuric acid 4N.
7. Oxidant solution – 80ml of 0.05M NaIO₄ and 10 ml of 0.005M KMnO₄ were mixed. pH adjusted to 7.5 with KOH and diluted to 100ml.

8. Acetyl acetone.

9. Nash reagent – 150 g of ammonium acetate, 3ml of acetic acid and 2ml of acetyl acetone were mixed and diluted to 1 litre.

**Procedure**

Plants were carefully removed from the soil without disturbing the root nodules. Roots with nodules were excised and was placed in a 100mL conical flask. The flask was sealed with rubber serum cap. 10ml of air was removed from the flask with an air tight syringe and 10ml of acetylene was injected in to the flask and incubated for 60 minutes at room temperature. 15ml of oxidant solution was taken in a 10ml conical flask sealed with rubber cap. 5ml of gas was transferred to it by using a syringe from the conical flask in which plant roots were incubated with acetylene. The 10ml conical flask was agitated vigorously in a rotary shaker at 300 rpm for 90 minutes at room temperature. 0.25ml 4M sodium arsenite and 0.25 ml 4N sulfuric acid was added and mixed to destroy excess oxidant and the absorbance at 412 nm was determined after 60 minutes. Standards containing known amounts of ethylene were carried through the analysis at the same time as the samples.
Calculation

Activity of nitrogenase = $\mu$mol ethylene per unit sample per unit time

3.11.4.2 Determination of Acid phosphatase activity in root nodules and rhizosphere soil

Acid phosphatase activity was determined according to the procedure suggested by Sadasivam and Manickam (1991).

Principle

At an optimum pH of 5.3, acid phosphatase hydrolyzes p-nitrophenol phosphate. The released p-nitrophenol is yellow in colour in alkaline medium and is measured at 405 nm.

Reagents

1. Sodium hydroxide 0.085N – 0.85g sodium hydroxide dissolved in 250ml water.

2. Substrate solution – Dissolved 1.49 g EDTA, 0.84g citric acid and 0.03g p-nitrophenyl phosphate in 100ml water and pH was adjusted to 5.3.

3. Standard – 69.75mg p-nitrophenol was dissolved in 5.0ml distilled water (100 mM).

4. Enzyme extract – 1g sample was homogenized in 10 ml ice cold 50mM citrate buffer (pH 5.3) in a pre chilled pestle and mortar. Filtered through four layers of cheese cloth. Centrifuged the
filtrate at 10,000g for 16 minutes. Supernatant was used as the enzyme extract.

**Procedure**

3ml of substrate was incubated at 37°C for 5 minutes. 0.5ml of enzyme extract was added to it and mixed well. 0.5ml was immediately removed and mixed with 9.5ml of 0.085N sodium hydroxide. This corresponds to zero time assay (blank). Incubated the remaining solution (substrate + enzyme) for 15 minutes at 37°C. 0.5ml sample was drawn and mixed with 9.5ml 0.085N sodium hydroxide solution. Measured the absorbance of blank and incubated tubes at 405 nm. 0.2 to 1.0 ml (4 to 20 mM) of the standards were taken and diluted to 10 ml with sodium hydroxide solution and the colour was read and standard graph was prepared.

**Calculation**

Specific activity was expressed as mmoles p-nitro phenol released per minute per mg protein.

3.11.4.3 Determination of Alkaline phosphatase activity in root nodules and rhizosphere soil

Alkaline phosphatase activity was determined according to the procedure suggested by Sadasivam and Manickam (1991).

**Principle**

Alkaline phosphatase enzyme hydrolyzes p-nitro phenol phosphate at pH 10.5. The released p- nitro phenol is yellow in colour in alkaline medium. The absorbance is measured at 405nm.

**Reagents**

1. Sodium hydroxide 0.085 N – 0.85g sodium hydroxide was dissolved in 250 ml water.

2. Substrate solution – 375 mg glycine, 10mg magnesium chloride and 165 mg P- nitro phenol phosphate was dissolved in 42 ml of 0.1N sodium hydroxide and diluted to 100ml pH was adjusted to 10.5.

3. Standard – 69.75mg P- nitro phenol was dissolved in 5.0ml distilled water (100mM).

4. Enzyme extract – 1g sample was homogenized in 10 ml ice cold 50mM glycine NaOH buffer pH 10.5. Filtered and centrifuged the filtrate at 10, 000 g for 10 minutes. Supernatant was used as enzyme source.

**Procedure**

3ml of substrate solution was incubated at 37⁰C for 5 minutes. 0.5ml enzyme extract was added to it and mixed. 0.5ml was immediately taken from it and was mixed with 9.5 ml 0.085N NaOH. This corresponds to zero time assay (blank). The remaining solution (substrate + enzyme) was incubated for 15 minutes at 37⁰c. From
this 0.5ml sample was taken and mixed with 9.5ml NaOH solution. Absorbance was measured at 405 nm. A standard graph was prepared using standards of varying concentration from 0.2 to 1ml (4 to 20 mM) diluted to 10ml with sodium hydroxide.

**Calculation**

Specific activity was expressed as mmoles P-nitro phenol released per minute per mg protein.

### 3.11.4.4 Determination of Total Carbohydrate content in root nodules of *Casuarina*

Total carbohydrate content was determined by Anthrone method suggested by Hedge and Hofreiter (1962)

**Principle**

Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxy methyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

**Reagents**

1. Hydrochloric acid 2.5N

2. Anthrone reagent – 200mg anthrone was dissolved in 100ml of ice cold 95% sulfuric acid (freshly prepared).

3. Standard glucose solution
Stock standard – 100 mg glucose was dissolved in 100 ml water.

Working standard – 10ml stock standard was diluted to 100 ml with distilled water.

**Procedure**

100mg of sample was taken in a boiling tube Hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 NHCl and cooled to room temperature. It was neutralized with solid sodium carbonate until effervescence ceased. The volume was made up to 100 ml and centrifuged. Collected the supernatant and 1ml aliquot was taken for analysis. Standards were prepared by taking 0.2 ml to 1 ml of working standards. All tubes were made up to 1 ml with distilled water. 4 ml anthrone reagent was added to all the tubes. A blank was prepared without sample and with same quantity of reagents added. Boiled for 8 minutes and cooled rapidly and the green colour developed was read at 630nm. A standard graph was prepared and from the graph, the amount of carbohydrate present in the sample was calculated.

**Calculation**

Amount of carbohydrate present in 100 mg sample

\[
\text{Amount of carbohydrate present in 100 mg sample} = \frac{\text{mg of glucose}}{\text{volume of test sample}} \times 100
\]
3.11.4.5 **Estimation of Total Protein content in root nodules of *Casuarina***

Lowry’s method developed by Lowry *et al.*, (1951) was adopted for the estimation of protein.

**Principle**

The blue colour developed by the reduction of the phosphomolybdic – phosphotungstic components in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with alkaline cupric tartrate are measured in this method.

**Reagents**

1. Reagent A – 2% sodium carbonate in 0.1 N sodium hydroxide

2. Reagent B – 0.5 % copper sulphate in 1% potassium sodium tartrate.

3. reagent C- Alkaline copper solution – mixed 50 ml reagent A and 1ml reagent B prior use.

4. Folin – Ciocalteau reagent.


Stock standard - 50mg bovine serum albumin was dissolved in distilled water and made up to 50ml.
Working standard – 10 ml of stock solution was diluted to 50 ml with distilled water. One ml of the solution contains 200µg protein.

**Procedure**

**Extraction of protein from sample**

500mg sample was ground well with a pestle and mortar in 5-10 ml of phosphate buffer. Centrifuged and the supernatant was used for estimation.

**Estimation of protein**

0.2 ml to 1ml of working standards were taken in a series of test tubes. 0.2 ml of sample extract was taken. Volume of all the tubes were made up to 1ml. A tube with 1ml of water was taken as blank. 5ml of alkaline copper solution (Reagent C) was added to all the tubes including blank. Mixed well and kept for 10 minutes. 0.5ml Folin – ciocalteau reagent was added to all the tubes. Mixed well and was incubated for 30 minutes at room temperature in dark. A blue colour developed was read at 660 nm. A standard graph was plotted with concentration along x axis and absorbance along y axis from which the protein concentration was calculated.

**Calculation**

The amount of protein was expressed in mg / 100 g sample.
3.11.4.6 Estimation of Total Free Amino Acids in root nodules of *Casuarina*

Total free amino acids in root nodules were estimated according to the procedure of Misra *et al.*, (1975).

**Principle**

Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha–amino acids and yields an intensely coloured bluish purple product which is calorimetrically measured at 570 nm.

\[
\text{Ninhydrin} + \text{alpha–aminoacid} \rightarrow \text{Hydrindantin} + \text{decarboxylated amino acid} + \text{Co}_2^+ + \text{NH}_3
\]

\[
\text{Hydrindantin} + \text{ninhydrin} + \text{ammonia} \rightarrow \text{purple coloured product} + \text{water.}
\]

**Reagents**

1. Ninhydrin solution.
2. Citrate buffer 0.2M, pH 5.0.
3. Diluent solvent – mixed equal volumes of water and n-propanol.
4. Stock Standard: 50 mg leucine was dissolved in 50 ml distilled water.
5. Working standard – 10 ml stock solution was diluted to 100ml
**Procedure**

**Extraction of amino acids**

500mg sample was ground in a mortar and pestle with a small quantity of acid washed sand. To the homogenate 5-10 ml 80% ethanol was added. Centrifuged and supernatant was taken. Extraction was repeated twice with the residue and pooled all the supernatants. The volume was reduced by evaporation and the extract was used for the estimation of total free amino acids.

**Estimation**

To 0.1ml of extract 1ml ninhydrin solution was added. It was made up to 2ml with distilled water. Tubes were heated in a boiling water bath for 20 minutes. 5 ml of diluent was added and mixed. Kept for 15 minutes and the intensity of purple colour was read against a reagent blank at 570 nm. Reagent blank was prepared as above by taking 0.1ml 80% ethanol instead of the extract. A series of volume from 0.1 to 1 ml of working standard solution was taken that gives a concentration range 10µg to 100µg and was proceeded as that of the sample.

**Result**

A standard curve was drawn with absorbance versus concentration. From the graph concentration of total free amino acids in the sample was found out.
3.11.4.7 Estimation of Proline Content in root nodules of *Casuarina*

Proline content in root nodules were estimated according to the procedure of Bates *et al.*, (1973).

**Principle**

During selective extraction with aqueous sulphosalicylic acid, proteins are precipitated as a complex. Other interfering materials are also presumably removed by absorption to the protein sulphosalicylic acid complex. The extracted proline is made to react with ninhydrin in acidic condition to form the chromophore and read at 520 nm.

**Reagents**

1. Acid ninhydrin – 1.25 g ninhydrin was warmed in 30ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation until dissolved.
2. 3% aqueous sulphosalicylic acid.
3. Glacial acetic acid.
4. Toluene
5. Proline

**Procedure**

Extracted 0.5g of plant material by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Filtered the homogenate through
Whatman No.2 filter paper. 2ml of filtrate was taken and 2ml glacial acetic acid and 2ml acid ninhydrin were added. Heated in a boiling water bath for one hour. The tubes were then kept in ice bath. 4ml toluene was added to the reaction mixture and stirred well for 30 seconds. The toluene layer was separated and warmed to room temperature. The red colour intensity was measured at 520nm. A series of standards with proline were prepared and standard curve was plotted. From the standard curve the amount of proline in the test sample was calculated and expressed in mg/g.

**Calculation**

\[
\mu \text{ moles of proline per gram tissue} = \frac{\mu \text{g proline/ml} \times \text{ml toluene}}{115.5} \times \frac{5}{\text{g sample}}
\]

### 3.11.4.8 Estimation of Total Phenol Content in root nodules of *Casuarina*

Total phenol content was estimated by the procedure of Aparna Busarbarua (2000).

**Principle**

Phenol in alkaline medium react with phosphomolybdic acid of Folin – Cocalteau reagent producing a blue coloured complex.

**Reagents**

1. 80% ethanol
2. Folin – ciocalteau reagent

3. 20% sodium carbonate

4. standard solution

stock standard – 10mg catechol was dissolved in 100ml distilled water.

Working standard – stock diluted 10 times with distilled water.

**Procedure**

**Extraction procedure**

1g plant sample was ground in a mortar and pestle with 10ml 80% ethanol. Centrifuged at 10,000 rpm for 20 minutes. Decanted the supernatant to a 50ml beaker. Re-extracted the residue with 5ml of 80% ethanol twice, centrifuged and the supernatant were pooled. Evaporated the supernatant to dryness and added 5ml of distilled water to dissolve the residue.

**Estimation procedure**

1 ml of extract was taken and was made up to 3ml with distilled water and shaken well. 0.5ml of Folin- Ciocalteau reagent was added and shaken vigorously. After 3 minutes, 2ml 20% sodium carbonate solution was added. Mixed well and was kept for 1 hour. The absorbance was read at 630 nm against reagent blank. A standard curve was prepared using different concentrations of catechol.
**Calculation**

The concentration of phenol in the sample was found out from the graph and expressed in mg/100g.

**3.11.4.9 Estimation of Indole Acetic Acid in root nodules**

Indole acetic acid content in root nodules were estimated by the procedure of Tien *et al.*, (1979).

**Principle**

Indole acetic acid, when treated with Salper’s reagent forms a pink coloured complex, which is measured spectro photometrically at 535nm.

**Reagents**

1. Methanol
2. Hydrochloric acid 1N
3. Diethyl ether.
4. Salper’s reagent :- 1 ml of 0.5M ferric chloride was mixed with 50ml of 35% perchloric acid.

**Extraction**

5g freeze-dried plant material was ground with 10ml methanol to a fine suspension. Filtered the homogenate. Extracted the material on the filter twice by adding 10 ml methanol and then once with 5 ml. Evaporated the filtrate to an aqueous residue. The pH of the filtrate was adjusted to 2.8 with NHCl. Equal volume of cold (4°C) diethyl
ether was added. The contents were shaken in a separating funnel and allowed to stand for 4 hours at 4°C with intermittent shaking. The aqueous phase was then separated from organic phase. At 4 hour intervals two more ether extractions were done and organic phase were pooled and evaporated to dryness in the dark. The residue was dissolved in 2 ml methyl alcohol for the quantitative analysis.

**Procedure**

To 0.5ml sample, 1.5 ml of distilled water was added, followed by 4ml Salper’s reagent and incubated in darkness for 1 hour at 28°C. The intensity of the pink colour developed was read in a spectrophotometer at 535nm.

**Calculation**

A standard graph was prepared using a series of indole acetic acid solutions of known concentration as the standard and the quantity of IAA in the sample was calculated.

**3.12 0 Statistical analysis**

Observations recorded for all the characters were statistically scrutinized by the procedures suggested by Gomez and Gomez (1984) by the ANOVA table. Wherever the results were found to be significant, critical difference were worked out at 5% level (p=0.05).