CHAPTER 3

Materials and Methods

3.1. Isolation and Identification of *Frankia*

3.1.1. Germplasm collection

Germplasm was collected from different sites of Darjeeling (West Bengal), India in the month of February and November. The nodules were collected from *Alnus nepalensis* trees found in various regions of Darjeeling. The samples were traced on the way from Siliguri to Darjeeling via Mirik namely Thurbo, Fatak, Pashupati, Ghoom, and Darjeeling (See Figure.3.1 at the end of this chapter). In the field, the plant species were first located, essential data like its general information, habit, habitat, area of vegetation, specific collection site, soil type and nodules were recorded (See Figure.3.2 at the end of this chapter). Some healthy and young trees were chosen for nodule collection. Photographs of *Alnus nepalensis* and the surrounding vegetation were taken (PlateIII). The vegetation around the tree of our interest was studied and recorded as follows (See table 3.1 at the end of this chapter)

During collection of samples from all the sites one thing was strictly followed i.e. only the nodules of light brown colour were collected since the light colour of nodule is an indication of its active growth and youngness (Myrold 1994).

The percentage of nodulation was calculated by the following formula (Raman & Elumalai 1991.)

$$\% \text{ of nodulation} = \frac{\text{Number of plants with nodules}}{\text{Number of plants observed}} \times 100$$

In both the cases collected nodules were stored in plastic bags containing moistened paper towel to maintain favorable water potential and kept in an icebox containing ice to limit tissue deterioration and slow down the growth of contaminating microorganisms throughout the journey. Soil samples were also collected in much the same manner. Samples of nodules and soil were stored at -20°C immediately after bringing to the laboratory.

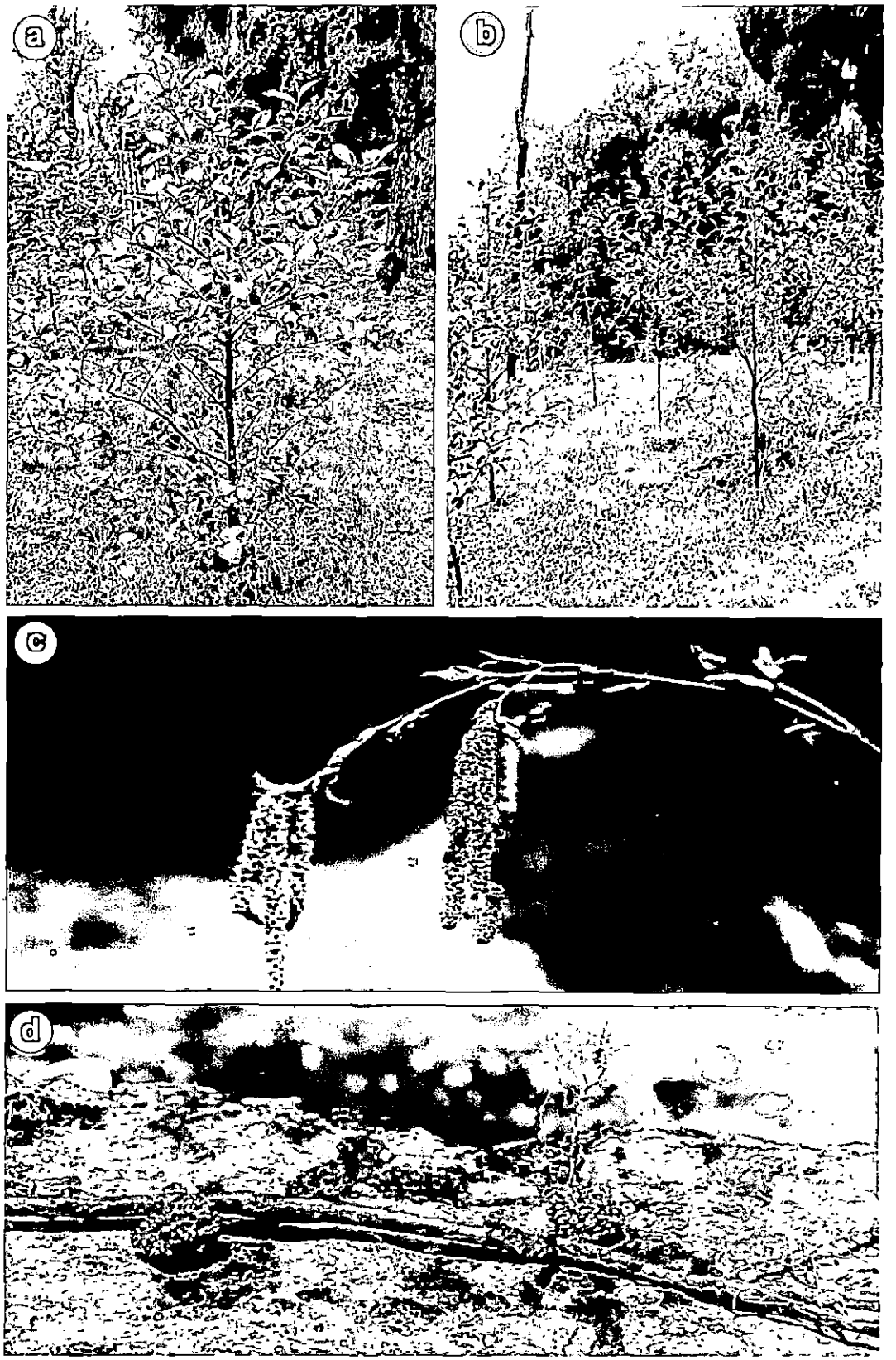

Plate III

(a&b) *Alnus nepalensis.* Two collection sites of *Frankia* germplasm of Darjeeling. (c) An inflorescence of *Alnus nepalensis* (d) A root nodule clump from *Alnus nepalensis.*

3.1.2. Isolation of endophyte-the microsymbiont

3.1.2.1. Nodule sterilization

Nodules were cleaned in plain water to remove soil and organic debris. Nodule lobes were separated and further cleaned by washing several times in a mild detergent such as Extran (E Merck, India cat#MA02). Lastly they were washed several times with distilled water and then with sterilized distilled water. Since *Frankia* is a slow growing organism, a chance of contamination of its culture by soil borne fast growing microorganisms is more (Lechevalier 1994). To overcome this problem surface sterilization of nodules were done by following methods:

- Nodules were treated with 0.1% $HgCl_2$ (E Merck India Cat# 17524) for 2 minutes and washed 6-7 times with sterile double distilled water.
- Nodules were treated with 30% H_2O_2 (E Merck India Cat# 1.07209.0250) for 5 minutes and then washed several times in Petri plates with sterile double distilled water.

3.1.2.2. Isolation of *Frankia* in liquid culture

After proper sterilization of the nodules following isolation techniques have been tried.

- A single surface sterilized nodule lobe was taken on a sterilized slide.
- The upper epidermal part was peeled off using two sterilized needles.
- The nodule lobe was then washed twice with sterile distilled water.
- The peeled nodule lobes were crushed on the side of the culture tubes containing liquid growth medium for *Frankia*.
- Surface sterilized nodules were chopped into small pieces and put into liquid medium. The tubes were incubated at 28-30°

3.1.2.3. Common media for isolation

Although there exists a range of isolation medium for *Frankia* e.g. DPM used by (Baker and O Keefe 1984), F medium used by (Simonet et al. 1985), OS –1 by (Dobritsa and Stupar 1989) and Q- mod by (Lalonde & Calvert 1979) but the best medium like

DPM by (Baker and O Keefe 1984), was finally used after few trial and error methods (See appendix I for composition of media).

3.2. Plant infectivity tests

3.2.1. Germination of seed and seedling cultivation

Surface sterilization of seeds was done with 30% H_2O_2 for 10 *minutes* and it was rinsed several times with sterile distilled water. The seeds of *Alnus* were placed on a sterile moist filter paper and kept in the BOD incubator at 26 ± 3^{0}C for germination.
Fifteen days old seedlings were transferred to sterile pouches containing different concentrations of Hoagland solutions (1/4, 1/8, 1/16) without nitrogen (See appendix I for composition). The following three sets were prepared for each isolate:

- Seedling inoculated with crushed nodule suspension (+ve control) (Section 3.1.2.3. for preparation of nodule suspension).
- Seedlings inoculated with 100µl of 30 days old *Frankia* culture under test.
- Un-inoculated seedlings were used in each set as –ve control.
- Each set contained twenty seedlings. The *Alnus* seedlings were allowed to grow in a plant growth chamber at 26°C with approximately 90% humidity and 1100 lux illumination.

3.3. Nitrogenase activity of *Frankia* in pure culture

Four cultures two from Thurbo (AnTB1& AnTB2) and two from Ghoom (AnGM2& AnGM5) were tested for nitrogenase activity.
Acetylene reduction assay (ARA) (Burris, 1967) was employed to measure nitrogenase activity of the isolates. For measuring the nitrogenase activity 3 ml of 30 day old subculture of each isolate was taken in 9 ml stoppered vials, 0.9ml of air was replaced with equal volume of acetylene. The vials were incubated at 28±1°C and were shaken time to time. Ethylene produced from acetylene was measured with a "Perkin Elmer-8700" gas chromatograph fitted with Porapak–T column. The chromatograph used hydrogen as a fuel and nitrogen serves as carrier gas.

3.4 Post isolation workup

3.4.1. Incubation and sub culturing

The vials thus inoculated were protected by properly sealing them with Parafim M (American National Can™ Chicago.60631) and were incubated in the dark at 28°C for several weeks to one month. The cultures obtained were initially studied under phase contrast microscope for the characteristic sub-surface sporangia or presence of vesicles. Selected colonies were aseptically withdrawn from liquid culture and subcultured in fresh medium. Colonies selected for sub culturing need to be thoroughly homogenized. This can be done by grinding the colony with sterile buffer or medium in small tissue grinder. Homogenization increases the number of actively growing hyphal tips and also helps to release spores from sporangia. The homogenized suspension is then inoculated into the fresh medium.

3.4.2. Maintenance of culture

After successful isolation of *Frankia* in pure culture, the isolates were named as per the conventional nomenclature systems of Frankia, i.e. first two letters from the Genus and species of the host plant with the first one Capitalized and the subsequent two letters from the collection site. For instance, culture from the nodule collected from Thurbo was named as AnTB1, AnTB2 etc. The cultures were maintained in DPM medium. To maintain active culture, sub-culturing was done every 2 to 6 months. Cultures were homogenized while transferring them to break apart hyphae and promote more rapid growth.

3.4.3. Decontamination

Because of slow growing nature, *Frankia* cultures sometimes become contaminated. Decontamination was achieved in several ways. Different antimicrobial agents were used like 500µg/ml of cyclohexamide (Actidione, Sigma, USA. Cat#C-2609) for removing of fungal contaminants. Actidione inhibits protein biosynthesis in eukaryotic cells by binding 80S ribosome. Since *Frankia* is gram +ve bacteria, Nalidixic acid (10µg/ml) (1-ethyl-4-dihydro-7methyl-4-oxo-1, 8-napthyridine-3-carboxylic acid,

(Sigma, USA. Cat#N-3143) was used. The efficiency of either decontamination approach is enhanced by re-culturing on an N-free medium (DPM) and further streaking this enrichment on N-free agar to re-isolate *Frankia*.

3.5. Structure and ultra structure

3.5.1. Light Microscopy

Root nodules, were fixed in FAA (90ml of 70% ethanol, 5ml glacial acetic acid and 5ml Formalene). They were then dehydrated in a series of extra pure acetone (E Merck India) and embedded in paraffin wax. Transverse and longitudinal sections 6-10μm thick were cut with a microtome (Leitz 1512, Germany). The safranin- fast green staining procedure (Sass 1958) was used to stain the cortical and xylem cells differentially in order to study their relative arrangement. The *Frankia!* culture was stained according to the procedure of Gram's staining protocol (Cappuccino/Sherman 1996).

3.5.2 Scanning Electron Microscopy (SEM) of *Frankia in vivo & in vitro*

The SEM provides information on specimen surface (fine surface topography). It offers a three-dimensional surface view of solid tissue. Both pure cultures as well as *Frankia* within nodules were taken for SEM. General requirements of SEM of *Frankia* (both *in-vivo* & *in-vitro*) are as follows:

3.5.2.1 Chemical fixation

Fixation offers the best way to preserve the cells as close as possible to their in-vivo condition with respect to volume, morphology and spatial relationships of organelles and macromolecules.

3.5.2.1.1 For *Frankia* in pure culture

One-month-old *Frankia* culture was taken in a micro centrifuge tube and allowed to settle down. The upper aqueous phase containing the medium was decanted carefully. The cells were fixed by adding 1ml of 3% glutraldehyde and kept for 4 hours.

Glutraldehyde was then decanted carefully and the cells were stuck on glass stubs with the help of egg albumin. The cells were then washed thoroughly in 0.1 M cacodylate buffer (see appendix II for composition) and post fixation was done in 1% OsO4 for 2 hours. It is conclusive that although the osmolality, pH and total time allowed for fixation are all critical in obtaining optimal preservation, there is a range of satisfactory conditions for particular specimen.

3.5.2.1.2. For *Frankia* inside nodules

Nodule lobes were taken, each nodule separated and washed thoroughly with distilled water. The nodule lobes were then fixed with 3% glutraldehyde for 4 hours and then washed with 0.1M cacodylate buffer. They were then post fixed in 1% OsO_4 for 2 hours.

3.5.2.2. Dehydration

A dry specimen is required by the design of scanning electron microscope. Drying in air causes gross distortion of cells due to the action of surface tension. So, to achieve distortion free drying, critical point drying is necessary. Since water is immiscible with most of currently used transitional fluids necessary to the critical point method, removal of water is done by the use of dehydration fluids; most commonly used among them is acetone or alcohol in ascending concentrations. Therefore in both cases dehydration was done with a series of acetone (30%, 50%, 70%, 80%, 90%, 95%), keeping then in each grade for 30 minutes with two changes followed by one hour in dry acetone with two changes. However, nodules were kept for 24 hours in each grade for proper dehydration. After dehydration, nodules were embedded in paraffin wax and thin sections (10μm thick) were prepared with the help of microtome (Leitz 1512, Germany). The sections were then mounted on glass stubs and made wax free with the help of xylene.

3.5.2.3. Critical point drying

Critical point freeze drying of the dehydrated *Frankia* cells was done using a Polaron E 3000 apparatus. The drying was carried out at 31.5°C at 1100 PSV. Critical

point drying which achieves transition from liquid to the vapor phase without the passage of a phase boundary and the associated deforming forces due to surface tension is the most popular method of drying specimens for SEM. After drying the cells were mounted for coating.

3.5.2.4. Coating

Coating means deposition of a heavy metal onto the surface of the specimen to make it electrically and thermally conductive. Conductivity is necessary for two reasons:

- To avoid image artifacts commonly referred to as charging and
- To acheive, a high yield of secondary electrons upon electron bombardment of the specimen and good resolution in the scanning electron microscope.
- Metal coatings can be established by evaporation or sputtering. For *Frankia* cells, it was done by gold sputtering.

3.5.2.5. Gold sputtering

Gold coating of *Frankia* cells and nodule sections was done with JEOL Fine Coat JFC-1100 ion sputter under vacuum in an inert atmosphere. Coating of uniform thickness was obtained by evaporating gold on the cells kept at specific distance at specific time. The specimen was then fixed on metal tablets with Dolite electroconductives and observed under scanning electron microscope (JEOL JSM – 35CF).

3.6. Isolation of genomic DNA

3.6.1. Isolation of genomic DNA from pure culture

Following protocol was followed for genomic DNA isolation of *Frankia* from pure culture:

- One ml of 1 month old *Frankia* culture was taken in a micro centrifuge tube
- It was centrifuged at 8500Xg for 10 minutes and the supernatant was discarded.
- The pellet was resuspended in 1ml of TE (pH 8.0) buffer (See appendix II for composition) and transferred to a 1.5ml micro centrifuge tube (Tarsons, India).

- It was centrifuged at 8500Xg for 15 minutes and the pellet was dissolved in 1ml of TE (pH 8.0) buffer.
- The solution was forced through 5ml sterile syringe needle of 0.56 X 25mm to break the colonies.
- To this solution 10mg/ml of molecular biology grade lysozyme (Sigma chemical Co., USA. Cat#L-6876) and a pinch of achromopeptidase (Sigma Chemical Co., USA. Cat#A3422) were added and was incubated at 20°C for one hour.
- 250µl of 20% SDS was added. It was incubated at 60°C for 30 minutes and at room temperature for 15 minutes.
- The solution was then divided into two equal parts, 625µl/tube.
- To each tube equal volume of molecular biology grade equilibrated Phenol (SRL Cat#1624262) was added and mixed gently.
- The tubes were then centrifuged at 8500Xg for 10 minutes and the upper aqueous phase so obtained was taken in a fresh tube.
- An equal volume of Chloroform (600µl) (E Merck Ind. Ltd. Cat#822265) was added gently and centrifuged at 8500Xg for 5minutes.
- The upper aqueous phase was taken in a fresh tube and to it 360µl of Isopropyl alcohol (E Merck Ind. Ltd. Cat#17813) was added and the tube was kept at room temperature overnight.
- The tubes were then centrifuged at 4°C for 20minutes at 12000Xg.The supernatant was discarded.
- The pellet was washed with 70% alcohol.
- Finally the pellet was dried in vacuum desiccators and the DNA was resuspended in 10µl of Pyrogen free water.

3.6.2. Isolation of genomic DNA from Alder root nodules

Ten different *Alnus nepalensis* trees were chosen from different collection sites; one from Pashupati, three from Fatak, three from Thurbo, two from Ghoom and one from New Fatak. From each tree ten nodules were collected. The nodules were numbered as

AnP from Pashupati, AnG from Ghoom, AnT from Thurbo, AnF from Fatak and AnNF from New Fatak. These samples were taken for RFLP based diversity studies. The surrounding vegetation of desired tree was cleared off and the digging of the soil was initiated at the base of the stem and worked towards the root tip. The root nodules were found at a reachable depth of 3"-12" and carefully excavated along with a small portion of the attached root system.

Various factors like size of the nodules and the presence of plant phenolics make the isolation of DNA from alder root nodules a tedious process. We adopted the method of Rouvier *et al.* (1996) with few modifications as mentioned below:

- A few nodule lobes were first washed with distilled water and then surface sterilized with 30% Hydrogen Peroxide for 1 minute. They were then washed in autoclaved distilled water twice.
- The epidermal part of a single nodule lobe taken was peeled in PVPP peeling buffer (See appendix II for composition) with the help of two fine needles.
- Each of these peeled nodule lobe was then crushed in a 1.5ml centrifuge tube containing 300µl of pre-warmed DNA-Extraction buffer (See appendix II for composition).
- After complete crushing, the final volume of the extraction buffer was made up to 500µl and it was then incubated at 65°C for 1 hour. This solution was then centrifuged for 10 minutes at 10000Xg at 4°C.
- The supernatant was carefully pippetted out & transferred to a fresh 1.5ml tube and extracted twice with equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation at 12000Xg for 10 minutes, the aqueous phase was taken in a fresh 1.5ml tube.
- The supernatant containing DNA was then precipitated with double volume of absolute alcohol (E Merck Germany Cat#K29824783). The solution was then centrifuged at 12000Xg for 20 minutes at 4°C. The pellet was washed with ice cold 70% EtOH and dried in vacuum desiccators.
- DNA pellet was then dissolved in 20µl of pyrogen free water. This method yields DNA preparation, which was clear in appearance.

Further modifying Rouvier *et al* (1996) method, we did RNase treatment of the isolated DNA to digest the RNA present in the DNA.

3.7. RNase treatment:

To this DNA, RNase (Sigma Cat# R-4875) was added at a rate of 50μgml^{-1} and incubated for 1 hour at 37°C in a cooling dry bath (Genei, India).

- To remove RNase the DNA was extracted with an equal volume of PCI (25:24:1) and centrifuged at 8500Xg for 5 minutes at room temperature.
- The upper aqueous phase was re-extracted with CI (24:1) and again centrifuged at 8500Xg for 5 minutes at room temperature.
- The supernatant so obtained was precipitated with double volume absolute alcohol and the pellet obtained was washed with 70% alcohol, vaccum dried and dissolved in 20μl Pyrogen free water.

3.8. Agarose Gel Electrophoresis of Genomic DNA both from pure culture and root nodules.

The size of the DNA was analyzed by using the following protocol:

- Agarose gel was prepared using Molecular biology grade, DNAase free 0.8% agarose (gelling temperature 36°C) (Sigma Cat#A 9539). O.24gm of agarose was dissolved in 30ml of 1X TBE buffer. (See appendix II for composition).
- This was heated in a microwave oven. (LG make).
- After cooling it to a temperature of 40°C, 0.5μg/ml Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenaanthridinium bromide, $C_{12}H_{20}BrN_3$, M_r 394.33, (Sigma Cat#E 8751) was added to the gel for staining DNA.
- The gel was cast on a gel platform (100×70mm) (Tarsons Cat#7024). An eight well comb was inserted to make wells.
- After solidification, the gel platform along with the solid gel was put into a Mini Submarine Gel Electrophoresis Unit (Tarsons Cat#7030) keeping the well ends

towards the cathode side. The gel was submerged in tank by adding 1X TBE buffer (see appendix II for composition).

- 2µl of DNA was mixed with 3µl loading buffer (type III, Sambrook *et al.* 2001) (for composition and preparation see appendix II) and was loaded into the wells.
- Lambda DNA *Hind III* was used as a molecular marker (Bangalore Genei Cat# MBD 2L).
- The DNA was made to run at a constant volt of 35V applied with Electrophoresis Power Supply Unit (Tarsons Cat#7090).
- After 1 hour the gel was viewed on a UV Transilluminator. (Bangalore Genei Cat#SF850).

3.9. DNA estimation

The yield and the purity of DNA were checked with the help of Spectrophotometer (Thermospectronic UV1) from the absorbance data of DNA samples at 260nm and 280nm. 2µl of DNA was diluted to 2ml with the help of pyrogen free water. The solution was then measured in a spectrophotometer at both 260nm and 280nm. Purity of DNA sample was calculated from ratio of O.D 260/O.D 280 and the quantity of DNA was estimated assuming 1 $O.D_{260}$=50µg.ml^{-1}.

3.10. PCR Amplification of genomic DNA both from the nodules and cultures

3.10.1. DNA Amplification

Double stranded DNA amplification was performed and the proximal, middle, distal and *nif* IGS-HD of the total genomic DNA of *Frankia* were amplified with the help of *Frankia* specific primers synthesized from Sigma-Genosys. The list of the primers is given in a tabular form (See table 3.2 at the end of this chapter).

The primers were dissolved in 1X TE (See appendix II for composition) buffer for making 100µM mother stock and were used at a final concentration of 0.5µM.

Different concentrations of template DNA like 10-50ng was used to amplify different gene regions (See table 3.3 at the end of this chapter).

The reaction was performed with the GeneAmp 2400 thermal cycler (Perkin Elmer, USA) under the following conditions: initial denaturation for 5 min. at 94°C, 35 cycles of denaturation (1 min. at 94°C), annealing (1 min.) and extension (1 min. at 72°C), and a final extension at 72°C. The reaction volume was 50µl.

Annealing temperature depends upon the size and nature of the primers and was calculated with the following formula.

Annealing temp. (°C)= {2(A+T)+4(G+C)}-5

Though both the primers (Forward and Reverse) may be having different annealing temperature, in such case the lowest one was used.

3.10.2. Gel electrophoresis

5µl of the amplified product was analyzed by performing the gel electrophoresis as mentioned above.

3.10.3. Gel documentation

Photographs of the amplified PCR products were taken with Kodak digital Science DC-120 Digital Camera.

3.11. Purification of amplified PCR products

Purification of the amplified PCR products was done using the Sigma GenElute™ PCR Clean-Up Kit as per the manufacturer's protocol. This purified amplification product was further used for cloning and RFLP studies.

3.12. Cloning of PCR products

A part of PCR product has been cloned into T-Vector with the help of TA Cloning kit supplied by Bangalore Genei, India.

3.12.1. Properties of T vector:

- Lac promoter: Expression of lac Z (α fragment) that helps in α-complementation. (Blue/White screening).

- LacZ α-fragment: Encodes for the first 146 aminoacids of β-galactosidase.
- Ampicillin resistance gene: Selection and maintenance in E.Coli.
- pMBI: The origin of replication, maintenance and high copy number in E. coli.
- T-7 Promoter: *in vivo/in vitro* transcription of RNA.
- SP6 Promoter: Sequencing of insert.
- f1 origin: Rescue of strand for mutagenesis and single strand sequencing.
- Multiple cloning sites: selection of restriction sites for cloning.

  (See figure 3.3 at the end of this chapter)

3.12.2. Ligation into T-Vector:

Two µl (50ng) of T-Vector was used for each reaction. Amount of PCR product to be used was calculated by following formula.

$$\frac{\text{Size of PCR product (bp)} \times \text{50ng vector}}{\text{3000bp (size of the vector)}}$$

The concentration of PCR product has been determined by spectrophotometer as mentioned before.

3.12.3. Ligation of reaction:

Set up of ligation reaction was as follows.

- PCR product-Xµl
- 10X ligation buffer-1µl
- T-Vector-2 (50ng) µl
- T4 DNA ligase-1µl

The volume was made upto 10 µl with distilled deionized P.F.water. The ligation reaction was incubated at 16°C in a ligation bath (Bangalore Genei Cat # LIB-1) for 4-6 hours followed by freezing the ligation mixture in 20°C until further use.

3.12.4. Preparation of Bacterial Transformation

3.12.4.1. Preparation of competent cells:

- An overnight grown culture of DH5∝ was taken in a prechilled centrifuge tube and centrifuged at 5000-7000rpm for 15minutes at 4°C.

- The supernatant was discarded and the pellet was suspended in 100mM $CaCl_2$ solution.
- It was then kept on ice for 30 minutes and the cells were spinned down at 500rpm for5 minute at 4°C.
- The pellet was resuspended in 5ml 100mM $CaCl_2$ and kept on ice for 30 minutes and was used for transformation.

3.12.4.2. Bacterial Transformation:

- The water bath was kept ready at 42°C.
- The solid L.B. medium plates (See appendix I for composition) containing antibiotic, X-gal & IPTG were prepared. X-gal and IPTG were added only at the time of plating the transformed cells.
- 100 µl of competent cells were at first thawed on ice and 10 µl of ligation mix was added directly into it and then mixed gently.
- Vials were incubated on ice for 30 minutes.
- Heat shock at 42°C was given to the mixture and then placed it on ice immediately.
- To each of these vials 400 µl of L.B. medium (For composition see appendixI) was added.
- The vials were incubated on 37°C shaker at 225 rpm for 1 hour.
- 250 µl of it was spread on to the antibiotic, Xgal and IPTG containing L.B. plates.
- The plates were then incubated overnight at 37°C. For colour development

3.12.5. Screening and analysis:

From the plates that developed blue and white colonies, 10-20 white colonies were taken into 5ml L.B. medium containing 100µg/ml of ampicillin and grown overnight.

Plasmid isolation was performed with the help of Silica column based plasmid mini prep. kit (GenElute™ Sigma Aldrich USA Cat# PLN70) as per the manufacturer's protocol.

The distal part of 16S rRNA gene fragment was digested out with *Nco* I (See Table 3.4 at the end of this chapter) since the insert was flanked by two *NcoI* sites and the fragment (Distal part of 16S rRNA gene does not have any *NcoI* sites.

Region of *nif* H-D IGS however had *NcoI* sites. Therefore this fragment was digested out with the help of two restriction enzymes, *PstI* and *ApaI*. Reaction conditions and the assay buffer for these two enzymes were different (See Table 3.4 at the end of this chapter). Therefore we first cut the plasmid with *PstI*, the product was purified with Sigma Spin™ post reaction clean up column and then cut with *ApaI*.

3.13. Restriction digestion of amplified regions.

3.13.1. Preparation of Restriction digestion reaction mix:

- 10µl of PCR amplified product of *nif* IGS-HD and other regions as described in section H.1 was taken in a sterile microcentrifuge tube.
- 2µl of 10X restriction enzyme digestion buffer (supplied with Bangalore Genei Pvt Ltd. restriction enzymes), was added to it and mixed thoroughly.
- 5U of restriction enzymes (Bangalore Genei Pvt. Ltd.) was used for restriction digestion. The enzymes used are shown in Table 3.5 (See table 3.5 at the end of this chapter).
- The tubes were incubated in a cooling dry bath at indicated reaction temperatures for 1 hour.
- The reaction was stopped by adding 0.5M EDTA (pH 8.0) to a final concentration of 10mM. The digested DNA was stored at -20°C until further use.

3.13.2. Analysis of restriction fragments (RFLP)

RFLP analysis was done performing the gel electrophoresis of the digested product.

A 3% (w/v) molecular biology grade high resolution agarose (Sigma, USA) gel was prepared in 1X TBE for a 15X10 cm casting tray. Electrophoresis was done in midi submarine electrophoresis unit (Tarsons Cat#7050). Twenty µl of the digested DNA was electrophoresed in 3V/cm electric field.

Lambda DNA *Hind* III digest Bangalore Genei, Cat#MBD2S) and Gene Ruler™ 1Kb ladder (MBI Fermentas, Lithuania Cat#SM0311) were used as molecular wt. standards. The gel was visualized on a UV transilluminator (Gibco-BRL) and photographed using Kodak digital Science DC-120 Digital Camera.

3.13.3. Scoring of data

The PCR RFLP data were scored as present (1) or absent (0) using the SIMQUAL program and DICE coefficients. A matrix was computed and phonograms developed by a UPGMA clustering of the matrix NTSYS-pc (Rohlf 1994).

3.14. The influence of host on nitrogenase activity

In many ecosystems plant growth is limited by the availability of nitrogen suggesting that dinitrogen fixing plants should have a substantial advantage. In fact nitrogen fixing symbioses involving both actinorhizal plant and *Frankia* have evolved and play a permanent role in the wide variety of ecosystem. Though it is the Frankia which actually fix the atmospheric Nitrogen, earlier work revealed that the host may play a greater role in developing the symbiosis (Sougofara *et al.* 1992). In our earlier experiments we found that amount of Nitrogen fixation may vary significantly from one plant to its neighbouring one.

This may be due to several factors like the age of the host plant, pH of the soil, the age of the nodules, genetic variation among the host and the symbiont etc. In our present study we have chosen an Alder stand in Tadong area of Gangtok (Sikkim). These trees are young and are of same age since they are all planted at the same time. Fresh light brownish nodules were collected in the month of April from 10 different trees of same Alder stand (Section 3.1.1). From the site seventeen healthy trees were dug for the nodule collection and the soil samples were collected for pH evaluation. In around 60% of the trees nodules were found. The plants were chosen carefully so that they are equally healthy and were of same height. Along with the nodules the temperature was recorded. Ten nodules from each plant were used for ARA. All the other general information about the site of collection was also collected as mentioned in 3.1.1 section. The selected site

vegetation as studied also included few more plant species other than *Alnus nepalensis* e.g. among the tree species were *Erythrina arborea, Exbucklandia populnea, Elaeocarpus sikkimensis* etc. and among shrubs most common were *Cesternum auranticum, Osbeckia chinensis and Melastoma paniculata* etc as identified and recorded at the site of collection.

For ARA each nodule was surface sterilized carefully and weighed. A vial was taken and its volume was measured (7.672ml). The vial was then fitted with a tight serum stopper. A small moist strip of paper 2cm/2cm were placed in the vial and the nodules were placed on top of it. The vial was sealed and C_2H_2 was injected. The amount of C_2H_2 was 0.76ml (10% of the volume of air was removed through the stopper prior to the addition of C_2H_2 and incubated at ambient conditions for 1-2 h. Sampling of C_2H_2 was done using a gas tight hypodermic syringe and injected into the "Perkin Elmer-8700"gas chromatograph. The amount of acetylene reduced to ethylene was calculated and expressed as nano mol/g/hr (Burris 1967). Each of the nodules which were used in ARA experiment were subjected to total DNA amplification as per the previous protocol discussed. DNA of the samples was amplified with different primers (See Table 3.2 at the end of this chapter). The amplified products were subjected to restriction digestion using the enzymes as mentioned in (See Table3.5 at the end of this chapter). These data were scored as (1) and (0) further analyzed with the help of a UPGMA software NTSYSpc (Rohlf 1994).

Table 3.1 Associated spp. of *Alnus nepalensis*

FAMILY	GENUS	SPECIES
Asteraceae	*Anaphelles*	*contorta*
Cyperidaceae	*Cyperus*	sp.
Asteraceae	*Eregeron*	*Karwinskianus*
Asteraceae	*Eupatorium*	*glandulosum*
Apiaceae	*Hydrocotyl*	*himalaica*
Polygonaceae	*Persicaria*	*nepalensis*
Urticaceae	*Pauzolzia*	*hierta*
Rubiaceae	*Rubia*	*manzit*
Caprifoliaceae	*Vivernum*	*eruvescens*

Table3.2: Primers used to amplify different regions of *Frankia* genome. (Primer numbers for rRNA genes are as per *E.coli* numbering (Embley *et al*, 1988) and for *nif* genes are as per *K.pneumoniae* numbering (Normand *et al*, 1988)).

Sl.#	Target Regions	Primer sequences	References
1.	*nif* HD-IGS	FGPH-750-5'GAAGACGATCCCGACCCCGA3' FGPD-826'-5'TTCATCGACCGGTAGCAGTG3'	Simonet *et al.*, 1991
2.	Distal part of 16S rRNA gene	FGPS-989ac 5'GGGGTCCGTAAGGGTC3' FGPS-1490'-5'AAGGAGGGGATCCAGCCGCA3'	Bosco *et al.*, 1992 Normand *et al.*,1996
3.	Middle part of 16S rRNA gene	FGPS-485-5'CAGCAGCCGCGGTAA3' FGPS-910-5'AGCCTTGCGGCCGTACTCCC3'	Normand *et al.*,1996
4.	Proximal part of 16S r RNA gene	FGPS-6-5'TGGAAAGCTTGATCCCTGGCT3' FGPS-505'-5'GTATTACCGCGGCTGCTG3'	Normand *et al.*,1996

Table3.3: PCR mix

Component*	**Amount**	**Final Concentration**
Water	30.4µl	
10X PCR Buffer	5µl	1X
10mM dATP	1µl	200µM
10mM dCTP	1µl	200µM
10mM dGTP	1µl	200µM
10mM dTTP	1µl	200µM
Forward Primer (5µM)	5µl	0.5µM
Reverse Primer(5µM)	5µl	0.5µM
Taq Polymerase (5U/µl)	0.6µl	0.06U/µl

* All the components are from Sigma Chemical Co., USA.

TABLE: 3.4 List of Restriction enzymes used after cloning and their reaction conditions:

Restriction enzyme with Cat# (Genei,India)	Sequence	Reaction Volume	Reaction temp. (°C)	Amount of RE Used
NcoI (MBE 15S)	C↓CATGG	20µl	37	5units
PstI (MBE 12S)	CTGCA↓G	20µl	37	5units
ApaI (MBE 17S)	GGGCC↓C	20µl	37	5units

Table3.5: List of Restriction enzymes used in present RFLP study

Restriction enzyme with Cat# (Genei,India)	Sequence	Reaction Volume	Reaction temp. (°C)	Amount of RE Used
*Alu*I (MBE-17-S)	AG↓CT	20µl	37	5units
*Taq*I (MBE-7S)	T↓CGA	20µl	65	5units
*Hinf*I(MBE-21S)	G↓ANTC	20µl	37	5units
*Hae*III(MBE-10S)	GG↓CC	20µl	37	5units
*Hha*I (MBE-14S)	GCG↓C	20µl	37	5units
*Mbo*I (MBE-27S)	↓GATC	20µl	37	5units
*Msp*I (MBE-31S)	C↓CGG	20µl	37	5units
HindIII (MBE-6S)	A↓AGCTT	20µl	37	5units
*Eco*RI (MBE-3S)	G↓AATTC	20µl	37	5units

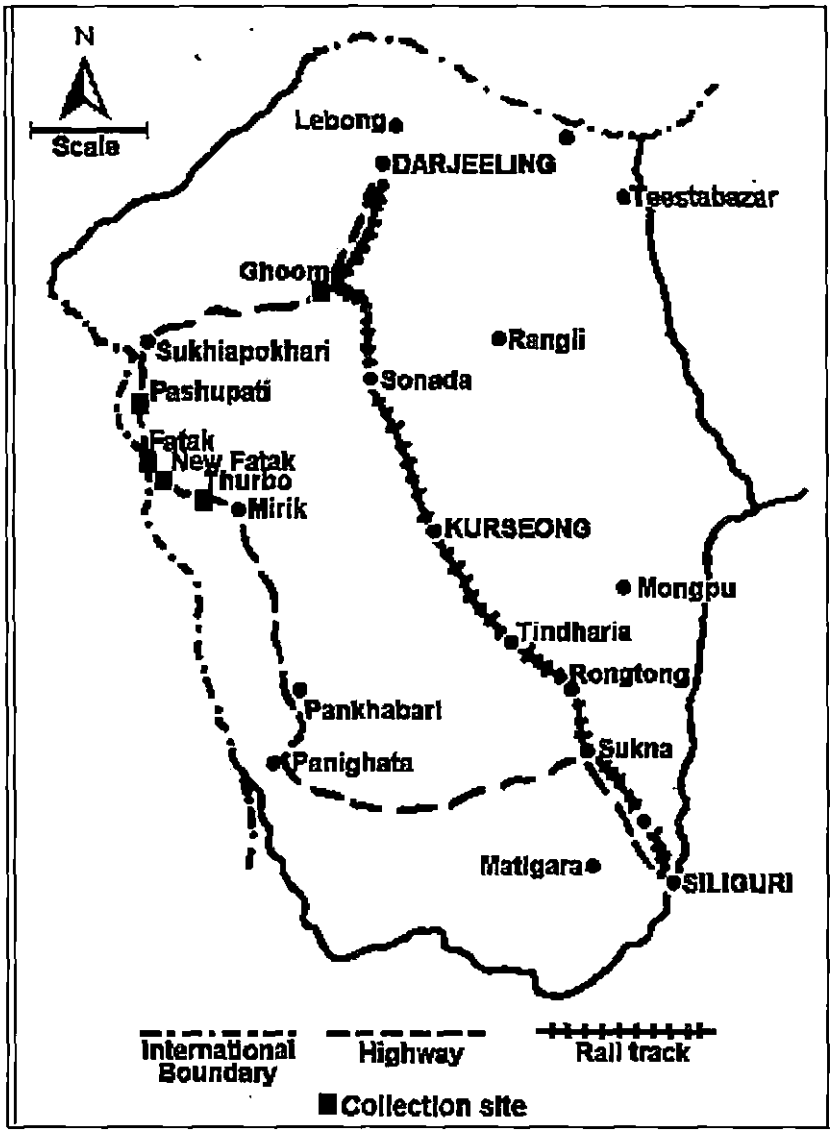

Figure 3.1: Map of Darjeeling District showing collection sites. Scale corresponds to 10 KM.

COLLECTION DATA SHEET

SAMPLE NO. K-2

GENERAL INFORMATION

1. Collection site and state : ST. Alphonsus School
2. Date : 29/2/2000 2A. Time: 2.00 p.m
3. Scientific name: Alnus nepalensis 4. Local name : Uotis
5. Nodules : (Present) / absent
6. Nodules collected : (Yes) / No
7. Root collected : Yes / (No)
8. Seed collected : Yes / (No)
9. Soil collected : (Yes) / No
10. Twig collected : (Yes) / No

B . HABITS

1. (Tree) / Shrub / herb
2. Flowering time : .....
3. Seeding time : .....
4. Planting time : .....

C. HABITAT AND AREA OF THE VEGETATION

1. Rainfall Seasons : From ..... to .....
2. Altitude : 1599 Feet / metre
3. Temperature : (a) Soil: 12 °C (b) Air: 16 °C
4. Topography : Swamp / Plain / (Hilly) / Mountain / Others.
5. Vegetation type : Natural forest / Road side / (Social forest) / others.
6. Management : Cutting / Burning / Natural / (Habitat preserved)
7. Associated species : .....

D. SPECIFIC COLLECTION SITE

1. Site cover : Bare upto 20 % / 21- 40 % / 41- 60 % / (61-80 %) / 81- 100 %
2. Soil pH : 6.2
3. Colour : Red / Yellow / Brown / Grey / Reddish brown
4. Drainage : Flodded / Poorly drained / (Well drained)

F. NODULES

1. Location : Crown area / Tap root / (Lateral root)
2. Growth form : (Clumped) / Scattered

COLLECTED BY : Balwinder Bajwa **SIGNATURE** 29/2/2000

Figure 3.2: Sample datasheet of Darjeeling for collection of *Alnus nepalensis* root nodules in the field.

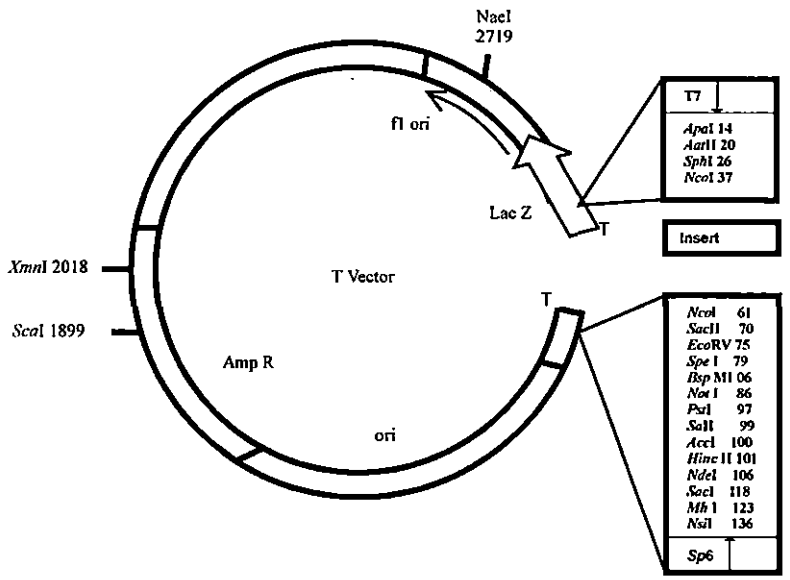

Figure 3.3: Diagram showing T-Vector used in cloning. (Supplied by Genei,India)