Methodology
3.1 EXPERIMENTAL SITE DESCRIPTION

Soil bio-diversity experiments were undertaken in soil from Karion village, Chamba, Himachal Pradesh (Fig 1, 2). Chamba is a valley forming the north western district of Himachal Pradesh, in India (32.5619°N and 76.1206°E). It is situated in the bosom of the Himalayan Mountains. This valley has sub-tropical climate. Mean annual precipitation during the experimental tenure i.e. 2007, 2008, 2009 was approximately 44.45, 68.21 and 35.94mm of rainfall respectively. The data from the agro-ecosystem/cultivated land was compared with the data from natural ecosystem viz. soil from uncultivated land. Crop sown in the agricultural field was Maize. Maize based cropping systems are very popular in the tropics.

3.2 SOIL SAMPLING

Distribution of the soil fauna in soil is not uniform hence soil sampling was done randomly from five different locations in the field. Random samples were then mixed thoroughly to prepare a composite mixture. Rectangular sampler (5x5x10cm) (Bhattacharya, 1971) was used for sampling. Samples were collected two times a year (January month and May month) for duration of three years i.e. 2007, 2008 and 2009 (Table1). Soil samples were collected from the top layer (0-10cm). Plant materials and other debris were removed by hand picking from the samples. Next the samples were sieved through a sterile sieve (2mm mesh size). Samples were brought in the laboratory and were stored at 4°C and -20°C for enzymatic studies and molecular studies respectively, until further analysis. Cloning in order to assess bacterial diversity was done in the last year sample of the January month and May month.

3.3 METEOROLOGICAL CONDITION DURING EXPERIMENTAL TENURE

Temperature and rainfall were recorded throughout the experimental period (Fig 3).

3.4 PHYSIO-CHEMICAL ANALYSIS OF SOIL

Physio-chemical analysis of the soil samples (Particle size distribution, dry weight, pH, total soil organic matter, exchangeable sodium, exchangeable potassium, bulk density) were carried out as per standard and published methods (Table 2).
3.4.1 Particle size distribution

Determination of the particle size with the sedimentation procedure based on stroke’s law was conducted using an aspirator bottle. 25g soil was mixed, digested and centrifuged with 40ml of sodium acetate-acetic acid buffer (pH 5.0) in 250ml conical flask. Supernatant was discarded and soil was taken in graduated aspirator bottles filled up to 8th mark with water. It was then shaken vigorously. Bottles were placed undisturbed for at least 5 to 6h, until all sand and silt settled down and clay remained in suspension. After six hours the clay suspension was decanted. The process was repeated till supernatant liquid was in free suspension. The liquid was allowed to stand in the trough and 5 to 7g of NaCl was added. The solution was stirred and allowed to stand. Then, the clear water was decanted and the mass of the dry clay was found out. The solid soil left in aspirator bottle contained silt and sand only. It was dried and passed through 20µm sieve. The particle, which passed through it, was silt and that which did not pass was sand. Percentage was calculated using the following formula:

Percent Clay fraction = \( \frac{\text{Oven dried soil wt. (g)}}{\text{Initial wt. of soil}} \times 100 \)

Percent Sand fraction = \( \frac{\text{Weight of fraction on sieve (g)}}{\text{Initial wt. of soil}} \times 100 \)

Percent Silt fraction = \( \frac{\text{Weight of fraction under sieve (g)}}{\text{Initial wt. of soil}} \times 100 \)

Or

Percent Silt fraction = 100 − (% sand + %Clay)

3.4.2 Dry matter and water content of soil

Soil samples were dried at 105°C. Water and dry matter contents were determined from the weight loss (Schlichting and Blume, 1966). Triplicates of 20g moist field soil samples were weighed in the porcelain dishes and were dried to constant weight at 105°C for at least 3h in an oven. The dried samples were cooled in a desiccator, and weighed again.
Sampling Site

Fig 1: Uncultivated land of Chamba, Himachal Pradesh.

Fig 2: Cultivated land of Chamba, Himachal Pradesh with Maize plantation. Land fertilized with cow dung without the use of agrochemicals.
Table 1: Sampling Time

<table>
<thead>
<tr>
<th>Month</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>2007</td>
</tr>
<tr>
<td>May</td>
<td>2007</td>
</tr>
<tr>
<td>January</td>
<td>2008</td>
</tr>
<tr>
<td>May</td>
<td>2008</td>
</tr>
<tr>
<td>January</td>
<td>2009</td>
</tr>
<tr>
<td>May</td>
<td>2009</td>
</tr>
</tbody>
</table>
Table 2: Physio-chemical properties of cultivated and uncultivated soil, Chamba

<table>
<thead>
<tr>
<th>Soil location</th>
<th>pH</th>
<th>CEC Na⁺</th>
<th>CEC K⁺</th>
<th>Percent dwt</th>
<th>Percent water content</th>
<th>Percent organic matter</th>
<th>Particle size distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Sand</td>
</tr>
<tr>
<td>Himachal cultivated</td>
<td>7.56±0.01</td>
<td>9.40±0.06</td>
<td>300.27±2.02</td>
<td>92.15±0.40</td>
<td>6.75±0.40</td>
<td>2.11±0.17</td>
<td>61</td>
</tr>
<tr>
<td>Himachal uncultivated</td>
<td>7.37±0.03</td>
<td>10.43±0.19</td>
<td>156.72±1.34</td>
<td>73.92±0.18</td>
<td>26.08±0.18</td>
<td>1.15±0.09</td>
<td>56.5</td>
</tr>
</tbody>
</table>
Dry matter content was calculated as under:

\[ \text{Percent Dry Matter} = \frac{\text{Soil - Dry matter}}{\text{Initial wt. of soil}} \times 100 \]

\[ \text{Percent Water Content} = 100 - \text{Percent Dry matter} \]

### 3.4.3 Water holding capacity

Water holding capacity was evaluated using method described by Jaggi, 1976. 50g of field moist sieved soil was taken in cylinder. These were put in a container with water for a minimum of 1h. The water column was maintained as high as the soil column in the cylinder. This cylinder was then transferred onto sand bath for at least 3h. 20g of saturated soil was weighed onto porcelain dish and dried to constant weight at 105°C for at least 3h. Samples were allowed to cool in a desiccator and weighed again. Water holding capacity was determined using the following formula:

\[ \text{Percent WHC} = \frac{\text{Saturated soil} - \text{Dried Soil}}{\text{Dried Soil}} \times 100 \]

### 3.4.4 Soil pH determination

Saturated solution of soil (1:2.5:: soil:water) was prepared, and pH was determined using a glass electrode pH meter (Corning Incorporated, Corning, NY, USA).

### 3.4.5 Cation exchange capacity

#### 3.4.5.1 Exchangeable sodium

Triplicates of 5g soil were mixed with 25ml of neutral 1M ammonium acetate solution. This was centrifuged for 5 minutes at 1500rpm and then filtered immediately through a Whatman filter paper No.1. Supernatant solution was used for determination of sodium ion concentration using a flame photometer. The concentration of sodium in ppm was calculated by drawing standard curve of known concentration of sodium. Standard curve was drawn with the help of 200ppm stock solution of Na\(^+\) ions with NaCl diluted to 0, 5, 10, 15 and 20ppm of Na\(^+\) with acetate buffer.
3.4.5.2 Exchangeable potassium

Triplicates of 5g soil were mixed with 25ml of neutral 1M ammonium acetate and centrifuged till 5 minutes at 1500rpm. This was then filtered immediately through a Whatman filter paper No.1. Supernatant solution was used for determination of potassium ion using flame photometer. The concentration in ppm was calculated by drawing standard curve of known concentration of potassium. The standard curve was made by preparing a 100ppm stock solution of K\(^+\) ions with KCl and further making standard solutions of 0, 5, 10, 15 and 20ppm K\(^+\). The concentration of ions was calculated in Kg ha\(^{-1}\) using the following formula;

\[
\frac{\text{Available ion in soil Kg/ha}}{\text{Initial wt. of soil} \times 10^6} = \frac{R \times \text{Volume of extract} \times 2.24 \times 10^6}{\text{Initial wt. of soil} \times 10^6}
\]

Where, R = ppm of potassium ion in the extract (obtained from standard curve)

3.4.6 Soil organic carbon

Determination of soil organic matter was done by the Walkley and Black, 1934. Triplicates of 1 g soil samples were taken in 250ml conical flask and mixed with 10ml 0.5M K\(_2\)Cr\(_2\)O\(_7\). To oxidize total organic matter, 20ml of conc. H\(_2\)SO\(_4\) was added and shook for 30 minutes. Then it was treated (to increase the temperature and remove the interference of some elements) with 10ml of 85 percent H\(_3\)PO\(_4\) and 0.2g of NaF. Next, 80 drops of indicator (diphenylamine) solution were added. Now the volume was made up to 250ml with distilled water. Unreacted potassium dichromate was back titrated by taking 10ml of the aliquot with 0.1N ferrous sulphate or ferrous ammonium sulphate (Mohr’s salt). The end point was noted when the colour changed from turbid blue to green.

\[
\text{Percent organic Matter} = \frac{10 \times (B - T)}{B} \times \frac{0.003 \times 100}{\text{Wt. of Soil}}
\]

Where, B: Volume of FeSO\(_4\) solution required for blank titration

T: Volume of FeSO\(_4\) solution required for sample titration

0.3: Conversion factor (1ml of 0.1N Mohr’s salt correspond to 0.003g of C)
3.4.7 Bulk density

Soil bulk density is simply the dry weight of soil per volume. Core sampler was filled with oven dried soil until full. Weight of soil inside the sampler was calculated by weighing the sampler with soil and subtracting the weight of the empty sampler from it. Volume of sampler was determined. The Bulk density of soil was calculated using the following formula:

\[
\text{Bulk Density} = \frac{\text{Weight of soil sampler with soil} - \text{Weight of empty soil sampler}}{\text{Volume of soil sampler}}
\]

3.5 SOIL MICROBIAL ANALYSIS

3.5.1 Plate count technique

Agar nutrient mediums were prepared according to the directions for respective micro-organisms, sterilized in autoclave at pressure 1.05N cm\(^{-2}\) and temperature 120°C for 15 minutes and was cooled upto 40°C. About 20-25ml of the medium was poured in sterilized petri plates and allowed to solidify. Soil dilutions were prepared by diluting 1g of soil to 9ml sterilized distilled water. This stock solution was serially diluted to the concentrations at which the desirable organisms showed optimum growth. This concentration was \(10^{-5}\) for bacteria. 100µl of the respective dilutions were spread evenly using a sterilized glass spreader on the agar-medium plates, which were inverted and incubated for 5-7 days at appropriate temperatures. Incubation period and conditions varied, depending on the nature of the organism to be enumerated. After the suitable incubation period, the plates were removed from the incubator and the colony forming units (cfu) were counted manually ranging between 30 and 300.

Bacteria

*Thornton’s medium* was used for estimation of total bacteria in soil. The composition of the medium was as follows:

- \(\text{K}_2\text{HPO}_4\) 1g
- \(\text{MgSO}_4\cdot\text{H}_2\text{O}\) 0.5g
- \(\text{CaCl}_2\) 0.1g
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- NaCl 0.1g
- FeCl₃ 0.002g
- KNO₃ 0.5g
- Asparagine 0.5g
- Mannitol 1g
- Agar 15g
- Distilled Water 1L

The incubation temperature and pH was 31°C and 7.5 respectively.

3.5.2 Soil enzymes

3.5.2.1 Arginine deaminase activity

Arginine deaminase activity was determined by colorimetric technique as per methods described by Kandeler, 1995a. Triplicates of 5g naturally occurring moist soils were incubated at 37°C for 3h after adding 2ml arginine substrate solution (11.5M). Blank solution was similarly prepared, but incubated at -20°C. After incubation, 18ml 2M of potassium chloride solution was added to all the soil samples. This was shook for 30 minutes and then filtered. 1ml filtrate was then extracted and mixed with 3ml KCl (2M), 2ml sodium phenolate solution (0.12M), 1ml sodium hypochlorite (0.005M in 0.125M sodium hydroxide) and 1ml sodium nitroprusside (0.17mM). This was left for 30 minutes for colour development. Optical density was observed in a spectrophotometer against the blank at 630nm. Enzyme activity was calculated using a standard curve. Calculation was done using the following formula:

\[ \mu g \text{ N/g d wt. hour} = \frac{(T - B) \times 20 \times 100}{3 \times 5 \times 3 \times % d \text{ wt. of soil}} \]

Where, 
- \( T \) = mean value of the test material \( \mu g \) ml\(^{-1} \)
- \( B \) = blank \( \mu g \) N ml\(^{-1} \)
- 20 = whole volume of extract (ml)
- 3 = incubation period (h)
- 5 = soil weight (g)
- 100 %\(^{-1}\)d wt. = factor for dry weight.
3.5.2.2 Nitrate reductase activity

Nitrate reductase activity was determined by colorimetric technique as per methods described by Kandeler, 1995b. Triplicates of 5g soil samples were incubated with 4ml of 2,4-dinitrophenol solution (0.9 mM), 1ml potassium nitrate solution (25mM) and 5ml distilled water at 25°C for 24h. Similar set up was prepared for the control sample but it was incubated at -20°C. After incubation, 10ml 4M potassium chloride solution was added to all the soil samples including the control. This was shook for 30 minutes and filtered. To 5ml of the filtrate, 3ml of ammonium chloride buffer (0.19M, pH 8.5) and 2ml of colour reagent were added. This was left for 15 minutes for colour development. Optical density was observed in a spectrophotometer against the blank at 520nm. Enzyme activity was calculated using a standard curve. Calculation was done using the following formula:

\[
\text{µg N/g d wt. 24 hour} = \frac{(S - C) \times 20 \times 100}{5.5 \times \text{Percent d wt. of soil}}
\]

Where, 
- \(S\) = mean value sample (µg N) 
- \(C\) = control (µg N) 
- 20 = volume of extract (ml) 
- 5 = aliquot of filtrate (ml) 
- 5 = soil weight (g) 
- 100% \(^{-1}\) d wt. = factor for dry weight.

3.5.2.3 Urease activity

Urease activity was determined by colorimetric technique using the unbuffered method as described by Kandeler, 1995c. Triplicates of 5g soil samples were mixed with 2.5ml substrate (79.9mM). For the control sample 2.5ml of distilled water was pipetted instead of the substrate. The samples were then incubated at 37°C for 2h. After incubation, 2.5ml of substrate solution was added to the control samples and 2.5ml of distilled water was added to the soil samples. Ammonia produced was extracted with 50ml potassium chloride solution (2M) and determined by a modified Berthelot reaction (Kandeler and Gerber 1988). 1ml filtrate was mixed with 9ml of distilled
Methodology

water, 5ml reagent A \{100ml sodium hydroxide (0.3M) + 100ml sodium salicylate (1.06M) + 120g sodium nitroprusside\} and 2ml dichloroisocyanurate (39.1mM). This was shook and then left for 30 minutes for colour development. Optical density was observed in a spectrophotometer against the blank at 690nm. Sodium nitroprusside acts as a catalyst in the above process, increasing the sensitivity of the reaction by ten folds. Enzyme activity was calculated using a standard curve. Calculation was done using the following formula:

$$\mu g \text{N/g d wt.2 hour} = \frac{(S - C) \times 10 \times 5.5 \times 100}{5 \times \text{Percent d wt.of soil}}$$

Where, \(S\) = mean value sample (\(\mu g\ ml^{-1}\))

\(C\) = control (\(\mu g\ ml^{-1}\))

10 = dilution factor volume of extract (ml)

5.5 = volume of extract (ml)

5 = soil weight (g)

100 \%^{-1} d wt. = factor for dry weight.

3.5.2.4 Dehydrogenase activity

Dehydrogenase activity was determined by colorimetric technique using the method described by Öhlinger (1995). Triplicates of 5g air dried soil were mixed with 5ml of substrate i.e. 2,3,5-triphenyl-tetrazolium chloride (TTC). Concentration of substrate varied with the type of soil used. For all our samples i.e. sandy loam soil 0.1 – 2 percent TTC solution was prepared in 0.1M tris-buffer). For the control sample instead of the substrate 5ml of tris-buffer was added. These samples were then incubated at 30°C for 24h in dark, as tetrazolium compounds are light sensitive. After incubation formazan formed was extracted with 25ml acetone and was estimated spectrophotometrically at 546nm. Concentration of \(\mu g\) tri-phenyl formazan (TPF) in the filtrate was determined from calibration. Calculations of \(\mu g\ TPF\ g^{-1}\ d wt.\ 24\ h^{-1}\) was done using the following formula:

$$\text{TPF } \mu g /g\ d wt.24\ hour = \frac{(T - B) \times 100}{3 \times \text{Percent d wt.of soil}}$$
Where, \( T \) = mean value of sample (TPF)

\[
B = \text{mean value of control (}\mu\text{g ml}^{-1})
\]

\( 5 \) = soil weight (g)

\[
100 \%^{-1}\text{d wt.} = \text{factor for dry weight}
\]

3.6 **TOTAL SOIL DNA ISOLATION**

Total Soil DNA was extracted from 0.50g (dry weight) of soil, using bead beating method by soil DNA extraction kit (Ultra Clean™, Soil DNA Isolation Kit, Mo Bio Laboratories, CA, USA) according to manufacturer’s instruction, with minor modifications. All the extraction was done in triplicate. The quality of isolated DNA was evaluated by agarose gel electrophoresis in 0.8 percent w/v agarose (Fig 10) (GeNei™, Bangalore, India), visualized in Molecular Imager (Gel Doc™ XR+ Biorad, USA) and quantified using Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific, DE, USA).

3.7 **CLONING**

3.7.1 **PCR amplification of 16S rDNA**

From the extracted soil genomic DNA, bacterial 16S rDNA fragment was amplified on a PCR Thermocycler (2700, Applied Biosystems, USA) using primers 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 928R (5’-CCG TCA ATT CCT TTG AGT TT-3’), targeting approximately 920bp fragment of *E. coli* 16S rDNA. Each 25µl reaction contained 1X PCR buffer (GeNei™, Bangalore, India), 1.5mM magnesium chloride (GeNei™, Bangalore, India), 0.2mM of each deoxynucleoside triphosphates (GeNei™, Bangalore, India), 0.5µM of each primer (Sigma Aldrich Co., Bangalore, India) and 1.5U Taq DNA Polymerase (GeNei™, Bangalore, India) to which 1-10ng/µl template DNA was added. Thermal cycling was carried out by an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1minute, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes, followed by final elongation step at 72°C for 15 minutes, in order to get an A’ overhang for TA cloning. All reactions are prepared at 4°C in 200µl reaction tubes to avoid formation of primer dimers. Four independent PCR’s were done per sample and later pooled to avoid PCR
biases (Farrelly et al, 1995; Polz et al, 1998; Qiu et al, 2001; Tanner et al, 1998). Amplicons were checked on 0.8 percent w/v agarose gels stained with 0.5µg/ml ethidium bromide (Fig 11). Samples from separate PCR’s were then pooled for further analysis.

3.7.2 PCR amplification of the \textit{nifH} gene fragment

From the extracted soil genomic DNA, \textit{nifH} DNA was amplified on a PCR Thermocycler (C-1000\textsuperscript{TM}, Biorad, USA) using degenerate primers PolF and PolR (5’ TGC GAY CCS AAR GCB GAC TC 3’) and (5’ ATS GCC ATC ATY TCR CCG GA 3’) respectively (Poly et al 2001) were used to amplify a 360bp region between sequences 113 and 476 (referring to the \textit{Azotobacter vinelandii} \textit{nifH} coding sequence M20568). Each 25µl reaction contained 1X PCR buffer (GeNei\textsuperscript{TM}, Bangalore, India), 1.5mM magnesium chloride (GeNei\textsuperscript{TM}, Bangalore, India), 0.2mM of each deoxynucleoside triphosphates (GeNei\textsuperscript{TM}, Bangalore, India), 0.5µM of each primer (Sigma Aldrich Co., Bangalore, India) and 1.5U Taq DNA Polymerase (GeNei\textsuperscript{TM}, Bangalore, India) to which 1-10ng/µl template DNA was added. Thermal cycling was carried out by an initial denaturation step at 94\degree C for 5 min, followed by 30 cycles of denaturation at 94\degree C for 1 minute, annealing at 55\degree C for 1 minute, and elongation at 72\degree C for 2 minutes, followed by final elongation step at 72\degree C for 15 minutes, in order to get an A’ overhang for TA cloning. The quality and quantity of the amplified product was analyzed on 1.5 w/v percent agarose gel.

3.7.3 Transformation

16S rDNA/\textit{nifH} amplicon was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). Cloning was done using pGEM T Easy vector (Promega, WI, USA). pGEM-T Easy Vector is a linearized vector with a single 3’-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (Mezei, 1994; Robles, 1994). pGEM-T Easy Vector is approximately 3kb and is supplied at 50ng/µl. The appropriate amount of PCR product (insert) to include in the ligation reaction was calculated using the following equation.
Fig 10: Total soil DNA from soil sample Lane 1: Soil DNA from uncultivated soil, Lane 2 and 4: Empty, Lane 3: Soil DNA from cultivated soil M: Supermix ladder.

Fig 11: 16S rDNA amplification from soil DNA Lane 1: 16S rDNA amplified from uncultivated soil, Lane 2: 16S rDNA amplified from cultivated soil, M: 100bp ladder.
Fig 25: *nif*H amplification from soil DNA Lane1: *nif*H amplified from May month uncultivated soil sample, Lane 2: *nif*H amplified from May month cultivated soil, Lane 3: positive control, M: 100bp DNA Ladder
ng of vector × Kb size of insert
Kb size of vector × insert: vector molar ratio = ng of insert

Where, the insert to vector molar ratio was kept at 3:1.

Ligation mix was prepared using 2.5µl of 2X rapid ligation buffer, 25ng of pGEM-T easy vector, 1.5U of T4 DNA ligase and 100ng of PCR product (pGEM-T Easy vector kit promega). This was kept at 4°C overnight and later was transformed into electrocompetent *E. coli* DH5α cells by electroporation, using an electroporator (MicroPulser, Biorad, USA).

Successful cloning of an insert into the pGEM-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. Clones containing PCR products produce white colonies whereas clones without any insert produce blue colonies. Thus insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

Transformants were selected by standard protocols on Luria-Bertani agar with ampicillin (100mg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside) (0.5mM) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (80µg/ml) facilitating blue white selection (Fig 12).

False positive white clones were screened by vector specific M13F and M13R primers (M13F5' - GTA AAA CGA CGG CCA G 3'; M13R5'- CAG GAA ACA GCT ATG AC-3'). PCR amplification products were screened in 1.5 percent w/v agarose to check the insert of correct size (Fig 13). Clones providing amplicon of the right size were subjected to Restriction Fragment Length Polymorphism (RFLP) with 1.5U *EcoRI* restriction enzyme (Fermentas, USA) for 16S rDNA clones and *AluI* (Fermentas, USA) for *nif*H clones overnight at 37°C. Restriction profiling of clones was checked in 2 percent w/v agarose gel in 0.5X tris-borate-EDTA by using 50V/cm. Restricted bands and their respective fragment sizes were analysed in gel-doc (Bio-Rad, USA) (Fig 14). The RFLP patterns were compared with each other and the clones with unique patterns were considered as one operational taxonomic unit (OTU’s, and their dominance patterns were evaluated as abundance profiles.)
3.7.4 Sequencing and phylogenetic analysis

One representative from each of the unique OTU / phylotype was sent for sequencing to Macrogen Inc., S. Korea. Sequencing was done in 3730XL20-21147-008 (Macrogen, Seoul, Korea) using M13F and M13R primers.

3.7.5 Phylogenetic analysis

All 16S rRNA gene sequences were manually trimmed of vector and primer sequence prior to alignment and analysis. Sequences obtained from the clones were compared with the sequences available in the GenBank database using the Basic Local Alignment Search Tool, BLAST (Altschul, 1990), at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The presence of chimeric sequences was checked with the Bellerophon program (Huber, 2004). The sequences were aligned using the program ClustalW (Chenna, 2003); http://www.ebi.ac.uk/Tools/clustalw) was used for multiple alignments. Sequences aligned using ClustalW were imported into DNADIST in PHYLIP version 3.6 (Felsenstein, 2004) to generate distance matrices using the Jukes-Cantor correction for multiple substitutions and the neighbour-joining method (Saitou and Nei, 1987) was used for tree construction. Bootstrap values were estimated using 1000 replicates. Tree view software was used to visualize the tree.

3.7.6 Diversity analysis of clone libraries

Clone libraries and operational taxonomic units (OTUs) by RFLP banding pattern were analysed using different diversity indices. These indices included:

(i) library coverage calculated by the following equation:

\[
\text{Goods Coverage} = \left[ 1 - \left( \frac{n}{N} \right) \right] \times 100
\]

Where, \( n \) is the number of unique clones represented by single OTU
\( N \) is the total number of clones examined (Good, 1953)

(ii) species richness (S) or the total number of OTUs (Brewer, 1994)

(iii) Shannon–Weaver diversity index, calculated by the equation
Fig 12: Blue white selection of cloning plate after transformation of 16S rDNA gene in *E. coli* DH5α using electroporation
Fig 13: Screening of the false positive white clones obtained during cloning using vector specific M13F and M13R primers. Lane 1 and 2-28: Clones of the correct insert size. Lane 2: False positive white clone.
Fig 14: RFLP analysis: Restriction profiling of 16S rDNA clones to establish OTU’s. Lane 1-28: RFLP profile of 16S rDNA clones using EcoRI enzyme, M: 100bp marker
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\[ H = - \sum_{i=1}^{S} (p_i \ln p_i) \]

Where, \( S \) is the total number of species

\( p_i \) is the frequency of the \( i^{th} \) species (the relative contribution of an individual OTU to the whole library).

(iv) the Simpson’s index, calculated by the equation

\[ D = \sum_{i=1}^{S} \left( \frac{n_i (n_i - 1)}{N (N - 1)} \right) \]

Where, \( n_i \) is the number of individuals of species \( I \) which are counted,

\( N \) is the total number of all individuals counted.

The number of unique phylotypes (phylotype richness, \( S \)) was used for creation of rarefaction curves. Relative diversity between bacterial communities was evaluated by calculating the Shannon diversity index.

3.8 T-RFLP

We chose T-RFLP of the 16S rDNA molecule over other fingerprinting techniques because it has the advantage of being rapid and reproducible (Dunbar 2000, Osborn 2000).

3.8.1 PCR amplification of 16S rDNA

After extraction of total genomic DNA, bacterial 16S rDNA was amplified on a PCR Thermocycler (2700, Applied Biosystems, USA). The primers used were specific for conserved bacterial 16S rDNA sequences, 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 928R (5’-CCG TCA ATT CCT TTG AGT TT-3’), targeting approximately 919bp fragment of \( E. coli \) 16S rDNA (Liu et al, 1997). The forward primer 8F was labelled with 6-carboxyfluorescein (FAM). Each 25ul reaction contained 1X PCR buffer (GeNei\textsuperscript{TM}, Bangalore, India), 1.5mM magnesium chloride (GeNei\textsuperscript{TM}, Bangalore, India), 0.2mM each deoxynucleoside triphosphates \{dATP, dCTP, dGTP, and dTTP (GeNei\textsuperscript{TM}, Bangalore, India)\}, 0.5µM of each primer (Sigma Aldrich Co., Bangalore,
India) and 1.5U of Taq DNA Polymerase (GeNei™, Bangalore, India) to which 10-20ng of soil DNA was added. Amplification was carried out with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 2 minutes, followed by final elongation step at 72°C for 7 minutes. All reactions were prepared at 4°C in 200µl reaction tubes to avoid formation of primer dimers. Amplicons were checked on 1 percent w/v agarose gels. Three amplification products were prepared from each DNA sample and were then pooled for further analysis to reduce PCR bias. Care was taken to avoid bright sunlight, which reduces the fluorescence of samples. Amplicons were purified using Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA) as directed by the supplier, and eluted with 50µl sterile water.

3.8.2 Restriction digestion

Purified PCR products (approximately 200ng) were digested separately with 2.5U of tetrameric restriction endonucleases AluI [AG^CT], HaeIII [GG^CC] and MspI [C^CGG] (Fermentas, USA) respectively in three separate tubes with total reaction volume 20µl. 2.5µl of restriction buffer was added to this and remaining volume was made to 20µl with autoclaved MilliQ water. Use of multiple enzymes for T-RFLP analysis increases the resolution by increasing the number of different fragments observed and simultaneously decreasing the number of recorded similarities. Restriction digests were incubated at 37°C for 3h. The value of using multiple enzymes for TRF analysis is to increase confidence that the similarity relationships identified between samples are not the result of biases in the way that a single enzyme samples diversity.

To analyze the terminal restriction fragments (T-RF), 1µl of digested samples was electrophoresed on 7 percent polyacrylamide gel on an automated DNA sequencer. T-RFLP profiles were visualized using genemapper software (version 4.0, Applied Biosystems) which can detect the fluorescently labeled fragments of T-RF’s.

3.8.3 Analysis of T-RF peaks

For each sample, peaks over a threshold of 25U above background fluorescence were analyzed by manually aligning fragments to the size standard. To avoid detection
of primers and uncertainties of size determination, terminal fragments smaller than 50bp and larger than 825bp were excluded from the analysis. Reproducibility of patterns was confirmed for repeating T-RFLP analysis twice. Two additional binning criterions were used to screen pseudo peaks. Firstly, the fragments that differed by less than 2bp were considered identical and clustered together due to the systematic instrument error in determining fragment size. Secondly, the relative peak area ratio, which was calculated by dividing each individual peak area by the total peak area of each profile, must be equal or greater than 1 percent to be considered as a real peak to eliminate possible background noise. The relative abundance of T-RFs within the sections was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample.

T-RFs of different length were considered to be indicative of different bacterial operational taxonomic units (OTUs) present in the sample, and the relative peak heights were used as a measure for the relative abundance of the bacterial OTUs. Terminal restriction fragment (T-RF) lengths can be predicted from known sequences, using software like FRAGSORT, PAT etc; thus, the T-RFLP method can potentially identify specific organisms in a community based on their T-RF length. Same T-RF length may predict for multiple species of bacteria, but increased specificity can result from analysis of digests with multiple enzymes (Dunbar, 2001; Marsh, 1999).

Fragment Sorter 5.0 (Fragsort 5.0, Ohio State University, USA) was used to potentially identify specific organisms in a community based on their T-RF length. This downloadable windows program compares terminal restriction fragment sizes (TRFs) obtained from microbial community DNA amplified with a labeled primer, and digested with one, two, three, or four different restriction enzymes to the TRFs generated from in silico amplification and digestions of a gene sequence database (e.g. 16S rDNA gene database).

Custom database was generated by using the MiCA query function found at the MiCA. The MiCA website can be used to generate a database of predicted T-RFs for each species by using different primers or restriction enzymes.
The FRAGSORT program output is a list of micro-organisms and T-RF sizes that correlate with multiple experimentally generated T-RFLP profiles. The organisms are initially listed in descending order from the greatest to the least normalized T-RF peak area (or height). The output was sorted based on organism name. Fragment sizing error was adjusted to constant sizing value of 1.

3.9 DIVERSITY INDEX

Diversity indices summarize both species richness and relative abundance using a single number and thus they are useful as a first approach to estimate the diversity of bacterial species.

Results from FRAGSORT analysis were further used for calculating the diversity indices. Structural diversity between samples was evaluated by the Bacterial phylotype richness (S), Shannon-Weaver diversity index (H) (Shannon), and the reciprocal of Simson’s index (1/D) (Simpson). Richness (S) is defined as the number of unique T-RFs or OTU within each electropherogram. The Shannon-Weaver diversity index (H) and the reciprocal of Simpson’s index of diversity (1/D) was calculated as described earlier.

T-RFLP profiles from three separate restriction digests, *HhaI*, *MspI*, and *RsaI*, were processed using distance metrics i.e. Jaccard distance. A dendrogram was constructed from these distance metrics using MVSP software. The Jaccard coefficient considers the presence/absence of T-RFs and is equal to the ratio of the number of T-RFs in common between two profiles to the total number of T-RFs present in both profiles. The only problem was encountered in the analysis of the treatment 2 and 3, in which repeated unsuccessful PCR amplifications were observed with the T-RFLP technique.

3.10 MICROCOSM

This study was undertaken at the Greenhouse located at the Daulat Ram College, University of Delhi, Delhi. The experiments were set up in earthen pots which were autoclaved three times before use. All potted plants were subjected to uniform photoperiod, light intensity, relative humidity and temperature. The aim was to assess
and compare the effect of inorganic (NPK), organic (Cow dung and Corn cob) and combination of both on soil microbial components and chemical properties. Also one rhizobacteria was investigated for having plant growth promoting factors. The bacteria was nifH positive detected by colony PCR using nifH specific primers. Sequence analysis of the bacteria showed 98 percent BLAST similarity Rhodococcus erythropolis. This bacteria was selected as it had genes for nitrogen fixing enzyme, nitrogenase and thus could be used as a potential plant growth promoting bacteria.

3.11 EXPERIMENTAL SET UP

Microcosm experiments (including 13 treatments) were conducted in greenhouse in duplicate under controlled conditions (Table 3). Pots were autoclaved three times before use. Each pot was filled with 600g of air dried soil, from Chamba, Himachal Pradesh. Soil was sandy loam (sand 60 percent, silt 26.5 percent and clay 13.5 percent) with bulk density 1.54g cm$^{-3}$, pH 7.56 ± 0.01, percent organic carbon 0.45 ± 0.3, exchangeable sodium and exchangeable potassium 9.49 ± 0.11 ppm g$^{-1}$ d wt.$^{-1}$ and 301.27 ± 3.5 Kg ha$^{-1}$ respectively. Soil moisture was maintained by addition of autoclaved water to avoid any contamination for each pot. Surface seed sterilization was done to disinfect the seeds before sowing by soaking in 10 percent hydrogen peroxide solution for 30min. This also enhances the germination efficiency of seeds.

Amendments were added as per agricultural handbooks of Hill, IARI. Fertilizer applications were done at the rate of 0, 120 and 240Kg of N ha$^{-1}$ respectively. Nitrogen application was done in three doses. One third dose of nitrogen was applied basally, rest one third dose was applied after 20 days and the last dose was applied after 30 days. This was done to avoid the nitrogen losses from soil due to leaching. The Phosphorous and Potassium fertilizer were kept constant in the NPK microcosm. Phosphorous: Potassium, were added in a ratio of 60:40. This was done to see the effect of nitrogen gradient on plant and soil. Both organic amendments corn cob and cow dung application was kept between 15-20T ha$^{-1}$. Bacterial inoculation was $1.2 \times 10^8$ cells per pot.

Seed sowing date was taken as the zero day reading for the microcosm. Four seeds were sowed in each pot. Amendments of the individual treatments were added to
soil two weeks before planting the seeds. In each case, plants were thinned to one plant per pot 2 weeks after sowing. Sampling was done in duplicate from each amendment on the seventh day, thirtieth day and the forty fifth day of the microcosm, and was stored in -20°C until further analysis. Plant length data was regularly monitored during the whole experiment. Soil properties: i.e. pH, total Carbon and total Nitrogen, soil microbial biomass, enzyme assays (Urease, Arginine Deaminase, Nitrate reductase, Dehydrogenase), microbial diversity using T-RFLP were determined using standard methods described earlier in thesis.

3.12 STATISTICS

Each sample was prepared in triplicate and mean of three repeats was processed. Difference between treatments was ascertained by one way ANOVA. The correlation (Pearson coefficient) between soil health measuring parameters was conducted by using SPSS 16.0 for Windows. Pearson's correlation coefficient is a measure of the linear association between two variables.
Table 3: Amendments added in microcosm experiment conducted in greenhouse. Rate of application of different amendments is as follows: P: @ 60 Kg ha\(^{-1}\), K: @ 40 Kg ha\(^{-1}\), N: @ 120 Kg ha\(^{-1}\), NN: @ 240 Kg ha\(^{-1}\), Corn cob: @ 25 Tonns Ha\(^{-1}\), Bacteria: @ 3\times10^9 bacterial cells per pot.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amendment</th>
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<tbody>
<tr>
<td>Treatment 1</td>
<td>S + PK + B</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>S + NPK + B</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>S + NNPK + B</td>
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<tr>
<td>Treatment 4</td>
<td>S + NPK</td>
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<tr>
<td>Treatment 5</td>
<td>S + CCOB + B</td>
</tr>
<tr>
<td>Treatment 6</td>
<td>S + CDUNG + B</td>
</tr>
<tr>
<td>Treatment 7</td>
<td>S + CCOB</td>
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<tr>
<td>Treatment 8</td>
<td>S + CDUNG</td>
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<tr>
<td>Treatment 9</td>
<td>S + CCOB + NPK</td>
</tr>
<tr>
<td>Treatment 10</td>
<td>S + CDUNG + NPK</td>
</tr>
<tr>
<td>Treatment 11</td>
<td>Sterile Soil + B</td>
</tr>
<tr>
<td>Treatment 12</td>
<td>S + CCOB + NPK + B</td>
</tr>
<tr>
<td>Treatment 13</td>
<td>S + CDUNG + NPK + B</td>
</tr>
</tbody>
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