CHAPTER 3

MATERIALS AND METHODS

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3.1 Sample collection:

The different places of Manipur were selected as the site for collection of *Achyranthes aspera* L. leaves and stem sample. The samples were collected for the study during February 2013 to June 2013. The leaves and stem samples were collected from the following five districts (Figure 1) viz,

- Imphal west- 24.7828˚N latitude, 93.8859˚E longitude
- Imphal east- 24.7807˚N latitude, 93.9674˚E longitude
- Bishnupur- 24.0679˚N latitude, 87.3165˚E longitude
- Thoubal- 24.5436˚N latitude, 93.9674˚E longitude
- Senapati-24.4984˚N latitude, 94.0753˚E longitude

In the present study, four samples of each district were collected. The samples were collected using sterilized forceps and scapel and transferred immediately into the sterile polythene plastic bags and maintain in 4˚C in refrigerator until isolation process were done.

3.2. Isolation of endophytic bacteria:

3.2.1. Preparation of media:

The Yeast Extract Mannitol Agar (YEMA) (g/l): Yeast extract, 1.0; Mannitol, 10.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1, Agar, 15.0 medium was used for the isolation of endophytic bacteria. The medium was sterilized by autoclaving at 121^0C for 20 min, cooled to 40^0C and poured on petriplate. The pH of the medium

was adjusted to pH 7.0. Glycerol stock was used for storing the cultures for a longer time at refrigerated conditions (-4^{0}C).

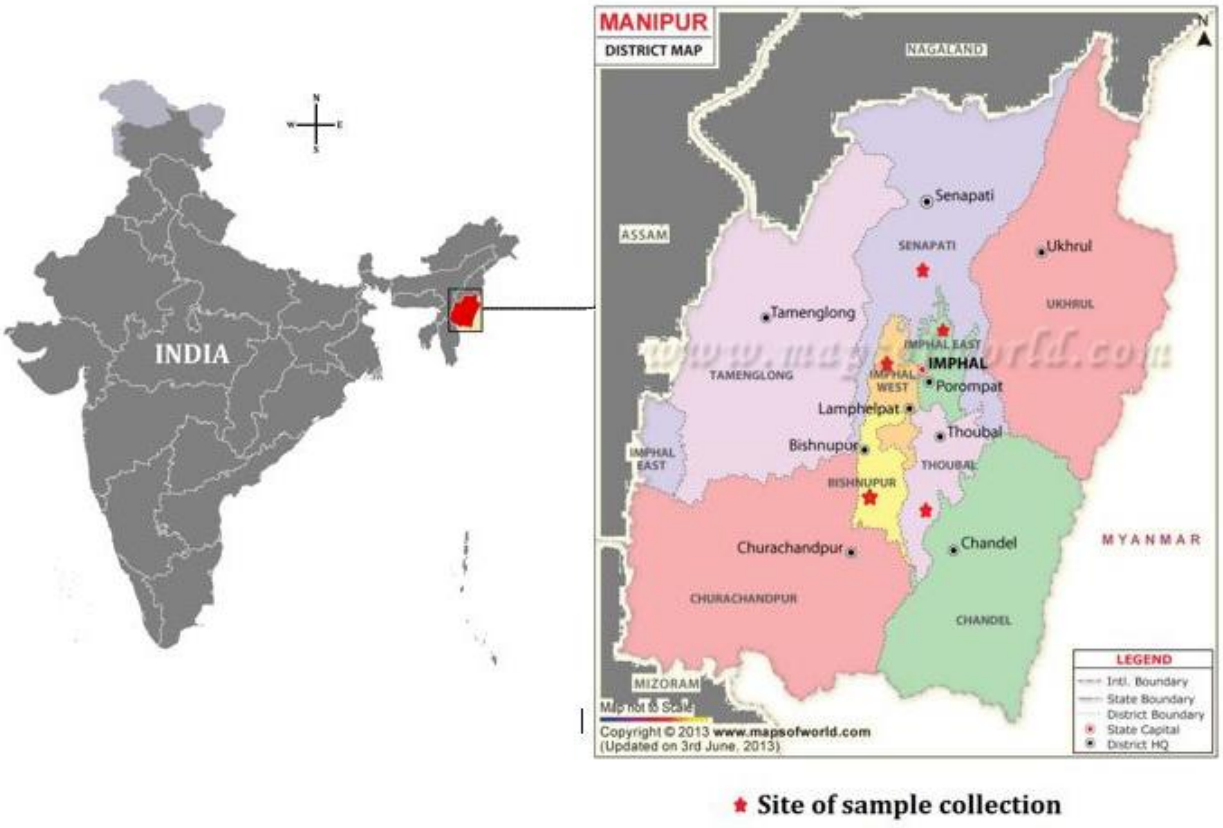

Figure 1. Map showing the site of sample collection.

3.2.2. Isolation of endophytic bacteria:

Fresh leaves and stem samples of *A. aspera* L. plants were collected from different parts of Manipur, India. The samples were washed with tap water and surface sterilized with 70% ethanol for 1 min followed by 0.1% mercuric chloride for 5 min. Samples were washed in water, and rinsed in phosphate buffer, macerated in mortar and pestle under aseptic condition. Suitable dilution of 1.0 g of macerated tissue was plated on Yeast Extract Mannitol Agar (YEMA) and

incubated at 30^0C for 3 days. The bacterial colonies were selected, subcultured, purified and used for further studies.

For sterility check, 0.1 ml aliquot from the final wash was inoculated on YEMA plate. Samples were discarded if any growth was detected if any.

3.2.3. Mantainance of Stock Cultures:

For the mantainance of stock cultures, bacterial cells were grown on nutrient broth (NB) and stored at -50˚C in 40% glycerol. Cultures for experimentations were revived on nutrient agar (NA) and stored at 4˚C. Cells were transferred to fresh NA plates on weekly basis and fresh cultures were made from the stock cultures and kept at -50˚C for retrival. The stocks were maintained in SEML laboratory of department of Microbiology, Assam University, Silchar.

3.3. Characterization of endophytic isolates:

3.3.1. Morphological Characterization:

The isolates grown on Yeast Extract Mannitol Agar (YEMA) were characterized for Colony morphology, margin, color, surface texture, surface diameter and opacity.

3.3.2. Biochemical Characterization:

The following biochemical tests were carried out. They were Gram staining, Indole, MR, VP, Urease, Catalase and Oxidase.

3.3.2.1. Gram staining:

A drop of normal saline was placed on a well labeled clean greased-free glass slide using a sterile inoculating loop. A colony of an overnight culture of bacterial isolate was emulsified with normal saline to make a thin smear. The

smear was air dried and then heat fixed. The slide was flooded with crystal violet (primary stain) for 1 min after which the stain was rinsed from the saline with water. The smear was flooded with Lugol's iodine to fix the primary stain. The iodine was rinsed with water after 1 min. the slide was then flooded with a decolourizer (acetone) and rinsed off almost immediately. The counter stain; safranine was added and left for 1 min before being rinsed off. The stained smear was air dried, and then observed under the microscope at 1000X magnification.

3.3.2.2. Indole test:

About 2-3 colonies were inoculated in a test tube containing 3ml sterile tryptone broth and incubated at 37˚C upto 48 h. 0.5ml of Kovac's reagent was added, shaked gently and allowed to stand for 5 min. Appearance of red colour indicates positive result.

3.3.2.3. Methyl red test:

A single colony was inoculated in 0.5ml sterile glucose phosphate broth and incubated overnight at 37˚C. A drop of methyl red indicator was added. Appearance of bright red colour indicates positive result.

3.3.2.4. Voges-Proskauer test:

About 2-3 colonies were inoculated in 2ml of sterile glucose phosphate peptone water and incubated at 37˚C for 48 h in a sterile test tube. 0.6ml of α-napthol and 0.2ml 40% potassium hydroxide reagent was added and shaked vigorously with aeration. It was allowed to stand for 1 h at room temperature. Appearance of pink red colour indicates positive result.

3.3.2.5. Urease test:

About 2-3 colonies were inoculated in sterile test tube containing 3ml sterile urea broth and incubated at 37˚C upto 7 days. A change in the colour of medium from yellow to pink is the indicative of positive result.

3.3.2.6. Catalase test:

A drop of 3% hydrogen peroxide was placed on a clean grease-free glass slide. About 2 colonies of the bacteria were picked from the culture plate using a sterile wire loop and placed on the hydrogen peroxide. Presence of bubbles indicated positive test.

3.3.2.7. Oxidaseas test:

A piece of filter paper was soaked in freshly prepared oxidase reagent. A single colony was smeared on the filter paper properly using a glass rod. Appearance of blue purple colour within 10 seconds indicates positive result.

3.3.2.8. Citrate Utilization test:

Slope of Simmons citrate agar was prepared in test tube (stored at 2-8˚C), the slope was then streaked with the culture and stabbed the butt. The tubes were incubated at 37˚C for 48 h. Visible blue colour of the medium from green indicates positive result.

3.3.2.9. Carbohydrates fermentation test:

Fermentative degradation of various carbohydrates such as glucose, sucrose, fructose, mannitol, maltose and cellulose by microbes was carried out in a fermentation tube that contains a Durham tube for the detection of gas production as an end product of metabolism. The following carbon compounds

viz., glucose, sucrose, fructose, mannitol, maltose, and cellulose were used at 0.5 percent level in the medium. Sterile fermentation tubes of glucose broth, sucrose broth, fructose broth, mannitol broth, maltose broth and cellulose broth containing phenol red indicator was inoculated with the isolates and incubated at 30^{o}C for 48 hours. Uninoculated tubes were maintained as control. Change in colour due to production of acid and appearance of bubbles due to production of gas was compared with the control tubes and was recorded.

3.3.3. Physiological Characterization:

The following physiological tests were done. They were oxygen requirement test, growth profile of bacterial isolates at different salt concentration and pH.

3.3.3.1. Oxygen requirement test:

Soft nutrient agar medium was prepared by incorporating 0.5% agar agar in nutrient broth. 5ml of the media were taken in test tubes, sterilized and allowed to solidify. Bacterial isolates were stabbed in each test tube and incubated at 37˚C for 24 h. The requirement of oxygen for growth was assessed by observing the growth along the stab.

3.3.3.2. Growth at different pH:

Growth of different isolates at different pH was determined by adjusting the pH values of nutrient broth (NB) to pH values of 4,5,6,7 and 8 using 0.1N HCL and 0.1N NaOH solution. 5 ml of the medium was taken into the test tubes before autoclaving. After autoclaved the pH was rechecked and inoculated with

the isolates. It was incubated at 30˚C for 48 h. Growth was assessed by observing the turbidity in the test tubes.

3.3.3.3. Growth at different NaCl concentration:

Growth of isolates at different NaCl concentration was determined by adjusting the NaCl concentration of nutrient broth to 0.5, 2, 5, 10 and 20. 5 ml of the autoclaved medium was inoculated with the isolates and incubated at 30˚C for 48 h. Growth was assessed by observing the turbidity in the test tubes.

3.3.4. Molecular characterization of endophytes:

3.3.4.1. DNA extraction and 16s rRNA amplification:

Genomic DNA was extracted from overnight cultures using the HiPurA Bacterial Genomic Purification Kit (Hi-Media). Briefly, after cultivation of the isolates for 24 h at 37^0C on YEMA plates, 5-10 colonies of the bacteria were suspended in TE buffer (10mM Tris-HCl, 1mM EDTA (pH-8) containing 5 µl lysostaphin (1.8 U/µl). After 1 h incubation at 37^0C, 25 µl of proteinase K (8 mg/ml) and 200 µl of buffer AL (containing reagents AL1 and AL2) were added. The suspension was incubated for 30 min at 56^0C and for 10 min at 95^0C, and after a spin for few seconds an amount of 420 µl was added to each sample and placed in a spin column. After centrifugation for 1 min, the HiPurA spin columns were placed in a clean collection tubes and the samples were washed twice with 500 µl of buffer AW. After a second wash and a centrifugation for 3 min, the the HiPurA spin columns were placed in a clean 2 ml microfuge tube and the DNA was twice eluted with 200 µl and 100 µl of buffer AE, respectively. The purified

DNA were stored at 4^0C for short term storage (24-48 h) and -20^0C for long term use.

3.3.4.2. Polymerase Chain Reaction (PCR) conditions:

The strains were further confirmed by amplification of 16S rRNA gene using a set of primer pairs F-CAGAGTTTGATCCTGGCT and R-AGGAGGTGATCCAGCCGCA. PCR amplifications were performed in a total reaction mixture of 25.0µl comprising 2X Master mix (GCC, biotech), 12.5 µl; 27F, 1.0 µl; 1492R, 1.0 µl; lysate DNA, 1.0 µl and nuclear free milli Q water, 9.5 µl. The Master mix contained 0.2 units/ml Taq DNA polymerase, 32mM $(NH4)_2SO4$, 130mM Tris HCl, 0.02% Tween 20, 3mM $MgCl_2$ and 0.4 mM dNTPs (dATP, dCTP, dGTP, dTTP) and inert red dye. The thermo cycling conditions involved an initial denaturation at 94^0C for 5 min, followed by 30 cycles of 94^0C for 30 sec, 55^0C for 30 sec, and 72^0C for 1.30 min and final extension at 72^0C for 10 min. After the amplification, the PCR products were separated by agarose gel electrophoresis.

3.3.4.3. Agarose Gel Electrophoresis:

Agarose powder (Hi-Media) was used in preparing the agarose gel used in this study at 1% concentrations. The agarose was dissolved in 0.5×TBE; Tris-Borate-EDTA and microwaved to dissolve the agarose and casted on gel plate putting the comb properly. The electrophoresis chamber was filled with running buffer (0.5×TBE) and casting tray was placed in the chamber removing the comb. The gel was totally submerged in the buffer. A tracking dye was added to the PCR products that were colourless to make them visible. The amplicon (10 µl) was

loaded in each well. A 100 bp molecular weight marker was loaded into the first well as a standard for estimating the size of the resulting DNA fragment. The electrophoresis chamber was connected to a power source and the DNA was run at 80V for 30-45 min. The separated DNA fragments were visualized by staining the gel with ethidium bromide for 15 min and then de-stained in water for 15 min. The DNA bands were viewed by illumination with UV light and images were recorded.

3.3.4.3. Phylogenetic analysis of 16srRNA sequences:

The amplified product was sequenced commercially (Xcelris, India). The sequence was compared with those available in the GenBank using Blast search. The sequence were subjected to nucleotide blast and >99% match score was accepted for the species identity. The sequences were submitted to the GenBank. Phylogenetic analyses were performed using the software package MEGA6 (Tamura *et al*., 2011). Phylogenetic distances were calculated with the Kimura two-parameter model (Kimura, 1983) and tree topologies were inferred using the neighbour-joining (NJ) method (Saitou and Nei, 1987). To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings (Felsenstein, 1985).

3.4. *In vitro* tests for plant growth promoting (PGP) traits by endophytes:

3.4.1. Siderophore production assay:

Siderophore production was monitored by formation of orange halos around bacterial colonies on Chrome Azurol S (CAS) agar plates kept incubated at 30^0C for 48 hours (Schwyn and Nielands, 1987). The cultures were inoculated

in iron deficient medium containing(g/l): K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; Succinic acid, 4.0 at 30^0C on a rotary shaker at 120 rev min^{-1}.Quantitative estimation of siderophores was done by CAS-shuttle assay. 1ml of culture supernatant was mixed with 1ml of CAS reagent (10mM HDTMA; 1mM $FeCl_3$ solution; 2mM CAS solution) and absorbance was measured at 630 nm against a reference, having 1 ml of uninoculated broth and 1 ml of CAS reagent (Payne 1994). The activity was recorded in percentage siderophore units calculated as $[(Ar-As)\times Ar^{-1})\times 100]$. Where, Ar = absorbance of reference at 630 nm (un-inoculated media + CAS reagent) and As = absorbance of sample at 630 nm (culture supernatant + CAS reagent). The type of siderophore produced (catecholate or hydroxamate) was determined according to Arnow's (1937) and $FeCl_3$ tests (Meyer *et al*., 1995). In the Arnow test, 1ml of culture supernatant was mixed with equal volume of 0.5 M HCl, nitrite-molybdate reagent and 1 M NaoH in a clear microcentrifuge tube, mixing vigorously between the addition of each reagent. The presence of catecholate type of siderophore was indicated by a pink colour change; the mixture remained clear if catecholate siderophores were absent. In the $FeCl_3$ test, 1 ml of culture supernatant was mixed with 200 µl of 100 mM $FeCl_3$ in 0.1 M HCl. The presence of hydroxamate type siderophore was indicated by colour change to orange or pink.

3.4.1.1. Effect of growth medium on siderophore production:

Production of siderophore was investigated on different media viz. Succinic medium (SM) (g/l): K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2;

$(NH_4)_2SO_4$, 1.0; Succinic acid, 4.0, Nutrient broth (NB) (g/l): Peptone, 5.0; NaCl, 5.0; Beef extract, 1.5; Yeast extract, 1.5, Luria broth (LB) (g/l): Casein enzymic hydrolysate, 10.0; Yeast extract, 5.0; NaCl, 10.0, Yeast Extract Mannitol Broth (YEMB) (g/l): Yeast extract, 1.0; Mannitol, 10.0; K_2HPO_4, 0.5;$MgSO_4.7H_2O$, 0.2; NaCl, 0.1. A loopful of culture from YEMA slants were separately inoculated in 100 ml of different medium and incubated at 28^0C, on a rotary shaker at 220 rpm. Siderophore production was monitored every 24 hours.

3.4.1.2. Effect of Sugars, Nitrogen sources and Organic acids on siderophore production:

The influence of different carbon sources such as glucose, sucrose, mannitol, maltose, fructose were studied on siderophore production by the isolate. All carbon sources were added externally at concentration 1% (w/v) to the Succinic medium (Sayyed *et al*. 2005) after replacing succinate. In case of Nitrogen Sources, influence of urea and sodium nitrate was studied by replacing ammonium sulphate in Succinic medium. Siderophore production in these media was compared with that of SM containing ammonium sulphate. In order to examine the influence of different organic acids on siderophore production, Succinic medium was supplemented with 0.4% (w/v) each of malic acid, oxalic acid and citric acid instead of succinate.

3.4.1.3. Effect of iron on siderophore release:

In order to determine the threshold level of iron for siderophore production, iron content of Succinic medium was varied by the addition of ferric chloride in the range of 0-30µM concentration. Bacterial strain was inoculated

and kept for incubation at 29^0C at 120 rpm and siderophore content was estimated as described above.

3.4.2. IAA production assay:

The indole acetic acid (IAA) production was determined as reported by Loper and Scroth (1986). The isolates were grown on YEMA medium for 5 days. A loopful of the culture was inoculated in different flasks having YEM broth, each supplemented with a different L-tryptophan concentration (0%. 0.2%, 0.4%, 0.6%, 0.8% and 1.0%) and incubated at shaker (150 rpm, 30^0C). Production of IAA was determined after every 24 h interval. The cultures were harvested by centrifugation (11000×g, 15 mins), 1 ml of the supernatant was mixed with 2 ml of Salkowski reagent (50 ml, 35% perchloric acid with 1 ml, 0.5 M $FeCl_3$) (Gordon and Weber, 1951). Optical density (OD) was measured at 530 nm and the amount of IAA produced was calculated by comparing with the standard curve prepared with IAA.

3.4.3. Phosphate solubilisation:

Phosphate solubilization assay was done using modified Pikovskaya medium (g/l): glucose, 10; $(Ca_3)_2PO_4$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4.7H_2O$, 0.1; KCl, 0.2; $FeSO_4.7H_2O$, 0.002, yeast extract, 0.5; $MnSO_4.2H_2O$, 0.002, agar, 20, pH-7containing bromophenol blue (2.4 mg/ml) (Nautiyal 1999). The halo and colony diameter were measured every 24 h upto 4 days of incubation at 30^0C. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilisation index (defined as the ratio of the total diameter to the colony diameter (Edi Premono *et al*. 1996). Quantitative

estimation of P content in the supernatant was estimated using the vanado-molybdate colorimetric method (Koenig and Johnson 1942).

3.4.4. Ammonia production:

The ability of bacterial strains to produce ammonia was assessed as described by Marques *et al*. (2010). In this method 20 µl of an overnight grown test culture was inoculated into 5 ml of 1% proteose peptone broth and incubated at 30^{0}C in a shaking water bath. After 24–48 h, 0.5 ml Nesseler's reagent was added to the culture and the color change was noted, a yellow coloration indicates the positive result while the intensity of color is indicative of the amount of ammonia produced by the test strain.

3.4.5. Nitrogen fixing ability:

Each isolated strain was inoculated in plates containing nitrogen – free medium (Jiang 2005): per litre containing mannitol,10g; KH_2PO_4,0.2g; $MgSO_4.7H_2O$, 0.2g; NaCl,0.2g; $CaSO_4.2\ H_2O$,0.1g; $CaCO_3$,5.0g; pH 7.0-7.5. Plates were incubated at 28°C for 7 days, and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation. Nitrogenase activity was determined by Acetylene reduction assay to confirm nitrogen fixation ability of isolate by using GC-FID (Hardy *et al*. 1971).

PCR amplification of *nifH* gene

For PCR analysis of the *nifH* gene, a set primer *nifH* F: 5'-CGTTTTACGGCAAGGGCGGTATCGGCA-3' and *nifH* R: 5'-TCCTCCAGCTCCTCCATGGTGATCGG -3' were used. PCR reaction conditions for the amplification of *nifH* gene fragment was as follows: initial step of denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C

for 1 min, primer annealing at 51 - 57°C for 30 s, and elongation at 72°C for 1 min followed by a final step of extension at 72°C for 5 min. Amplified PCR products were separated on 1% agarose gel.

3.4.6. ACC deaminase activity:

ACC deaminase activity was screened according to El-Tarabily (2008) using the nitrogen-free Dworkin and Foster's minimal salts agar medium (DF) (Dworkin and Foster, 1958). The medium was supplemented with either 2 g $(NH_4)_2SO_4$ or 3 mM ACC per liter as a sole nitrogen source.

3.5. Colonization study of isolates from experimentally inoculated plants and its effect on plant growth in Pot trial experiment:

Micropropagated plantlets of *A. aspera* were raised from the surface sterilized seeds on half strength Murashige and Skoog (MS) medium. The seeds of *A. aspera* were placed in the petri dishes containing 25 ml sterilized half strength of MS medium. The plates were incubated under humidity (60%), temperature (24 ± 2^0C) and light (1000 lux, 16 h light and 8 h dark). After four weeks, when the seedlings have cotyledons and roots, they were transferred to freshly prepared MS medium and allowed them to grow. After development of extensive root system and had 6 leaflets, the plantlets were gradually acclimatized to natural environment and finally planted in sterile soil under greenhouse conditions (26 ± 2^0C and 70% RH).

Bacteria were grown to the mid-log phase, pelleted by centrifugation (6000×g, 10 min, 24^0C), washed twice, and suspended in sterile double distilled water. It was maintained the $O.D_{600}$ at 1. To confirm inoculation density and purity, an aliquot of culture was serially diluted in sterile double distilled water

and plated on YEM agar medium. The plants were inoculated in triplicate when a minimum height of 7.5 cm was reached and the stalks were at least 0.5 cm in diameter, which corresponded to 75 to 80 days after seed germination. A 26-gauge needle attached to a tuberculin syringe containing a bacterial suspension was passed horizontally through the stem just above the first cotyledon leaves of the plant. A 10 µl droplet of suspension was formed at the tip of the needle, which was withdrawn through the plant stem.

The bacterial multiplication in the stem and leaves of plants were determined at 3 and 5 days after inoculation. Lower parts of stems and first leaves were collected from 3 replications. Each stem and leaves sample were weighed, surface sterilized for 15 sec in 70% ethanol. They were rinsed with double distilled water and macerated with 1 ml of sterile double distilled water. After 20 min, the macerated were settled down and the supernatant were serially diluted. They were plated on YEM agar amended with 25 µg/ml of kanamycin and kept incubated at 28^0C for 48 hours. As a control for the inoculation studies to test for the presence of indigenous endophytic bacteria, the control plants were same inoculated with sterile double distilled water by using the methods used for the experimental plants. All bacterial endophytes recovered were compared morphologically with control plants to distinguish growth of introduced bacteria.

Further, the acclimatized plantlets were transferred into bigger pot having diameter of $25cm^2$ and depth of 20 cm. The pot has the capacity of holding 5 kg of soil:sand (4:1) and kept at randomized block design. The plants were watered every alternative days. The plants were harvested after 150 days and different

growth parameters such as shoot length, root length, numbers of leaves, fresh leaf weight, fresh shoot weight, fresh root weight, dry leaf weight, dry shoot weight, dry root weight and area of the leaf were measured.

3.6. Availability and uptake of NPK:

The available N content of soil was determined using the boric acid method (Subbiah and Asija, 1956), P content by ascorbic acid method (Bray and Kurtz, 1945), K content by Flame photometer (Hanway and Heidel, 1952). Nitrogen uptake in *A. aspera* plants was determined by micro-Kjeldahl digestion method (Jackson 1958), total P and K by dry ashing method (Chapman and Pratt, 1961).

3.7. Experimental design and statistical analysis:

The pot trial experiment has two treatments (with and without bacterial inoculation) each with 5 plants and arranged in a completely randomized design. All data were subjected to one-way analysis of variance (ANOVA) followed by independent t-test at $P \leq 0.05$ using the SPSS 16 software (SPSS Inc).

3.8. Determination of *in vitro* Antioxidant activity of *Achyranthes aspera* L. by endophytes:

3.8.1. Quantitative analysis of total phenol content:

The amount of total phenols in the plant tissues (leaves) was estimated by the method proposed by Malik and Singh (1980). The sample (0.5g) was homogenized in 10 ml of 80% ethanol. The homogenate was centrifuged at 8000×g for 20 minutes. The extraction was repeated with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots were pipetted

out and the volume in each tube was made up to 3.0ml with distilled water. Folin-Ciocalteau reagent (0.5ml) was added and the tubes were placed in a boiling water bath for one minute. The tubes were cooled and the absorbance was recorded at 650 nm. The results were expressed as catechol equivalent in µg.

3.8.2. Scavenging effect on DPPH free radical:

The free radical scavenging activity of 50% aqueous ethanolic extract of *A. aspera* L. on stable radical 1, 1-diphenyl -2-picrylhydrazyl (DPPH) was evaluated by the method of Brand-Williams *et al*. (1995). Briefly, 2.0ml of extract at different concentrations (50µg/ml to 250µg/ml) was mixed with 2.0 ml of DPPH solution in methanol (0.004% w/v). The mixture was allowed to stand at room temperature in dark for 20 min. The mixture was vortexed and then absorbance was recorded at 517nm. Ascorbic acid was used as a reference standard and control consisted of DPPH solution without extract. The test was performed in triplicate and percentage scavenging of DPPH free radical by extract was calculated using the equation: $[(A_{control}- A_{test})A_{control}^{-1}] \times 100$. Here, $A_{control}$ was the absorbance of control and A_{test} was the absorbance in presence of extract or standard.

3.8.3. β-Carotene-linoleic acid assay:

β-carotene bleaching assay was done according Wettasinghe and Shahidi (1999). A 1ml of β-carotene solution (0.2mg/ml in chloroform) was pipetted into a round bottom flask containing 0.02ml of linoleic acid and 0.2 ml of 100% Tween-20. The mixture was evaporated in a rotary vacuum evaporator for 10 min to remove chloroform. After that, the mixture was immediately diluted

with 100ml of distilled water with vigorous shaking to form an emulsion. Varying concentrations of extract and standard (100µg/ml to 500µg/ml) was added to 5ml of the emulsion in different test-tubes and the mixture was kept at 37^0C for 1h. Absorbance of sample and control was measured at time t=0 and t=60 min. Total antioxidant activity was calculated based on the following equation: $AA=[1-(A_0 - A_t) \times (A_0^0 - A_t^0)^{-1}] \times 100$, Where AA is antioxidant activity, A_0 and A_0^0 are the absorbance values measured at the initial incubation time for samples and control respectively while A_t and A_t^0 are the absorbance values measured in the samples or standards and control at t=60 min.

3.8.4. Determination of reducing power:

The reducing power of leaves extract was determined by the method of Jayanthi and Lalitha. (2011). Substances possessing reducing power react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Varying concentrations of plant extract and standard (50µg/ml to 250µg/ml) were mixed with phosphate buffer (2.5ml) and potassium ferrocyanide (2.5ml). The mixture was kept at water bath at 50^0C for 20 min. After cooling 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000×g for 10 min. The upper layer (2.5 ml) of the resulting solution was mixed with distilled water (2.5 ml) and freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700nm. Ascorbic acid at various concentration was taken as standard. Increase in absorbance indicated the increased reducing power of extract and standard.