MATERIALS AND METHODS
3. MATERIALS AND METHODS

Present work was carried out in the Microbial Technology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi; Amity Institute of Microbial Technology, Amity University-Uttar Pradesh, Noida and the Department of Microbiology, Ch. Charan Singh University, Meerut, Uttar Pradesh.

3.1 Chemicals

The chemicals used in the present study were of analytical grade and obtained from standard companies. Acrylamide, bovine serum albumin (BSA), bis-acrylamide, bromophenol blue, commassie brilliant blue (R-250), 2-β-mercaptoethanol, Sodium Dodecyl Sulfate (SDS), N,N,N’,N’ tetra methylethylenediamine (TEMED), wide range protein molecular weight marker (6.5-205 kDa) for SDS-polyacrylamide gel electrophoresis were purchased from Sigma Chemical Company, St. Louis, USA. Hydrogen peroxide (30%) and Mercuric chloride were procured from Merck India Limited, Mumbai, India. Ammonium sulphate was obtained from Qualigens, Mumbai, India. Casamino acid, silver nitrate, vitamins (amino benzoic acid, biotin, nicotinamide, pyridoxal phosphate, riboflavin, thiamine hydrochloride for media preparation) were obtained from SRL Chemicals, Mumbai, India. Yeast extract, malt extract, peptone, agar-agar were obtained from Hi-media, Mumbai, India.

3.2 Symbiotic Fungi (Sebacinales) and Rhizobacteria

3.2.1 Procurement of Cultures

The pure cultures of two species of Sebacinales, Piriformospora indica and Sebacina vermifera var sensu, were obtained from School of Life Sciences, Jawaharlal Nehru University, New Delhi. The pure cultures of rhizobacteria were obtained from various authentic sources from India and abroad (Table 5).

Table 5. Cultures of rhizobacteria used in the present study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas fluorescense</em></td>
<td>IUB, Bremen</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>IARI, New Delhi</td>
</tr>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>IARI, New Delhi</td>
</tr>
<tr>
<td><em>Bradyrhizobium sp. (M2-97)</em></td>
<td>IARI, New Delhi</td>
</tr>
</tbody>
</table>
The pathogenic fungi used were *Magnaporthe* sp., *Alternaria solani* and *Dreschlera sorokiniana*. These were obtained from Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi.

### 3.2.2 Maintenance and Storage of Cultures
Circular agar discs (7 mm in diameter) infested with spores and actively growing hyphae of Sebacinales (*P. indica* and *Sebacina vermitera* sensu) (Verma *et al.* 1998) were placed on to Petri plates (90 mm, disposable, Tarson, India) containing 20 ml solidified Kafer or Aspergillus medium (Hill and Käfer, 2001; Pham *et al.* 2004b; Prasad *et al.* 2005) (Appendix-1). Inoculated Petri plates were incubated (Memmert type incubator) in an inverted position for 7 days at 28±2°C in dark. Usually 4-5 fully grown fungal agar discs (7 mm in diameter) were inoculated into each 500 ml Erlenmeyer flask containing 200 ml of Kafer broth medium. They were incubated at 28±2°C with constant shaking at 120 rev min⁻¹ on rotary shaker (GFL 3019, Federal Republic of Germany). The prepared inoculum was stored at 4°C until use.

The rhizobacterial inocula were streaked on 90 mm Petri plates containing sterile nutrient agar medium (Appendix-1) and incubated at 30°C in Memmert type incubator for 24 h. Later they were stored at 4°C until use. Further, the liquid culture inocula were also prepared wherever needed in nutrient broth.

### 3.3 Optimization of Various Physiological Parameters for Sebacinales
The optimization of different physiological parameters (from 3.3.1 to 3.3.5) as mentioned below was studied in different four media namely, Modified Melin Norkrans (MMN), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Kafer Agar (KF) were tested (Appendix-1). Further characterization study on *P. indica* and *S. vermitera* sensu was performed using Kafer medium.

#### 3.3.1 Selection of the Basal Medium
Growth performance of the test fungi on individual solid media was carried out by inoculating them on different medium plated in 90 mm Petri plates in five replicates. Linear growth (cm) and surface area (cm²) was calibrated every 24 h for
10 days. Incubation temperature was maintained at 28±2°C throughout the course of the experiment.

3.3.2 Effect of pH
Effect of hydrogen ion concentration (pH) on the growth of test fungi was studied on the Kafer broth medium. The initial pH of the medium was adjusted to 5.5, 6, 6.5, 7 and 7.5, respectively, before autoclaving. A volume of 25 ml of the broth of each pH condition was taken in 100 ml Erlenmeyer flask, sterilized and inoculated with 7 mm mycelium disc of the test fungi. Each treatment was replicated five times and the flasks were incubated at 28±2°C for ten days and growth of the test fungi. The dry weight was recorded after 15 days of incubation. The final total growth was measured on a single day for all treatments/replicates.

3.3.3 Effect of Temperature
Effect of different incubation temperatures on mycelial growth of the test fungi was studied on Kafer medium both in liquid and solid forms. pH of the medium was adjusted at the optimum and 25 ml of each was dispensed in 100 ml Erlenmeyer flasks. The flasks were then sterilized and incubated with 7 mm mycelial disc of the test fungi and incubated at five different temperatures 20, 25, 28, 30 and 35°C, respectively, for 10 days as shake culture. Each treatment was replicated five times. After incubation the mycelial mats were harvested to obtain their dry weight. Final pH of the culture filtrate was recorded with a dynamic digital pH meter.

3.3.4 Effect of Relative Humidity (RH)
This aspect was investigated at five different relative humidities viz., 75, 80, 85, 90 and 95%, respectively, during the course of incubation. For creating those RH levels, saturated solutions of NaCl, KCl, KH2PO4 and (NH4)2SO4 were, respectively, taken in desiccators loaded with silicon gel. This experiment was carried out on solid Kafer medium. The Petri plates were kept in desiccators containing the respective solutions and incubated at 28±2 °C for 10 days. Growth of the test fungi was recorded as radial growth.
3.3.5 Nutritional Requirements—Liquid Basal Medium

The Kafer medium supporting the best growth of the test fungi identified during growth dynamics study was selected as the basal medium for determining the nutritional requirements of the test fungi, Sebacinales.

(i) Utilization of Carbon Sources by the Test Fungi: For this parameter, ten different carbon sources were taken including pentoses, hexoses, and polysaccharides. Each carbon source was added in the liquid basal medium separately in place of glucose so as to maintain the same carbon concentration as in the basal medium. In case of starch and cellulose the concentration was taken 20g/L, irrespective of molecular weight equal to the quantity of glucose in one litre of basal medium. The pH of the modified medium was adjusted at the respective pH optimum of the test fungi by using 0.1 N HCl and 0.1 N NaOH. A volume of 25 ml, each of the aliquots, was dispensed in 100 ml Erlenmeyer flasks and sterilized at 1.05 kg/cm³ pressure for 20 min in autoclave. A control series without carbon source was also maintained. Each treatment was replicated five times and inoculations were made by adding 7 mm mycelium disc of the test fungi. The inoculated flasks were incubated at 28±2 °C for 15 days as shake culture before the mycelial mats were harvested and dry weight recorded. The final pH of respective culture filtrate was also recorded.

(ii) Utilization of Nitrogen Sources: Utilization pattern of 13 amino acids and 6 complex organic and inorganic nitrogen sources for mycelial growth of the test fungi was tested. The amino acids included L-arginine, L-cysteine, glycine, L-glutamic acid, L-lysine, L-histidine, DL-methionine, DL-Phenylalaanine, DL-serine, DL-tryptophan, DL-valine, L-asparagine and DL-isoleucine and the inorganic nitrogen sources used were urea, peptone, ammonium chloride, potassium, sodium and ammonium nitrates, respectively. The basal medium used was the same as used in the study on carbon sources, but the nitrogen sources replaced the nitrogen sources of the basal liquid medium. The pH was adjusted at the optimum for the respective test fungi. A volume of 25 ml of the modified
media was dispensed in the 100 ml Erlenmeyer flasks and sterilized at 1.05 kg/cm² pressure for 20 minutes.

A control was also maintained without any nitrogen source. Each treatment was replicated five times. The flasks were inoculated with 7 mm mycelial disc of the test fungi and incubated at their optimum incubation temperature. Rest of the process was the same as in the previous experiment.

(iii) Utilization of Potassium Sources: For this study, the same basal medium and five different potassium sources were taken viz., KCl, KNO₃, K₂HPO₄, K₂SO₄ and sodium potassium tartrate. The potassium sources were taken to replace KH₂PO₄ in quantities so as to maintain the same potassium concentration as in the basal medium. A control without potassium source was also run and five replicates were taken for each treatment. A volume of 25 ml of the modified media were dispensed in the 100 ml Erlenmeyer flasks and sterilized as earlier. Inoculation was done by adding 7 mm mycelial bit of both the test fungi. The inoculated flasks were incubated at respective optimum temperature of the test fungi for 15 days. The mycelial mats were harvested and dried to obtain the final dry mass.

(iv) Utilization of Phosphorus Sources: The study was carried out in the same basal liquid culture medium and different phosphorus sources taken were Na₂HPO₄, NaH₂PO₄, CaHPO₄, K₂HPO₄ and KH₂PO₄. One control was also run without any phosphorus source. The phosphorus sources were added in the medium singly in place of KH₂PO₄ so as to maintain the concentration of phosphorus the same as in the basal medium. The flasks after inoculation were inoculated and incubated at the respective optimum temperature for 15 days. The mycelia mats were harvested and dried at 60°C for 48 h to obtain their dry weight.

3.4 Hyphae and Spores Staining of Sebacinales

For the purpose of staining of Sebacinales (P. indica and S. vermisfera), hyphae grown in Kafer media were taken on glass microscopic slide and thereafter stained with 0.5% Trypan blue (Appendix-II). This was immediately followed by destaining with lactophenol (Appendix-II) and observed under Leica microscope (Model, 020-518.500, Germany).
3.4.1 Immuno-Fluorescence (Meyberg, 1988)

(i) Reagents: 3.7% paraformaldehyde, filtered through an 0.4μm Millipore filter and mixed with an equal amount of double strength buffer, washing buffer 1: PBS containing (Appendix-II), 100 mM glycine, permeabilizing buffer: 0.1% Triton X-100 in PBS, washing buffer 2: PBST, blocking buffer: 1% BSA, PBS buffer pH 7.4

(ii) Procedure: (a) Cell Culture: Cover glasses were immersed in 50% H₂SO₄ for 1h using a porcelain rack (Thomas Scientific). Cover glasses were washed for 1h in running tap water, rinsed three times in distilled H₂O and sterilized under UV light for 4 h. *P. indica* was grown in culture dishes with the cover glasses for 48 h. Culture media was drained-off and cover slips were rinsed with PBS.

(b) Fixation: Fixation was done in 3.7% para-formaldehyde in PBS for 15 min at room temperature and washed three times for 5 min each with PBS buffer containing 100mM glycine. The cells were permeabilized with 0.1% Triton X-100 in PBS for 4 min and rinsed with PBS again. The sample was incubated in 1% BSA in PBS buffer (pH 7.4) for 30 min to block unspecific binding and again washed with PBST, 3 times for 10 min each time. The samples were further incubated with primary antibody diluted 1:100 in 1% BSA solution in PBS buffer, pH 7.4 for 60 min and again washed with PBST, 3 times for 10 min each time. Incubated with FITC (Sigma Aldrich, F-0382), conjugated 2nd antibody developed in goat diluted 1:100 in 1% BSA in PBS, pH 7.4, 60 min at 37°C followed by washing with PBST, 3 times for 10 min each time. Samples were mounted in PPD-mounting medium (or 90% glycerol) and observed under fluorescence microscope (Olympus, model, FV-300).

3.5 Growth of Rhizobacteria

*Pseudomonas fluorescence* and *Azotobacter chroococcum* were inoculated on King’s B and Nutrient agar medium respectively under aseptic conditions (Appendix-I). *P. fluorescence* produced pigments on this medium.
3.6 Interaction of *P. indica* and Rhizobacteria

3.6.1 Preparation of Inoculum of *P. indica* and Rhizobacteria on Kafer Medium

*P. indica* inocula were spread uniformly on the surface of Kafer agar medium and incubated in dark at 28±2°C for 4 days. One loop full of rhizobacteria suspension was streaked on Kafer agar plates. The suspensions were spread uniformly on agar surface by rotation with an L-shaped glass rod. These were incubated at 28±2°C for 2 days.

3.6.2 Effect of Rhizobacterial Culture on the Growth of *P. indica*

For this purpose, rhizobacteria *Pseudomonas* and *Azotobacter*, individually as well as in combination, were co-cultivated with the fungus *P. indica* on Kafer medium. A circular disc (7 mm diameter) of mother culture with spores and hyphae of *P. indica* was placed centrally on agar plate. At the three sides of this Petri plate, above rhizobacteria were streaked at distance of 2-3 cm. Incubation was done at 28±2°C and observations on growth pattern of *P. indica* at regular intervals of one week.

3.6.3 Effect of Rhizobacterial Culture Filtrate on the Growth of *P. indica*

(i) Kafer Solid Medium: *P. indica* colonies were cultivated on Kafer agar medium in three different Petri plates in for 4 days at 28 ± 2°C in dark. One Petri plate was kept as controlled, one for co-cultivation with *Pseudomonas* filtrate and other one for *Azotobacter* filtrate. When small colonies of *P. indica* started appearing, one loop full of respective rhizobacterial species was streaked on the surface of fungus colonies in different plates. Incubation in the three Petri plates continued for further 3 days under the same conditions.

(ii) Kafer Broth Medium: The given bacterial culture filtrates were taken after 24 h on incubation of bacteria. They were centrifuged at 2000 rpm for 30 min under aseptic conditions and then culture filtrate was obtained *P. indica* inoculum agar discs were transferred into 25 ml of sterilized Kafer broth medium in each of the three Erlenmeyer conical flasks. After 7 days of the incubation, the fungus cultures were treated with 25 ml bacterial culture filtrate. The incubation was continued for
the next 5 days on Emenvve rotary shaker at constant speed (120 rpm) and temperature (28 ± 2°C) in dark.

3.7 Characterization of *Pseudomonas fluorescence*

3.7.1 Antimicrobial Activity

10g of soil collected from a garden at JNU, New Delhi, was mixed with 90 ml sterile distilled water in Erlenmeyer conical flask. Shaken well and dilutions were made upto $10^{-2}$. 1 ml of aliquot was taken from $10^{-2}$ dilution and pour plated on Trypton yeast extract agar medium in Petri plate (Appendix-I). The plates were incubated at 30°C for 48 h for microbial growth. The bacterial colonies developed were further spread on the same medium and the *Ps. fluorescence* was inoculated at various places of the plates. The plates were incubated at 30°C for 48 h and observations were recorded and photographed.

3.7.2 HCN Production (Bakker and Schipper, 1987)

Culture of *Ps. fluorescence* was streaked onto King’ B medium (Appendix-I). Whatman No.1 filter paper disc (9 cm diameter) soaked in 0.5% (w/v) picric acid in 2% (w/v) sodium carbonate solution was placed on the lid of each Petri plate. Plates were sealed with parafilm and incubated at 28°C for 4 days. Change of color of disc from deep yellow to orange and finally to orange brown to dark brown indicated the production of HCN.

3.7.3 Production of Ammonia (Dye, 1962)

*Ps. fluorescence* was grown in peptone water (Appendix-I) in test tubes which were incubated at 30°C for 4 days. Thereafter, 1 ml of Nessler's reagent was added to each test tube. Presence of faint yellow colour indicated the release/production of small amounts of ammonia and deep yellow to brownish colour indicated the production of high concentration of ammonia.

3.8 Interaction of *P. indica* with Pathogenic Fungi

The pathogenic fungi used during the study were *Magnaphorthe* sp, *Alternaria solani* and *Dreschiera sorokiniana*. Mother cultures of both the pathogenic fungi and *P. indica* were maintained on modified Kafer medium (Hills and Käfer 2001;
Pham et al. 2004 b) (Appendix-I). The cultures of *P. indica* and each of three pathogenic fungi used in the present study were grown in separate Petri plates as control. Then each of the pathogenic fungi was separately grown with *P. indica* in the same Petri plate. For the growth of the culture on agar medium, a fungal inoculum (7 mm diameter) was placed on the surface of the modified Kafer medium. The Petri plates were incubated at 28 ±2°C for 7 days in dark.

**3.9 Study of Plants-Microbes Interactions**

**3.9.1 Host Plants** (Source of seeds and plant)
Certified seeds of *Vigna radiata* L. (Pusa 9531) and *Zea mays* L. var white were procured from Indian Agriculture Research Institute, New Delhi. *Bacopa monniera* plant was collected from School of Life Sciences, Jawaharlal Nehru University, New Delhi. These plants were collected with purpose to investigate their interaction with fungus *P. indica* and rhizobacteria as given in Table 5 (above).

**3.9.2 Seed Surface Sterilization and Pre-germination**
The seed of test plants were soaked in sterile water overnight and surface sterilized by washing with 90% ethanol for a few seconds followed by 4% (v/v) NaOCl for 15 min. The seeds were further washed five times with sterile distilled water and rinsed with 70% (v/v) ethanol for 30 sec. This was followed by a quick treatment with 15% (v/v) NaOCl. Chemicals adhered were removed by repeated rinsing with sterile distilled water (Gamborg and Phillips, 1996). Surface sterile seeds were pre-germinated either on moistened germinating paper or on water agar plates (0.8%). The germinated seedlings were placed in the soil sand mixture hole (Varma and Schuepp, 1995).

**3.9.3 Growth Conditions in Culture Room**
The seedlings were transferred to culture bottles containing *P. indica* inoculated MMN_/10 and MS media (Appendix-I) after 3-4 days of the appearance of the plumule and radicle. Plants alone (control) or in co-culture with *P. indica* were allowed to grow for 10 days in culture room maintaining 16 h photoperiod, 1000 Lux light at 24±2°C temperature. Root infections were checked regularly using optical device.
3.9.4 Placement of the Fungus
As a regular practice, the explant was allowed to establish into the medium and the initiation of rooting system was observed. This practice helps to overcome "transfer shock". Fungus plug was cut-off to about 7 mm in diameter using back of the sterile Pasteur pipette. Plug was picked up with the support of platinum spatula and placed on the surface of the solidified medium in which the explant is growing. Initial distance between the partners (plant and P. indica) was normally kept about 2 cm apart.

3.9.5 Micropropagation of Bacopa monniera
Micropropagated plantlets of B. monniera were raised from the young shoot on Murashige and Skoog (MS) (1962) medium. 20 ml of medium was filled in each culture test tube and autoclaved at 15 lbs/inch² for 20 min. Raised B. monniera plants were cut into small pieces containing at least two node and two internodes parts of stem and placed on to the medium. The culture tubes were incubated in culture room under controlled humidity (60%), temperature (24±2°C) and light conditions (1000 lux; 16 h light and 8 h dark). After one month the plants were taken out for pot culture experiment in green house was maintained at 26±2°C with 70% humidity.

3.9.5.1 Scanning Electron Microscopy (SEM) of P. indica and Infected Root of B. monniera
(i) Reagent: 0.5% saline solution (NaCl); 0.1 M Phosphate buffer, pH 7.2; 2.5% glutaraldehyde in 0.1 M Phosphate buffer; acetone series 30-95 and 100%, respectively, dry acetone.
(ii) Procedure: Cleared mycelia and tissue cultured Bacopa root samples were washed 3-4 times with saline solution. Fixed in 2.5% glutaraldehyde for 2 h at 4°C, followed by washing three times with buffer, each for 2 h. Samples were dehydrated in acetone series 30 to 95% each for 30 min at 4°C and finally, dehydrated by dry acetone (100%) with 2 changes each for 1 h duration, once at 4°C and another at room temperature. Samples were dried in liquid CO₂ in critical
point drying apparatus and mounted on SEM stubs with silver paint and coated with metal (Au) approximately at 400Å (40 nm) by the sputter coater (Balzers). Specimens were examined in Philips SEM-501 B at 15 K Volts. SEM screening was done using the facilities of Indian Institute of Chemical Technology (IICT), Hyderabad and All India Institute of Medical Sciences (AIIMS), New Delhi.

3.9.5.2 Transmission Electron Microscopy (TEM) of *P. indica* Infected Root of *B. monniera*

Samples were fixed in modified Karnovsky's Fluid buffered with 0.1 M Sodium Phosphate buffer at pH 7.4. Fixation was done for 10-18 h at 4°C, after which the tissues were washed in fresh buffer, and post-fixed for two hours in 1% Osmium tetroxide in the same buffer at 4°C. After several washings in 0.1 M Sodium Phosphate buffer, the specimens were dehydrated in graded acetone solutions and embedded in CY 212 araldite. Ultrathin sections of 60-80 nm thickness were cut using an ultacut E (Reichert Jung) ultramicrotome and the sections were stained in alcoholic uranyl acetate for 10 min. and lead citrate for 10 min before examining the grids under a TEM (Philips, CM-10) operated at 60-80 kv (David *et al.*, 1973). TEM was done using the facilities at AIIMS, New Delhi.

3.9.5.3 Interaction of *P. indica* with *B. monniera* and Their Anti-oxidant Analysis

Shoot of *Bacopa* plant materials were dried at room temperature and powered coarsely. The powder material was macerated with petroleum ether to remove the fatty substances; the mass was further extracted from 50% aqueous alcohol for 3 day and filtered. The extract was concentrated under reduced pressure and lyophilized (Labconco, USA.) to get dry residue.

**Photochemiluminescence Assay**

(i) Methods: For the determination of the integral antioxidative capacity (AC) of the water soluble substances in *B. monniera* extract, the method of photochemiluminescence (PCL) was used. A new method for the application of the photochemiluminescence for the estimation of the radical scavenging activity of the plant extracts has been developed. This method is quick, sensitive and accurate
and may serve the purpose of biomarker in the standardization of the plant extracts and
their formulations.

(ii) Apparatus: Photochem® (Analytic Jena AG, Germany)

(iii) Standard kit: ACW (Analitik jena AG) - where the luminol plays a double
role of photosensitizer as well as the radical detecting agent.

The total antioxidative capacity of the water soluble compounds (ACW)
were measured by chemiluminescence assay using Photochem®. The extracts
(1 mg/ml in water) were mixed with reagent containing luminol. The antioxidants
were quantified by their inhibitory effects on luminescence generation by
comparison with ascorbic acid used as standard and the antioxidative capacity was
calculated as equivalents units of ascorbic acid (from the calibration curve of
ascorbic acid) and the results were expressed as nmoles ascorbic acid/g
equivalents.

(iv) Calculation: Photochem® apparatus and method allowed precise as well as
time and cost effective determination of the integral antioxidative capacity of the
substance. Free radicals are generated in the instrument by means of
photosensitizer. The free radicals thus generated were detected by their reaction
with a chemiluminogenic substance. Luminol acts both as photosensitizer as well
as the detecting reagent. In the presence of radical scavengers in the extract the
intensity of the PCL was attenuated as a function of concentration. In this way the
antioxidative capacity of the extract could be quantified.

The value is derived through the software automatically, PCL software and
is based on the calibration curve.

3.9.5.4 Biophysical Phenomies of P. indica with Bacopa Plants
Chl a fluorescence transients exhibited by any photosynthetic material are
measured by a plant efficiency analyzer (PEA) or Handy-PEA fluorimeter
(Hansatech Instruments, King’s Lynn, UK). The Handy-PEA fluorimeter used for
studies and the Biolyzer program to analyze the results
(http://www.unige.ch/sciences/biologie/bioen/bioindex.html).
3.9.6 Growth Conditions in Glass House

The plant-microbe interactions experiments were repeated in environmentally controlled glass house using sterile soil: sand mixture (3:1). The mixture was sterilized and filled in pre-washed and sterile plastic pots (1 kg). Small and large colonies of *P. indica* giving an appearance of "corals" were washed to remove the adhering chemicals. Inoculum (1% w/v) was thoroughly mixed with soil/sand mixture and pre-germinated sterile seedlings were sowed in each pot (4 seeds/pot). Efforts were made that the root system remained in direct contact with fungal inoculum. The plants green house conditions were: 25±2°C, 16 h light/8 h dark with light intensity 1000 Lux and relative humidity 70%. Plants were weekly supplied with 1/10 diluted Hoagland solution (Appendix-I) with supply of phosphorus and phosphorus deficient nutrients solution on alternate weeks. The plants were irrigated with tap water on alternate days to maintain about 70% soil moisture.

3.9.7 Leonard Jar Experiment: Mungbean-Microbe Interaction

(i) Materials: Leonard bottle-jar assembly, washed river sand, CaCO₃, lighting assemblies, seedling nutrient solution (1/5 strength), mungbean seed, test species of *Bradyrhizobium* and *P. indica*, wick (absorbent cotton), glass cover (removed after seedling emergence), water resistant paper bags (to cover assembly during sterilization, and to cover junction of bottle and jar).

(ii) Procedure: The top half of the unit consists of a bottle (round beer or spirits 800ml capacity), bottom of which were cut out to provide a level flat ground finish (Amber bottles are used as they protect the roots from lights). The lower half (the reservoir) consists of a jar of such dimensions that the inverted bottle sat snugly on its rim and the neck of the bottle comes to within 2-4 cms of the bottom of jar. A wick was provided to help the capillary rise of moisture from the reservoir to the top of the growth vessel. Absorbent cotton wool was used for the purpose. The wick was secured in the neck with a wad of cotton wool. Added moderately well washed coarse river sand, mixed with 1g/kg CaCO₃, to the bottle units to within 5cm of the top. The wick was kept roughly centrally and had reached practically to the surface. Seedling nutrient solution was prepared and diluted to 1/5th to moisten
the jar from the top until liquid reached to drain into the reservoir. Reservoir was filled with the same nutrient solution to within 2 cm range of the junction of the two vessels. Top vessels was covered with a Petri plate half, and the whole unit with moisture-proof paper was secured with rubber bands. Placed dry sterilize fine gravel or chips (about 4 mm) to cover the same in the units to a depth of 2-3 cm. Autoclaved whole units at 120.9°C and 15 lbs/inch² for two hours and kept covering intact until they are brought into use. Sterilized and prepared the seeds in the usual way (as in water agar Petri plate). Covering paper of bottle was removed leaving junction of bottle and reservoir protected. Seed were planted, with or without prior inoculation (according to purpose), just below the surface of the moist sand aseptically. Replaced the Petri plate half to protect against contamination and maintain the surface in moist conditions. The assembly was assembled in place favourable for germination and growth, i.e. under lighting assembly with overhead illumination. As soon as plants were established, and before their development was restricted by Petri plate, cover the sand with the dry gravel to depth of 2-3 cm. Inoculation was conveniently carried out immediately prior to this stage if it was not already done. The units were now left open. Uninoculated and nitrate (0.05/KNO₃ (w/v) controls were also put in the usual way. Units were arranged compactly with overhead illumination.

(iii) Maintenance: The diluted nutrient solution in the reservoir (Jar) is likely to need to be supplemented once or twice during the experiment, according to the nature of the plant, the amount of growth and conditions generally. This was done, with usual bacteriological precautions by separating the two containers sufficiently for the reservoirs to be filled.

3.9.8 Plants-Microbes Interactions and Plant Growth Parameters

(i) Aerial Length: The height of the plants was measured in cm at regular interval of 15 days using a thread along with the plant and then the thread was measured with the help of scale. Observations were recorded in three independent replicates.

(ii) Aerial Biomass: Each endophyte treated plant was replicated three times in pots. They were quantified for fresh and dry biomass for three times at interval of
15 days. Plants were wiped with tissue paper and air-dried for fresh weight. Later, they were dried at 80°C for 12 h in air circulation memmert type oven. Samples were desiccated at room temperature before weighing on a Mettler balance (AE 160). The process was repeated until constant weight was achieved.

(iii) Underground Length: Underground parts were thoroughly washed under running tap water to remove the adhering soil particles. The length of the underground parts was measured in three independent replicates with the help of a thin thread.

(iv) Underground Biomass: After repeated washing, the free surface water on the underground plant parts was blotted out with filter paper, then air-dried and weighed for fresh weight on a Mettler balance (model AE 160).

(v) Endophyte Dependency (ED): Endophyte dependency was determined using the formula given by Gerdemann, (1975) and modified by Plenchette et al. (1983). This gave a per cent increase of yield relative to that of without mycorrhizal plants.

\[
ED = \frac{\text{Parameter with mycorrhiza} - \text{Parameter without mycorrhiza}}{\text{Parameter with mycorrhiza}} \times 100
\]

ED was used instead of mycorrhizal dependency (MD) to designate endophyte dependency.

(vi) Percent Colonization of Plant Roots: *P. indica* and *S. vermicifera* sensu inoculum was added at the rate of 1% (w/w). Plants with an equal amount of autoclaved inoculum served as control. Randomly selected roots were thoroughly washed under running tap water. Segments were treated with 10% KOH at 60°C for 10 min followed by neutralization with 1N HCl and stained with Trypan blue 0.5% (Phillips and Hayman, 1970). Per cent colonization was calculated for the inoculated plants using the following formulae (McGonigle et al., 1990).

\[
\text{Percent colonization} = \frac{\text{No. of colonized root segments}}{\text{Total number of segments}} \times 100
\]
(vii) **Histological Analysis** (Dickson *et al.*, 1998; Phillips and Hayman, 1970)

**Reagents used:** Lactophenol (Appendix-II), 0.5 % Trypan blue (Appendix-II), 10 % KOH, 1N HCl

**Procedure:** The roots were thoroughly washed with tap water to remove the adhering soil particles. They were cut in small segments (1.0 cm approximately) and were heated in 10% KOH for 15 min. After washing with water, the root segments were neutralized with 1N HCl. They were stained with 0.5% Trypan blue overnight or at 60°C for 1 h and mounted on lactophenol for observation under light microscope (Leica Microscope, Type 020-518.500, Germany).

### 3.10 Molecular Studies

#### 3.10.1 Protein Profiling of Plant Roots and Symbiotic Fungi

(i) **Protein Extraction From Plant Roots** (Rosendahl, 1994; Pareek *et al.*, 1995)

Thoroughly washed and surface cleaned fresh roots were macerated by grinding in an ice-chilled mortar and pestle with cold extraction buffer (1 ml/g roots) (Appendix-II). The slurry was transferred to an Eppendorf tube and centrifuged at 12,000 rpm for 30 min at 4°C (Heraeus Sepatech Biofuge). To 300 μl of the supernatant 300 μl of chilled 10 % (w/v) TCA was added and centrifuged at 10,000 rpm for 20 min at 4°C. Repeated washings were performed with di-ethyl ether for several times to remove TCA. 50 μl of the sample buffer was added and vortexed to dissolve the pellet. The samples were stored at −80°C till use. Extracted proteins were used for the SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) to observe the difference in protein profiles of host roots colonized with *P. indica* and *S. vermicola* sensu (Laemmli, 1970).

(ii) **Estimation** (Bradford, 1976)

**Reagents:** Bradford reagent (Appendix-II), 80 mM PBS, pH 7.4 (Appendix-II)

**Procedure:** One ml of Bradford reagent was added to 20 μl of the test sample and 80 μl of 0.15 M NaCl and incubated for 10 min at room temperature. The absorbance was read at 595 nm (Spectrophotometer, Shimadzu, model 160A, Japan). A zero time control was taken to calibrate the spectrophotometer to zero.
The protein content was measured from a standard curve of BSA at concentrations 100 to 1000 μg/ml.

(iii) Protein Extraction From Sebacinales

**Reagents:** Extraction buffer pH 8.0 (Appendix-II)

**Procedure:** The mycelia of test organisms were harvested and washed twice in extraction buffer. The sample was homogenized in liquid nitrogen with the help of sterile mortar and pestle followed by sonication three times at 18 KHz for 30 sec with 1 min multiple interruptions to avoid heating of the sample. The entire operation was performed on ice. Then, the homogenate was centrifuged at 12,000 rpm (25000 g) for 20 min at 4°C (Plattner et al., 1999). Supernatant served as crude protein extract. The protein concentration was estimated by Bradford method as described above (para. 3.10.1 (ii)).

(iv) SDS-PAGE Analysis (Laemmli, 1970; Walker, 1994)

**Reagents:** As described in Appendix-II.

**Procedure:**

a. **Separating gel:** Separating gel was prepared using 10% polyacrylamide gel solution.

b. **Stacking gel:** After polymerization of the separating gel, 4% stacking gel solution was poured onto it. The comb was fitted and the solution was allowed to polymerize.

c. **Sample application:** 40 μl each of the test sample (containing 1 μl protein/μl) was mixed with 10 μl of 5X sample buffer and heated at 95°C for 5 min. The contents were loaded in the wells with the help of gel loading tips. In another set of experiment, *P. indica* proteins were run in a gel using a continuous well comb. The running conditions were similar in both the experiments, as described below.

d. **Electrophoretic conditions:** The proteins were stacked at a constant voltage of 70 V for 1 h and were separated at 100-120 V until the dye front reaches the bottom of the plate at 1 cm above the edge. The run was for total 5 h at room temperature (mini gel). The gel was stained with the Coomassie brilliant blue dye for 30 min to see the protein profile.