Chapter 3

Materials & Methods
3. Materials and Methods

3.1. Location of sampling sites

The study sites were located in the central parts of Tamil Nadu in South India. It included sites in Pudukkottai (Arasadipatti, Kummanukulum, Manjanviduthi), Thanjavur (Thirukkanurpatti, Vallamputhur, Thirumalaisamuthirum) and Trichirappalli districts (Meickalnaickanpatti, Valavanthi, Thalaimalaipatti, M.Kalathur). Fig. 1 shows the location of the study sites. Trichirappalli is located at 10.8050°N 78.6856°E. The average elevation is 88 metres (289 ft). It is located almost at the geographic centre of the state of Tamil Nadu. Trichirappalli experiences a moderate climate from August to October, tempered by heavy rain and thundershowers, and cool and balmy climate from November to February. Fog and dew are rare and occur only during the winter season. Pudukkottai district covers an area of 4663 Sq. Km. which has a coast line of 39 Kms. The district is located between 78.25' and 79.15' of the East of Longitude and between 9.50' and 10.40' of the North of Latitude. Thanjavur is situated beside the mighty River Cauveri, Thanjavur is geographically located in between 10.8º N and 79.15º E in the South Indian state of Tamil Nadu. Thanjavur has a tropical climate condition. During summers the average temperature of the city rises to 36.6 ºC, while during the winter season, the average temperature goes down to 22 ºC. The city of Thanjavur experiences heavy rain of about 111.37 cm during the rainy season.

3.2. Collection of root and soil samples

Extensive field survey was carried out in order to collect the root and rhizospheric soils samples of sunflower plants from different agro-ecological zones of Pudukkottai, Thanjaur, Trichirappalli districts of Tamil Nadu during the year 2011 -2012. Samples were collected randomly. Rhizospheric soils at a depth of 4-16 cm from 5 different locations in each study site were collected in sterile polyethylene bags. Approximately 500gm of rhizosphere soil was collected. Soil samples were air-dried and stored at 4 ºC for processing. A
Fig. 1. Map Showing the sampling sites of Southern Districts of Tamil Nadu, South India

Meickalnaickanpatti-S1,
Valavanthi -S2,
Thalaimalaipatti -S3,
M.Kalathur -S4,
Arasadipatti - S5,
Kummankum-S6,
Manjanviduthi -S7,
Thirukkanurpatti -S8,
Vallamputhur –S9,
Thirumalaisamuthirum- S10
portion of the soil samples was analyzed for soil physio-chemical parameters like pH, electrical conductivity, available phosphorus, and available potassium. The remaining soil samples were used to isolate, plant growth promoting Rhizobacteria, *Trichoderma* and AM fungal spores. The root samples were washed thoroughly with running tap water to remove the adhered soil particles. Then roots were cut into small pieces of about 1cm and used for the assessment of percentage colonization of AMF.

3.3. Analysis of physico-chemical properties of soil samples

3.3.1. Physical parameters

3.3.1.1. Determination of soil pH

In a clean conical flask 20 g of air-dried soil was taken and 100 ml distilled water was added for making 1:5 soil suspension. It was shaken for one hour at regular intervals. After shaking, the suspension was filtered through Whatmann No. 42 filter paper. The pH of the sample was determined using a pH meter (Sartorius brand pH meter).

3.3.1.2. Estimation of soil electrical conductivity (Levine, 2001)

To 1ml of water, 1g of soil was mixed that is 1:1 suspension. The suspension was filtered using suction. A round Whatman no. 42 filter paper was put in the Buchner funnel, and the filter paper was moistened with distilled water and made sure that it was tightly attached to the bottom of the funnel and all holes were covered. The vacuum pump was started. The suction was opened and the suspension was added to Buchner funnel. The clear filtrate was transferred into 50 ml bottle and the conductivity cell was immersed into the solution and the readings were noted.

3.3.2. Chemical parameters

3.3.2.1. Estimation of available nitrogen

Nitrogen of soil is mainly present in organic form together with small quantities of ammonium and nitrate forms. The nitrogen supplying ability of the
soil was determined by distilling soil with alkaline potassium permagnate solution. During the distillation, easily utilizable and amino- N hydrolyzed nitrogen liberated as ammonia, is measured. This serves as an index of nitrogen status of soil. Alkaline potassium permagnate method (Subhaiah and Asija, 1956) was followed to estimate available N of soil samples.

In 1000 ml round bottom distillation flask (Kjeldahl flask), 20g soil was taken. To this 20ml distilled water was added. Then 100ml each of 0.32% potassium permanganate and 100 ml 25% NaOH solution were mixed and immediately connected to Kjeldahl assembly. The froth during boiling was prevented by adding liquid paraffin (1ml) and bumping by adding a few glass beads. The contents were distilled in a Kjeldahl at a steady rate and the liberated ammonia was collected in an Erlenmeyer flask (250 ml), containing 20 ml of 2% boric acid solution with methyl red and bromocresol green indicator. With the absorption of ammonia, the pinkish colour turns to green. After 30 minutes it was titrated with 0.02 N H2SO4 till the colour changed from green to original shade (pink). Blank (without soil) was run simultaneously. Available nitrogen was calculated from the following formula,

\[
\% \text{ of Available nitrogen} = \left( \frac{100}{Wt. \text{ of soil (g)}} \right) \times (A-B) \times N \text{ of acid} \times 0.014
\]

Where,

1. Wt. of soil sample - Wt.
2. Volume of std. acid required for soil - A ml
4. Normality of Sulphuric acid. - N

3.3.2.2. **Estimation of available phosphorus (P)**

Soil available phosphorus is found as orthophosphate in several forms and combinations, but only a small fraction of it may be available to plants. Available phosphorus was estimated by Olsen’s method (Olsen, *et al.*, 1954)
modified by Watanbe and Olsen (1965). The reagent for Olsen’s P was 0.5 M NaHCO₃ (pH 8.5) prepared by dissolving 42 g NaHCO₃ in distilled water and made upto 1 lit. The pH was adjusted to 8.5 with 20% NaOH solution. 2.5 g of air dried soil was weighed into 150 ml Erlenmeyer flask, 50 ml of Olsen’s reagent (0.5 M NaHCO₃ Solution, pH 8.5) and one teaspoonful of activate charcoal were added.

The flasks were shaken for 30 minutes on the electrical shaker and contents filtered immediately through Whatman filter paper (No. 41). 5 ml of the filtrate was pipetted out into 25 ml of volumetric flask and was neutralized with 1: 4 H₂SO₄ using paranitrophenol as indicator. The volume was made up by adding distilled water. Colour developed when few crystals of stannous oxalate were added. The solution was shaken well and intensity of blue colour was read in photoelectric calorimeter within 10 min. at wavelength of 730 to 840 μm. A blank was run without soil.

**Standard curve**

Analytical grade potassium dihydrogen orthophosphate (KH₂PO₄) was dried in hot air oven at 60 °C for 1 hr and allowed to cool. Exactly 0.439 g of KH₂PO₄ was dissolved in 500 ml of distilled water. 25 ml of 7N H₂SO₄ was added and made upto 1 lit. with distilled water. This gives 100 ppm standard stock solution of KH₂PO₄. From this by diluting it 5 times it was made upto 2ppm P solution. For the preparation of standard curve different concentrations of P 0,2,4,6,8 and 10 ml of 2ppm P solution were taken in 25 ml volumetric flask separately, which corresponds to 0, 0.16, 0.32, 0.48, 0.64 and 0.80ppm P respectively. To these 5ml of the extracting reagent 0.5 (NaHCO₃) was added to each flask and pH was adjusted as above. The content was diluted with 20 ml water and 4ml reagent (Dickman and Brays reagent). Volume was made up and intensity of blue colour was read in photoelectric calorimeter using 730 - 840nm filter or using red filter (660nm). Graph was constructed by plotting reading on X-axis and concentrations of P on Y axis.
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Concentration of phosphorus                             0.32
Factor (F) =   –––––––––––––––––––––––––––––––––––––––– = –––––– = 0.01
Corresponding reading of the above concentration       30

= 1 colorimeter reading = 0.01 ppm phosphorus (P)

**Calculations:**

The amount of phosphorus was estimated by using the formula

\[
\text{Factor (F)} = \frac{\text{ppm P in aliquot total}}{\text{Aliquot taken in (ml)} \times \text{volume of extract} \times \frac{1}{\text{wt. of soil (g)}}} \times (\text{RxF})
\]

3.3.2.3. **Determination of available potassium (K)**

Only small fraction of total K is held in exchangeable form, while the rest remains in fixed or non-exchangeable form. When the crop exhausts the supply of exchangeable K, more K is released from the fixed reserve. Exchangeable K, is therefore, also referred to as ‘available K’. The flame photometric method (Jackson, 1958) was employed to estimate available K of samples. 5g of air dried sample was taken in 150 ml Erlenmeyer flask and 25 ml of 1 N ammonium acetate was added to the flask. The contents were shaken for 5 minutes on a mechanical shaker and filtered immediately through a dry filter paper (Whatman No.1). The filtrate was collected in a beaker. 5ml of filtrate was diluted with 25 ml with distilled water. The above diluted extract was Atomized to flame photometer to note the reading. The amount of potassium was estimated by the following formula:

\[
\text{Available K} = \frac{(\text{Rx F}) \times \text{Vol. of extract} \times \text{DF} \times 2.24 \times 10^6}{\text{Soil wt} \times 10^6}
\]

Where
R = Reading
F = Con. of K / corresponding reading
DF = Dilution factor
3.3.2.4. Determination of organic carbon (C)

Organic matter plays an important role in supplying nutrients and water and provides good physical conditions to the plants. The quantity of organic carbon of the soil was estimated by the method described by Jackson, (1967). 1g finely ground soil sample passed through 0.5 mm sieve without loss was taken into 500 ml conical flask, to which 10ml of 1 N potassium dichromate and 20 ml Con. H$_2$SO$_4$ were added with measuring cylinder. The content was shaken for a minute and allowed to set aside for exactly half an hour. Then 200 ml distilled water, 10 ml orthophosphoric acid and 1 ml diphenylamine indicator were added. The solution was titrated against std. ferrous ammonium sulphate (FAS) or ferrous sulphate, till colour flashes from blue violet to brilliant green. The blank titration was carried at the beginning without soil.

1. Weight of soil taken
2. Vol. of 1N Potassium dichromate added
3. Vol. of 0.5N FAS required to neutralize
   
   \[ 10\text{ml of 1 N Pot. Dichromate solution (blank without soil)} = \]

4. Vol. of 0.5N FAS required for soil
5. Vol. of 1 N H$_2$Cr$_2$O$_7$ solution used for the oxidation of organic carbon present in the sample. \( = 10(B-T) \)

The organic carbon % was calculated by the following formula

\[
\text{Organic Carbon } \% = \frac{10 \times (B- T) \times 0.003}{B} \times \frac{100}{\text{wt. of soil (g)}}
\]
3.3.2.5. Estimation of magnesium

About 10 - 20 ml of soil saturation extract was pipetted out, and diluted to 20 - 30 ml with distilled water. Then 3-5 ml of buffer solution was added. After that few drops of erichrome indicator was added. This solution was titrated with 0.01 N EDTA until the color changed from red to blue (Cresser et al., 1993)

Calculation:

Percent of total magnesium (meq/L) = Ca + Mg (meq/L) - Ca (meq/L)

3.3.2.6. Estimation of zinc, copper, iron

These estimations were carried out according to Whitney (2011). 1.97 g of diethylene triamine pentaacetic acid (DTPA), and 1.1 g calcium chloride (CaCl$_2$) was weighed in a beaker, dissolved with distilled water and then transfered to a 1-litre volumetric flask. In another beaker, 14.92 g of triethanolamine (TEA) was weighed and dissolved with distilled water and the pH was adjusted to exactly 7.3 with 6 N hydrochloric acid (HCl), and the final volume was made upto 1-litre with distilled water. The final extractant solution was 0.005 M DTPA, 0.1 M TEA, 0.1 M CaCl$_2$. A series of standard solutions for micronutrients in DTPA extraction solution was prepared.

Fe: 0, 1, 2, 3, 4, 5 ppm;
Zn: 0, 0.2, 0.4, 0.6, 0.8, 1.0 ppm;
Cu: 0, 1, 2, 3, 4 ppm;

10 g of air-dried sieved soil (2-mm) was weighed into a 125-ml Erlenmeyer flask. 20 ml of extractant solution was added. It was shaken for 2 hrs on a reciprocal shaker. The suspension was filtered through a Whatman No. 42 filter paper. Zn, Fe and Cu were measured directly in the filtrate by using an Atomic Absorption Spectrophotometer.
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Calculation:

Percent of total Zn, Cu, Fe, (ppm) = \frac{(ppm \text{ in extract} - \text{Blank}) \times A}{Wt}

Where: 
- A = Total volume of the extract (ml)
- Wt = Weight of air-dry soil (g)

3.4. Isolation of PGPR from Rhizosphere soil

The Rhizosphere soil samples from sunflower were collected in polyethylene bags and transported to the laboratory and kept in refrigerator (4°C) for further process. Bacteria were isolated using serial dilution technique, 1gm of soil sample was suspended in 100ml autoclaved distilled water. After sedimentation of solid particles, dilution was made upto10^{-7}. 0.1ml of each dilution was spread by L-shaped glass rod on nutrient agar medium. The plates were incubated at 26°C -28°C for 24 hrs. On the basis of colony morphology, the bacterial colonies were randomly selected and further purified by streaking on the same medium. Colonies were picked from these plates and maintained as pure culture with periodic transfer to fresh media and stocked for further use. All the isolates were identified at genus level based on morphological and biochemical characteristics.

3.4.1. Production of Indole Acetic Acid (IAA)

Indole acetic acid production was detected as described by Bric et al., (1991). Bacterial isolates were inoculated in nutrient agar amended with L-Tryptophen and incubated at 37°C for 48hrs. Fully grown cultures were centrifuged at 3000rpm for 30 minutes, the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowaski reagent (50 ml 35% of perchloric acid, 1ml of 0.5mFeCl₃ solution). Development of pink colour was indicative of IAA production.
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3.4.2. Ability for Phosphates solubilization

Phosphate solubilizing ability of the isolate was checked on Pikovskaya (PVK) medium (Pikovskaya, 1948), incorporated with tricalcium phosphate (Ca$_3$ (PO$_4$)$_2$). The isolates were spot inoculated on PVK medium. Formation of transparent halo zone around the developing colonies indicated phosphate solubilizing ability of the isolates.

3.4.3. Assay for Ammonia production

The rhizobacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water and incubated at 30 °C for 48 hrs. Nessler’s reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappucino and Sherman, 1992).

3.4.4. Hydrogen Cyanide production (HCN)

Production of HCN was determined using the modified procedure of Millar and Higgins (1970). All the bacterial strains were grown on Trypticase Soy Agar (TSA) plates. Sterilized Whatman No. 1 filter paper strips were soaked in picric acid solution (2.5 gm of picric acid, 12.5gm of Na$_2$CO$_3$, in 1000ml of distilled water). The strips were placed in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28 °C ±1 °C for 48 hrs. A change in colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.

3.4.5. Screening for siderophore production

Screening for production of siderophores by bacterial strains was performed by plate assay according to Schwyn and Neilands, (1987). The tertiary complex Chrome azural S (CAS) / Fe$^{3+}$/ hexadecyl trimethyl ammonium bromide served as an indicator. Forty eight hour old culture of the strains was streaked onto the Succinate medium amended with indicator dye. The formation
of bright zone with yellowish fluorescent colour by the culture in the dark colored medium indicated siderophore production. The result was scored either positive or negative to this test, based on the colour change of the medium from blue to fluorescent yellow while no colour change indicated the absence of siderophore production.

**Medium preparation**

<table>
<thead>
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<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>Succinic acid</td>
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<tr>
<td>Di potassium hydrogen orthophosphate</td>
<td>3.0g</td>
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<tr>
<td>Ammonium sulphate</td>
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<tr>
<td>Dist water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
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</table>

**Preparation of dye**

CAS – 60.5 mg in 50 ml dist. Water + 10 ml iron solution (1 mM FeCl₃.6H₂O in 10 mm HCl). While constantly stirring this solution hexadecyl trimethyl ammonium bromide (72.9 mg of HDTMA in 40 ml water) was added. The resultant dark colored medium solution with agar was used as the growth medium.

3.4.6. Cell wall degrading enzyme production

3.4.6.1 Cellulase activity

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC (Carboxy Methyl Cellulose) agar containing (g L⁻¹) KH₂PO₄ 1.0, MgSO₄.7H₂O 0.5, NaCl 0.5, FeSO₄.7H₂O 0.01, MnSO₄.H₂O 0.01, NH₄NO₃0.3, CMC 10.0, Agar 12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M
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3.4.6.2 Chitinase activity

A minimal salt medium containing colloidal chitin as sole carbon and energy source was used. The medium consisted of Na$_2$HPO$_4$, 6g; KH$_2$PO$_4$, 3.0g; NH$_4$Cl, 1g; NaCl, 0.5g; yeast extract, 0.05g, colloidal chitin 1.0% (w/v) agar, 15g and distilled water, 1000 ml and incubated at 30°C. Colloidal chitin was prepared by the method of Hsu and Lockwood, (1975) from crab shell chitin (Sigma). Colonies showing zones of clearance against the creamy background were recorded as chitinase-producing PGPR.

3.4.6.3. Protease activity

Protease activity was determined by casein degradation in skimmed milk agar medium (Cattelan *et al*., 1999). An ability to clear the skimmed milk suspension in the agar plates was taken as evidence for the secretion of protease.

3.4.6.4. Isolation of *Macrophomina phaseolina*

Diseased plant tissues were washed under running tap water to remove surface soil and other contaminant. Infected tissues were cut into small pieces and placed in 1% sodium hypochloride for five minutes. After washing with distilled water they were placed on PDA plates and incubated at 28°C. The cultures were purified by hyphal tip method (Sinclair and Dhingra, 1985) and maintained on PDA slants by storing it under refrigeration (4°C). Phytopathogens isolated from infected tissues were identified based on morphological and microscopical charters as described by Gillman, (1998).

3.4.7. *In vitro* Antagonistic activity

The bacterial isolates were screened for their ability to inhibit *M. phaseolina* by employing dual culture method (Rabindran and Vidyasekaran, 1996) on PDA plates. Individually a loopful of 2 days old bacterial cultures grown in nutrient agar media was streaked on one side leaving 1 cm from the

NaCl for 15 min. Clear zone around the colony was indicative of cellulase enzyme production (Berlemont, 2009).
margin, and then 6mm disc of fungal pathogen culture was placed at the other side. Plates without antagonist served as control. The plates were incubated at 25±2°C for 4-5 days. Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist from the fungal radius. The percent inhibition was calculated using the formula:

\[
\% \text{ inhibition} = \frac{(R - r)}{R} \times 100
\]

Where ‘r’ is radial growth of the fungal colony opposite the bacterial colony and, R is the radial growth of the pathogen in control plate.

3.4.8. Molecular characterization of the potential isolate

3.4.8.1. Isolation of genomic DNA (Pospiech and Neumann, 1995)

The genomic DNA isolation was performed using the reagent PrepMan (PE Applied Biosystems, CA, USA). A loopful of bacterial cells grown on Luria-Bertani broth was suspended in 245 μl 0.1M TE. (10 mM Tris Cl, 0.1 mM EDTA; pH 7.00) The cell suspension was incubated with 5 μl (50 mg/ml) of lysozyme solution at 56 °C for 45 min The following reagents were then added: 196.2 μl of 0.1M TE, 5 μl of dithiothreitol (1 M), 20 μl of EDTA (0.25 M), 25 μl of sodium dodecyl sulphate (10%) and 3.8 μl of Proteinase K (20 mg/ml). The reaction mix was incubated at 37 °C for 1 hr. A 500 μl of PrepMan solution was then added and incubated at 56 °C for 30 min. The reaction mix was finally heated at 100 °C for 8 min and centrifuged at 8500 ×g for 2 min. The supernatant obtained was diluted at a ratio of 1:10 with nuclease free sterile de-ionized water.
Luria-Bertani (LB)-agar

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</tr>
<tr>
<td>Yeast extract</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>5g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
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<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
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</tr>
</tbody>
</table>

3.4.8.2. Agarose gel electrophoresis

The genomic DNA was resolved on 0.8% agarose gel. The agarose gel was prepared in 1× TAE buffer. The PCR amplified DNA and restricted DNA fragments were separated on 2% sieving agarose gel prepared in 1× TAE buffer. The electrophoresis was performed in 1× TAE buffer for 5 to 6 hrs at a constant voltage of 140 V at 25°C. The DNA samples were visualized by staining with 0.6 μg/ml of ethidium bromide. The agarose gel DNA profiles were observed and photographed in UVI gel documentation (UVItec, Cambridge, UK). The data analysis was done with UVI photo V.99 and UVI band / map V.99 software (UVItec.). All the thermocycling reactions were processed in Geneamp 2400 PCR system (Perkin Elmer, USA).

3.4.8.3. Sequencing of the genes encoding 16S rRNA

The Microseq™ kit which is a complete system for identification of the cultured bacterial isolates. The partial gene encoding 16S rRNA was sequenced with Microseq™ 500 bacterial sequencing kit (PE Applied Biosystems, USA).

3.4.8.4. Partial 16S rDNA sequencing

Amplification of the partial 16S rDNA sequences

The 500 bp sequences of 16S rDNA were amplified with Microseq™ 500 16S rDNA-PCR module (PE Applied Biosystems, USA). A volume of 1 μl of the genomic DNA was diluted in 24 μl of nuclease free sterile de ionized water. The various reagents of PCR; primers 8f (5'AGAGTTTGATCCTGGCTCAG3')
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and 1492r (5’GGTTACCTTGTTACGACTT-3’), dNTPs, AmpliTaq Gold DNA polymerase, MgCl₂ and buffer were pre-mixed into a single tube as the “PCR master mix”.

A 50 μl of the reaction mixture was prepared which consisted of 25 μl of the diluted genomic DNA (1 ng/μl) and 25 μl of the PCR master mix.

The cycling conditions for the amplification reaction were as follows:
Initial denaturation 95 °C for 10 min

This was followed by 30 cycles of
Step I 95 °C for 30 sec
Step II 60 °C for 30 sec
Step III 72 °C for 45 sec

A rapid thermal ramp of 1 °C/sec was maintained between the steps
Final extension 72°C for 10 min
Final temperature 4°C

A 5 μl of the amplified 16S rDNA was confirmed on a 2% agarose gel.

3.4.8.5. Purification of the amplified 16S rDNA

The PCR products were purified with Microcon 100 PCR centrifugal filter device. The Microcon column was hydrated by adding 500 μl of sterile MilliQ water to the column. The column was spinned at 500×g in a fixed angle microcentrifuge for 6 min. After hydration of column, 400 μl sterile MilliQ water was added to the column and then 45 μl of the PCR product was loaded on to the column. The column was spined at 500 × g in a fixed angle microcentrifuge for 15 min. Collection tube was removed and discarded. The column was now inverted and attached to a new collection tube. 25 μl Sterile MilliQ water was added to the inverted column and spinned at 10000 × g for 3 min to collect the purified DNA in the collection vial. The purified DNA was recovered in 25 μl of de-ionised water.
3.4.8.6. Cycle sequencing of the amplified 16S rDNA

The amplified 16S rDNA was subjected to cycle sequencing with Microseq™ 500 16S rDNA sequencing module. The forward and reverse sequencing reactions were assembled in separate reactions.

The 20 μl of the reaction mixture consisted of 3 μl Purified PCR product and 13 μl of sequencing reaction mix. The final volume of 20 μl was made up by 4 μl of de-ionised water.

The cycling conditions were as follows:

25 cycles of

- **Step I**: 96 °C for 10 sec
- **Step II**: 50 °C for 5 sec
- **Step III**: 60 °C for 4 min
- **Final**: 4 °C soak

A rapid thermal ramp of 1 °C/sec was maintained between the steps.

3.4.8.7. Analysis of the DNA sequences

The cycle sequenced DNA was resolved by ABI PRISM™ 310 genetic analyzer (PE Applied Biosystems). The DNA samples were sequenced with the short capillaries (5-47 cm × 50 μm) and long capillaries (5-61 cm × 50 μm). The electrophoresis was performed with 1× electrophoresis buffer with EDTA and performance optimized polymer (POP6).

The parameters set for the electrophoresis in ABI PRISM™ 310 genetic analyzer was as follows:

- **Temperature**: 50 °C
- **Current**: 4 μA
- **Voltage**: 12 KV
- **Argon ion Laser power**: 9.7 MW
3.4.8. Identification of the isolates

The identification of the isolates was assessed based on the partial 16S rRNA gene sequences. To identify unknown bacterial isolates, the 16S rDNA sequences obtained were subjected to basic local alignment search tool (BLAST) search. This search was performed with Microseq identification and analysis software “Microseq™ Analysis software v. 1.40, Microseq™ 16S rDNA Sequence Databases v. 1.01” (PE Applied Biosystems, USA). The sequences were also analyzed with the BLAST (N) search against the non-redundant Genbank+EMBL+DDBJ+PDB databases using NCBI web service: www.ncbi.nlm.nih.gov/blast.

3.5. Isolation of Trichoderma spp

The fungal antagonist Trichoderma spp were isolated from the rhizosphere soil of sunflower using serial dilution and pour plate technique on potato dextrose agar medium (PDA). The antagonist were purified and identified based on morphological and microscopical characters. The isolates were maintained on PDA slants at 4 °C throughout the study.

3.5.1. Growth inhibition assay by dual culture method

Interaction between antagonistic fungi and pathogenic fungi were determined by the method of Dennis and Webster, (1971). A 5 mm diameter mycelial disc from the margin of the Trichoderma one week-old culture and the pathogen M. phaseolina, were placed on the opposite side of the plate at equal distance from the periphery. In control plates (without Trichoderma), a sterile agar disc was placed at opposite side of the pathogen M. phaseolina inoculated disc. The plates were incubated at 28 ± 2°C and observed after 7 days.

3.5.2. Assay for volatile metabolites of Trichoderma spp

Productions of volatile metabolites by Trichoderma spp were assayed as described by Dennis and Webster, (1971) and Goyal et al., (1994) with slight modifications. The Trichoderma isolates were centrally inoculated by placing
3mm disc taken from three days old cultures on the PDA plates and incubated at 28 ± 2°C for three days. The top of each petridish was replaced with bottom of PDA plate inoculated centrally with the pathogen. Petridish with PDA medium without *Trichoderma* spp at the lower lid and the upper lid with pathogen was maintained as control. The pair of each petridishes were sealed together with paraffin tape and incubated for 4-6 days. After incubation the inhibition of mycelial growth was calculated.

3.5.3. Assay for non volatile metabolites of *Trichoderma* spp

The effects of non volatile metabolites produced by the *Trichoderma* spp were determined by following the method of Dennis and Webster, (1971). The isolates of *Trichoderma* spp were inoculated in 100ml sterile potato dextrose broth in 250ml conical flasks and incubated at 28 ± 2°C for 15 days. After incubation the cultures were filtered through Millipore filter and culture filtrates were added to molten PDA medium (40°C) to obtain a final concentration of 10% (v/v). The medium was poured into petriplates and after solidification, 3mm disc of the pathogen was placed centrally and incubated at 28 ± 2°C. Control plates were maintained without amending the culture filtrate. The percent of growth inhibition in all the above experiments were calculated by the formula

\[ I = \frac{C-T}{C} \times 100 \]

Where

- \( I \) = Percentage of inhibition
- \( C \) = Growth of mycelium in control
- \( T \) = Growth of mycelium in treatment

3.5.4. Assay for enzyme activity

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) containing the following components (in
grams per liter): MgSO₄·7H₂O, 0.2; K₂HPO₄, 0.9; KCl, 0.2; NH₄NO₃, 1.0; FeSO₄·7H₂O, 0.002; MnSO₄, 0.002 and ZnSO₄, 0.002. The medium was supplemented with the appropriate carbon source for cellulose and chitinase assay (commercially available Carboxy methyl cellulose, Chitin). The pH was set to 6.3 with 50mM phosphate buffer and autoclaved at 15 lbs for 20 min. The medium was inoculated with a spore suspension to give a final concentration of ~5 ×10⁶ conidia per milliliter and placed on a rotary shaker at 150 rpm at 25°C for different time intervals. The cultures were harvested at 24, 48, 72 hrs of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4°C to get cell-free culture filtrate which were then used as enzyme source.

3.5.5. Assay for cellulase activity

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Co.) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrates of different Trichoderma isolates. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulose activity is defined as the amount of enzyme that catalyzed 1.0 μ mol of glucose per minute during the hydrolysis reaction.

3.5.6. Assay for chitinase activity

It was measured using colloidal chitin as substrate (Bindo et al., 2005). Enzyme solution (0.5 ml) was added to 0.5 ml of substrate solution, which contained 1% colloidal chitin in phosphate buffer (0.05 M, pH 5.2) and 1 ml distilled water. The mixture was then incubated in shaking water bath at 50°C for 10 min, thereafter 3 ml of 3, 5-dinitrosalicylic acid reagent was added. The mixture was then placed in a boiling water bath for 5 min, after cooling, the developed color, as indication to the quantity of released N-acetylglucosamine
(NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from the standard curve of NAGA.

3.6. **Diversity measurements of AM fungi in rhizosphere soil of sunflower**

3.6.1. **Isolation of AM fungi**

AM spore were collected from the soil samples by wet sieving and decanting method (Gerdemann and Nicolson, 1963) to obtain viable and debris free AM spores. Soil mass 100gm was suspended in 500ml of distilled water, heavier particles gradually settle down the bottom of the container. The suspension was passed through the series of sieves of the following pore dimensions 710mm, 425mm, 250mm and 45mm. Residues from the last two sieves were washed and collected in the beaker. After 5 minutes the supernatant was filtered through Whatmann No. 1 filter paper. The filter paper containing the residues was placed on the Petri plates and was observed under light binocular microscope for spore counting. The healthy and fresh spores were isolated with the help of needles and were placed on the slide and were mounted in Poly Vinyl Lacto Glycerol (PVLG) to make permanent slides.

3.6.2. **Identification of AM fungi**

Based upon microscopic characters, the AM spores were identified. For identification and nomenclature, synoptic keys of the following authors were used: Mortan and Benny (1990); Schenck and Perez (1990); Walker and Trappe (1993). For species code, Perez and Schenck (1990) was followed. Classification was based on size, shape, color, surface structure, general nature of the contents, hypal attachment and wall details. Photomicrographs were taken with the help of Leitz-Diaplan and Nickon Optiphot microscope.

3.6.3. **Estimation of root colonization**

The technique of Phillips and Hayaman, (1970) modified by Koske and Gemma, (1989) was used throughout the research work for determining the AM infection in sunflower roots growing under field condition. This procedure included various steps i.e. depigmentation of roots by 10% KOH, washing with tap water,

Materials and Methods

Acidification of roots with 2N HCl, staining root with 1% trypan blue. Segments of 0.5 cm length were placed in slides and observed under Microscope. Mycorrhizal infection (vesicles, arbuscules) in each segment was observed in order to estimate the mycorrhizal infection levels. The root infection percentages were calculated from the number of infected segments out of total root segments.

\[
\text{Root colonization (\%) } = \frac{\text{Number of AM positive segments}}{\text{Total number of segments observed}} \times 100
\]

3.6.4. Statistical analysis

Ecological measures of diversity used to describe the structure of AMF communities included spore density, species richness, relative abundance, isolation frequency, Shannon-Wiener index of diversity, Simpson’s index of dominance (Simpson, 1949, Franke- Synder et al., 2001; Zhang et al., 2004). The formula used to calculate these parameters is given in the Table 1. Spore density reflects the biomass of AMF species, at least to some extent. Relative abundance was defined as the percentage of spore number of a species, which indicated the sporulation ability of different species of AMF. Isolation frequency was defined as the percentage of soil samples in which a species occurred, which revealed the extent of distribution of given AMF species in an ecosystem. The degree of diversity was reflected by Shannon-Wiener index of diversity. Since the isolated spores lacked distinguishable fine taxonomic character, few species were not identified to species level and were not included in the statistical analysis, except as part of total spore density. The Pearson correlation coefficient was employed to determine the relationship between Spore density and Species Richness, Relative abundance and Isolation frequency.
Table 1. Diversity measures used to describe AM communities

<table>
<thead>
<tr>
<th>Measure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore density (SD)</td>
<td>The number spores in 100gm soil</td>
</tr>
<tr>
<td>Species richness (SR)</td>
<td>Number of identified AMF species per soil sample</td>
</tr>
<tr>
<td>Relative abundance (RA)</td>
<td>Spore number of a species (genus) x 100</td>
</tr>
<tr>
<td></td>
<td>Total number of identified spore samples</td>
</tr>
<tr>
<td>IF (Isolation Frequency)</td>
<td>The number soil samples in which AMF species occurred x 100</td>
</tr>
<tr>
<td></td>
<td>The total number of soil samples</td>
</tr>
<tr>
<td>Simpson’s index of dominance</td>
<td>D= ∑ (n_i / N)^2</td>
</tr>
<tr>
<td>Shannon –Wiener index of Diversity (H’)</td>
<td>H’= -∑ P_i ln P_i</td>
</tr>
</tbody>
</table>

P_i is the relative abundance of each identified species per sampling site and calculated by the following formula,

P_i = n_i / N, where n_i is the spore numbers of a species and N is the total number of identified species per sampling sites

3.6.5. Correlation analysis between AM fungal spore number and physicochemical characteristics of rhizosphere soil of sunflower

Correlation analysis was carried out to study the impact of physicochemical parameter on AM fungal spore density using statistica analysis tool of MS- Excel 2008.

3.7. Mass multiplication of AM fungi in the roots of onion

Three dominant indigenous AM fungi such as *Glomus mosseae*, *G. fasciculatum*, *Acalospora scrobiculata*, were used for inoculum production. The starter inoculum of AM fungus (*G. mosseae*, *G. fasciculatum*, *A. scrobiculata*) was raised by ‘Funnel Technique’ (Menge and Timmer, 1982) using Onion (*Allium cepa* L) as host. Onion plants grown in funnels were
transplanted into pots after 20 days of germination. The pots were kept in greenhouse (30±1°C) and watered regularly. The infectivity of onion roots by the AM fungi were checked at interval of 15 days. After three months, the pot cultures were harvested by pruning onion plants to the soil level. The soil mass was removed from the pot and the mycorrhizal roots were chopped into small pieces. The percentage AM colonization was determined by Philips and Hayman (1970) method and the spore number in the rhizosphere soil was assessed by wet-sieving and decanting method (Gerdemann and Nicolson, 1963).

3.8. Pot and Potting Mixture

Pots of 35×25 cm size were selected for the experiment. Pots were filled with sterilized sand: soil (1: 3). A layer of inoculum consisting of AM colonized root pieces and soil containing spores, 500/ gm of soil were spread over the pot mixture. The sunflower seeds were surface sterilized with 0.1% mercuric chloride solution, containing Tween -80 as surfactant, for three minutes and washed with sterile water. Five seeds were sown in each earthen pot separately. Seedlings without mycorrhizal inoculums severed as control. The following treatments were studied in triplicates.

1. Control (uninoculated plants)
2. *Glomus mosseae* alone
3. *Glomus fasciculatum* alone
4. *Acaulospora scrobiculata*
5. *Glomus mosseae* + *Glomus fasciculatum*
6. *Glomus mosseae* + *Acaulospora scrobiculata*
7. *Glomus fasciculatum* + *Acaulospora scrobiculata*
8. *Glomus mosseae* + *Glomus fasciculatum* + *Acaulospora scrobiculata*

3.8.1. Evaluation of Morphological characteristics

The test plant samples were collected at 40, 60 and 85 days after inoculation (DAI) and used for various parameter analyses like the shoot, root length (cm), fresh and dry weight (mg /g. fw), the number of leaves per plant,
number of seeds per head, head diameter, hundred seeds weight. All the data were analyzed using ANOVA and Duncan’s Multiple Range Test (DMRT) using SPSS software (11.5).

3.8.2. Evaluation Biochemical characteristics

3.8.2.1. Estimation of chlorophyll content by Arnon method (1949)

1gm of fresh leaf sample was ground in a mortar and pestle with 20 ml of 80 percent acetone. The homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was saved. The pellet was re-extracted with 5 ml of 80 per cent acetone each time, until it become colorless. All the supernatants were pooled and utilized for chlorophyll determination. Absorbance was measured at 645 and 663nm using a spectrophotometer (Systronics). The chlorophyll content was determined by using the following formulae.

Chlorophyll ‘a’ (mg/g f. wt.) = (0.0127) × (OD663) - (0.00269) × (OD645)
Chlorophyll ‘b’ (mg/g f. wt.) = (0.229) × (OD645) - (0.00488) × (OD663)
Total chlorophyll (mg/g f. wt.) = (0.0202) × (OD645) - (0.00802) × (OD663)

3.8.2.2. Estimation of protein by Lowry’s method (1951)

A plant material (0.5 mg) was macerated with a pestle and mortar with 10 ml of 20 percent trichloroacetic acid. The homogenate was centrifuged for 15 min at 6000 rpm. The supernatant was discarded. To the pellet, 5 ml of 0.1 N NaOH was added and centrifuged for 5 min. The supernatant was saved and made upto 10 ml with 0.1 N NaOH. This extract was used for the estimation of protein. From this extract, 1 ml of sample was taken in a 10 ml test tube and 5 ml of alkaline cupper sulphate solution was added. The solution was mixed well and kept in dark for 10min. Later 0.5 ml Folin-phenol reagent was added and the mixture was kept in dark for 30 min. The absorbance of the sample was read at 660nm using a spectrophotometer. Blank prepared without protein sample was used for zero setting. The absorbance value was referred to the standard graph of proteins prepared by using Bovine’s serum albumin.
Materials and Methods

3.8.2.3. Estimation of carbohydrate (Hedge and Hofreites, 1962)

Root tissues were cut into small pieces and 1.0 g of fresh plant sample was weighed and immersed in 10 ml of boiling ethanol, allowed to boil for 5-10 minutes on a steam bath. The contents were cooled and the tissue was crushed thoroughly in a pestle and mortar and filtered through cheese cloth. The extraction procedure was repeated to ensure the complete removal of alcohol soluble substances. Both the pooled extracts were filtered through Whatman No. 41 filter paper. The volume of the extract was reduced by evaporating on hot water bath to represent 5-10 ml of the extract for every gram of tissue. The extract was dried on hot water bath to remove the traces of alcohol, the volume was made upto 10 ml with distilled water and this was used for estimating sugar content. One ml of the aliquot was taken in a test tube. The volume was made up to 2.5 ml with distilled water. All the test tubes were kept in the ice bath to which, 5.0 ml of anthrone reagent was added slowly. Contents were stirred gently with a glass rod and heated on boiling water bath exactly for 7.5 minutes and cooled immediately on ice bath. After cooling, the absorbances of the solutions were measured at 630 nm against the blank in a spectrophotometer. Sugar content was calculated from the standard curve prepared using glucose.

3.8.2.4. Estimation of acid and alkaline phosphatase enzyme activity on the experimental plant roots (Sharma et al., 2004)

Crude enzyme extract was prepared by homogenizing 1gm of fresh tissue with 10 ml of 50mM Acetate buffer (pH 5.3) in a pestle and mortar. This content was filtered and centrifuged at 10,000 rpm for 10 minutes. The collected supernatant was used as acid phosphatase enzyme source. For alkaline phosphatase 50mM Glycine - NaOH buffer (pH- 10.4) was used for extraction. The enzyme phosphatase hydrolyses p – nitro phenol phosphate. The released p – nitro phenol is yellow in color in alkaline medium and is measured at 405nm. Immediately, after adding enzyme fraction (0.2 ml) to the substrate, the contents were mixed gently and the time was noted. Test tubes were covered and placed
in the dark for 20-25 minute incubation at 25 °C. The standard graph was prepared using 200mM p-nitrophenol.

3.9. Evaluation on defence related compounds induced by AM fungi in sunflower

3.9.1. Preparation of sand maize medium

Charcoal root-rot pathogen was isolated from infected root bits of sunflower collected from different study localities as mentioned earlier. Mass multiplication of *M. phaseolina* was done on sand maize meal medium in the ratio of 19:1 (Riker and Riker, 1936). This mixture was kept in polypropylene bags and autoclaved at a pressure of 15lbs pressure for one hour for three consecutive days. The following day, an agar disc from the periphery of a four days old culture of *M. phaseolina* on PDA was transferred aseptically to the mixture and incubated for 2 weeks at 28°C. The inoculum so prepared was incorporated in the soil (10gm/pot) and mixed well.

3.9.2. Determination of Pathogenicity

Pathogenicity of *M. phaseolina* was carried out to select the potential isolate. Sunflower cultivar CO(SVF)5 purchased from TNAU, Coimbatore was used. Pots of 35×25 cm size were filled with sterilized soil and artificially infested with *M. phaseolina*. Surface sterilized sunflower seeds were sown as five seeds per pot (El-Barougy et al., 2009).

3.9.3. Experimental design

Earthen pots of 35×25 cm size were filled with sterilized soil and were artificially infested with potential charcoal-rot pathogen *M. phaseolina*. A layer of inoculum consisting of AM colonized root pieces and soil containing spores, 500/gm of soil were spread over the pot mixture. Surface sterilized sunflower CO(SVF)5 cultivar seeds were sterilized and sown as five seeds per pot. The following combination was prepared and each treatment was replicated thrice.
1. Control (uninoculated plants)
2. *G. mosseae* + *M. phaseolina*
3. *G. fasciculatum* + *M. phaseolina*
4. *A. scrobiculata* + *M. phaseolina*
5. *G. mosseae* + *G. fasciculatum* + *M. phaseolina*
6. *G. mosseae* + *A. scrobiculata* + *M. phaseolina*
7. *G. fasciculatum* + *A. scrobiculata* + *M. phaseolina*
8. *G. mosseae* + *G. fasciculatum* + *A. scrobiculata* + *M. phaseolina*
9. *M. phaseolina* alone

3.9.4. Statistical analysis

After various growth periods 40DAI, 60DAI and 85DAI disease severity was analyzed and various defence related compounds were estimated. All the data were analyzed using ANOVA and Duncan’s Multiple Range Test (DMRT) using SPSS software (11.5).

3.9.5. Assessment of disease severity

Disease severity was assessed by using the rating scale of 1-5 given according to Shokes *et al.*, (1996) as follows, 1=healthy, 2=lesion on stem only, 3=25%, plant symptomic, 4= 26-50% plant symptomic, 5= more than 50% plant symptomic.

3.9.6. Determination of total phenolic content (Sadasivam and Manikam, 1992).

Root tissue was extracted with 10ml of (80%) ethanol. 1ml of extract was added to 1ml of 20% sodium carbonate and 0.5ml of Folin - phenol reagent and kept in a boiling water bath at 100 °C for 10 minutes. Total volume was made upto 10ml by adding distilled water and absorbance was read at 650nm. The amount of phenols present in the sample was calculated from a standard curve prepared from catechol.
3.9.7. Assay of peroxidase (POX)

Peroxidase activity was determined according to the method of Hammerschmidt et al., (1982). Root samples (1gm) were homogenized in 2ml of 0.1 M-phosphate buffer, pH 7.0 using mortar and pestle. The homogenate was centrifuged at 6000rpm for 15min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.1 ml of enzyme extract and 0.5ml of 1% H₂O₂. The reaction mixture was incubated at room temperature (28±2 ºC). The change in absorbance at 470 nm was recorded at 30 second time interval for 3min. One unit of peroxidase activity was defined as the amount of enzyme that catalyses an absorbance increase of 0.01 per min at 470nm.

3.9.8. Assay of poly phenol oxidase (PPO)

Root samples (1gm) were homogenized in 2ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16000 rpm for 15min at 4 ºC. The supernatant was used as enzyme source. The reaction mixture consisted of 0.1ml of enzyme extract and 1.5ml of 0.1 M phosphate buffer ((pH 6.5). To initiate the reaction, 0.2 ml of 0.01 M catechol was added and the change in absorbance at 495 nm was recorded at 30 second time interval for 3 min. The enzyme activity was expressed as change in absorbance per min⁻¹ g⁻¹ fresh root tissue (Mayer et al., 1965).

3.9.9. Determination of phenylalanine ammonia-lyase activity (PAL)

Phenylalanine ammonia-lyase was assayed by using an assay modified from Godwin et al., (1996). The reaction mixture contained 100Mmol/L Tris-HCl buffer, pH 8.5, 1Mmol/L 2-mercaptoethanol, 15 Mmol/L L-phenylalanine and 100µl enzyme extract. The reaction mixture was incubated at 30 ºC for 15 min and reaction was terminated by the addition of 6 Mol/L HCl and then measured at 290nm. One unit represents the amount of enzyme that produces 1µmol of cinnamic acid per hour.
3.9.10. Electrophoretic studies of isoenzyme analysis (Laemmli, 1970)

To study the expression pattern of different isoforms of polyphenol oxidase in different treatments, gel electrophoresis was carried out. For native anionic polyacrylamide gel electrophoresis, resolving gel of 8 percent acrylamide concentration and stacking gel of 4 percent acrylamide concentration were prepared. After staining, the gel was washed with distilled water and photographed.

3.9.10.1. Sample preparation

500 mg root sample was homogenized in 1ml phosphate buffer of 0.1M concentration (pH 7.0) in a prechilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min. and the supernatant was used for analysis.

Materials

Stock acrylamide solution

- Acrylamide: 30g
- Bis acrylamide: 0.8g
- Distilled water: 100ml

Separating gel buffer (pH 8.8)

- Tris-HCl: 23.4g
- Distilled water: 100ml

Stacking gel buffer (0.5M, pH 6.8)

- Tris-HCl: 7.8g
- Distilled water: 100ml

Polymerizing agents

- APS (Ammonium per sulphate) 0.1g/10 ml, freshly prepared
- TEMED (N,N,N,N-Tetramethyl ethylene diamine) fresh from refrigerator
Electrode buffer (pH 8.2 - 8.4)
- Tris base 0.90g
- Glycine 4.32g
- Distilled water 300ml

Sample loading buffer (5x concentration)
- Tris HCl Buffer (pH 6.8) 1.0 ml
- Glycerol 1.6ml
- Mercaptoethanol 0.8ml
- Bromophenol blue 0.8mg
- Distilled water 2.6ml

3.9.10.2. Procedure
Thoroughly cleaned glass plate and spacers were assembled properly and were clamped in an upright position on a gel casting unit with 2% agar on bottom to seal the chamber leak proof between glass plates.

Separating gel composition (8%)
- Acrylamide 2ml
- Distilled water 3.62ml
- Tris (pH 8.8) 0.15M 1.87ml
- APS 25µl
- TEMED 5µl

The gel solution was poured in the chamber between glass plates carefully leaving 4 cm from the top and a layer of distilled water was added on top of the gel and then allowed to polymerise for 30-60 min.

Stacking gel composition (4%)
- Acrylamide 0.65ml
- Distilled water 3.25ml
- Tris (pH 6.8) 0.05M 1.25ml
- APS 25µl
- TEMED 5µl
After removing water from the gel, stacking gel (4%) was added, comb was placed and kept for polymerizing (30-60 min). After polymerization the comb was taken out and gel plate was removed from the casting unit to the electrophoresis apparatus.

3.9.10.3. Preparation of sample for loading

50µl of the supernatant obtained from root extract was mixed with 25µl of dye prepared before, and this 75µl was used as the sample to be loaded in the wells. The gel electrophoresis apparatus was filled with electrode buffer in the tank and each sample was loaded in respective well. Cathode was connected at the top and the anode at the bottom and the DC power pack was turned to a constant 15V current. The whole process of gel run takes place under ice cold condition. After this the gel was removed from the unit and separated from the glass plates for staining.

Staining solution

**Polyphenol Oxidase (PPO)**

- Phosphate buffer (pH 7.0) 0.1M
- Phenylidiamine 0.1M
- Catechol 10Mm

The gel was removed from electrophoresis unit and kept in a staining box containing 0.1 M phosphate buffer to which 0.1 M phenyl diamine was added as the blocking agent. The gel box was kept in shaker for 30 min. followed by addition of catechol which was used as the substrate for appearance of bands.