CHAPTER 3

MATERIALS AND METHODS
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3.1 Isolation of *Bacillus* spp. and fluorescent *Pseudomonas* spp. from the rhizospheric soil and roots of cocoa

3.1.1 Collection of samples

Plots having adult bearing cocoa trees were selected from the four states, Kerala, Karnataka, Tamil Nadu and Andra Pradesh, where cocoa cultivation is taken up on a large scale under different agroclimatic conditions. The cocoa trees were grown as mixed crops in coconut and arecanut gardens. Rhizosphere soil and root samples of adult cocoa trees were collected from two districts of Tamil Nadu (Coimbatore & Pollachi), two from Karnataka (Tumkur and Dakshina Kannada), three districts from Kerala (Kasaragod, Kozhikode and Wayanad) and one district of Andhra Pradesh (East Godavari). The soil sample was collected from the base of cocoa tree at a distance of 0.5 M from the trunk after removing the top litter layer along with the roots and placed in sterile polythene bags. For each sampling, the soil was taken from three spots within the rhizosphere of the same tree and mixed. From each field site, 5 to 7 composite samples of rhizosphere soil (roots and adhering soil) were collected. Observations on variety, age, yield of the crops, cropping system, and nutrient inputs were taken. Samples were immediately brought to the laboratory in a clean polythene bag and pH of the soil samples were tested using pH meter (Eutech, Singapore). Samples were refrigerated at 4°C until analysis. The composition of reagents, different media and buffers used in this study are given in Appendix I. Details of sample collection are given in Appendix II and Fig.1.

![Map of South India showing locations of sample collection](image-url)

Fig. 1. Map of South India showing locations of sample collection
3.1.2 **Isolation of rhizospheric fluorescent *Pseudomonas* spp.**

Ten gram of rhizosphere soil from each sample was transferred to 90 ml of sterile water and shaken in a refrigerated incubator shaker (Innova 4335; USA) for 20 minutes at 240 rpm at 30°C. Immediately after shaking a series of ten-fold dilutions of the suspension were made for each sample. 0.1 ml of the $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilutions were spread plated on King’s B Agar (KBA) and S2 for the isolation of fluorescent pseudomonads. Petri dishes were placed in an incubator at 30°C for 24 to 48 hours. After incubation, fluorescence was observed under UV (UV trans-illuminator, Genei, India). Fluorescent colonies were enumerated and morphologically different colonies were selected from each sample and further cultured to establish pure cultures. Pure cultures were maintained in KBA slants at 4°C for further studies. Glycerol stock of each isolate was prepared in KB supplemented with 15% glycerol.

3.1.3 **Isolation of rhizospheric *Bacillus* spp.**

For isolation of endospore forming *Bacillus* spp., the $10^{-1}$ dilutions of the soil samples were heated in water bath at 80°C for 20 minutes. Dilutions (up to $10^{-5}$) were prepared, spread plating was done on nutrient agar and incubated at 30°C for 48 to 96 hours. Enumeration was done and morphologically different colonies were selected for purification. Pure cultures were maintained on NA slants at 4°C for further studies. Glycerol stock of each isolate was prepared in NB supplemented with 15% glycerol.

3.1.4 **Isolation of fluorescent *Pseudomonas* spp. from roots**

Ten gram of cocoa root samples were surface sterilized using 0.1% HgCl$_2$ for 1 minute. Excess HgCl$_2$ was removed by washing in sterile water and again kept in 95% ethanol for one minute. This procedure was found optimal for removing the entire external microflora. The sterilized roots bits were ground in sterile mortar and pestle and added to 90 ml sterile water blank to obtain $10^{-3}$ dilution and shaken for 20 minutes at 30°C. Fluorescent pseudomonads were isolated as described in section 3.1.2. Sterility checks were also included to monitor the efficiency of the disinfection procedure for each sample by transferring one milliliter of the final washing to 9 ml nutrient broth. Samples were discarded if growth was detected in the nutrient broth after 24 hours of incubation.
3.1.5 Isolation of *Bacillus* spp. from roots

The sample diluted to $10^{-1}$ from the above procedure (section 3.1.4) was heated at 80°C for 20 minutes for isolating endospore forming *Bacillus* spp. (section 3.1.3).

3.2 *In vitro* characterization tests for analyzing functional diversity and selection of efficient isolates

3.2.1 *In vitro* characterization tests for analyzing functional diversity

All the isolates were characterized for beneficial phenotypical/functional traits, which directly or indirectly help the plant growth.

3.2.1.1 Growth on nitrogen free medium

The ability of isolates to grow on nitrogen free medium was determined by streak plate method. Isolates streaked on Jensen’s agar (Jensen, 1942) were incubated at 30°C for 4-7 days. Isolates which were able to form glistening colonies in the N-free media were designated as positive.

3.2.1.2 Phosphate solubilization

Isolates were spotted on Pikovskaya’s agar (Pikovskaya, 1948) and incubated at 30°C for 3 days. Phosphate solubilizing bacteria will grow and form a clear zone around the colony. Presence or absence of clearing zone was recorded and zone sizes were measured.

3.2.1.3 Ammonia production

Production of ammonia was detected according to Cappuccino and Sherman (1992). Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48 to 72 hours at 30°C. 30 µl of each sample was placed in cavity slides and development of brown to yellow colour in the presence of Nessler’s reagent (Merck) was taken as positive test for ammonia production. The intensity of colour and precipitation was recorded as Low (L), Medium (M) and High (H).
3.2.1.4 Indole acetic acid (IAA) production

Production of IAA was detected based on the method of Brick et al. (1991). Isolates were spot inoculated in the centre of the Luria Bertani (LB) plate. Sterile Whatman No.1 filter paper was placed on the inoculated plate. After 3 days of incubation, the filter paper was transferred to another petriplate containing saturated filter paper dipped in Salkowsky-reagent. Development of a pink colour within 30 minutes on filter paper was considered as positive result.

3.2.1.5 ACC deaminase production

1-Aminocyclopropane carboxylic acid (ACC) deaminase production was detected in DF salts minimal medium (Dworkin and Foster, 1958) implemented with 3 mM ACC as the sole source of nitrogen. The medium was poured and plates were spot inoculated with isolates and incubated at 30°C for 24 hours. Ability to grow on this medium was taken as positive (Klee et al., 1991). According to the plenty of growth, results were recorded as Low (L), Medium (M) and High (H).

3.2.1.6 Siderophore production

The Chrome azurol S (CAS) assay of Schwyn and Neilands (1987) was followed for the detection of siderophores. CAS agar plates were spot inoculated with the isolates to be tested and incubated at 30°C for 24 to 48 hours. Presence of orange halo around the colonies was recorded and halo size was measured.

3.2.1.7 HCN production

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Bakker and Schippers (1987). Briefly, King’s B agar (KBA) amended with 4.4 g glycine/l was streaked with the isolates to be tested. Whatman No.1 filter paper soaked in cyanide detection solution was placed on the lid of the plate. Plates were sealed with parafilm and incubated at 30°C for 7 days. Development of orange to red colour indicated HCN production.

3.2.1.8 Chitinase production

Chitinase activity was tested as given by Renwick et al. (1991) in a defined medium. Isolates were spotted on minimal medium containing colloidal chitin and
incubated at 30°C for 3 days. Presence of the clearing zone was measured and recorded.

### 3.2.1.9 Antibiotics production

The capability of isolates to produce antibiotics was detected by agar well technique (Fuhrmann, 1994). Isolates were grown in NB/KB for 24 hours and the culture supernatant were collected. Supernatant of the culture was added to the wells made on Trypticase Soy Agar (TSA), which already had been spread plated with appropriate soil dilution (1:10). Plates were incubated for 24 hour. Sterile water served as control. Presence of inhibition zone around the well was observed.

### 3.2.2 Selection of efficient isolates

#### 3.2.2.1 Assessment of isolates

All the isolates were assessed and scored in a scale of 1 to 3 based on their performance in *in vitro* characterization studies. Maximum 3 points were given to HCN production, growth on N-free medium, high ammonifiers and good growth on minimal media containing ACC. Antibiotic producers were assessed based on the inhibition zone size, siderophore producers based on the diameter of orange halo, and phosphate solubilizers and chitin degrading isolates based on the size of clearing zones in a scale of 1 to 3.

#### 3.2.2.2 Seedling bioassay (PGC assay)

Isolates were tested on short duration test crop cowpea (Kairali variety), for determining their plant growth promotion potential. Cowpea seeds were surface sterilized by 0.1% HgCl₂ for 4 minutes and washed with sterile distilled water five times. Surface sterilized seeds were allowed to dry in airflow for a few minutes. Bacterization of the seeds was achieved by soaking seeds in the broth for 10 minutes. Seeds treated with sterile broth served as control. Seeds were aseptically transferred to petriplates with soft water agar (0.7% agar in water). Three replications were maintained for each treatment. Seedling bioassays were done in a versatile Plant Growth Chamber (PGC) (Sanyo, Japan) at 25°C, 85% RH, 10 hours light (5000 Lx/40 µmol.m⁻².s⁻¹ PPFD) and 14 hours dark cycle for seven days. Shoot and root length were recorded.
3.2.2.3 Green house studies

Isolates were evaluated for their plant growth promotion potential on cowpea seedlings in green house. Surface sterilized cowpea seeds were sown in plastic cups (four seeds /cup) filled with unsterile soil: sand mixture in 3:1 ratio. Isolates having cell density of $10^8$ cfu ml$^{-1}$ used as inoculum. After germination, seedlings were thinned to 2 per cup. Plants were kept under natural photoperiod (16 hours light/8 hours dark) in the greenhouse. Control was maintained by placing sterile seeds without culture. Twenty days after inoculation, seedlings were uprooted and shoot (SL) and root lengths (RL), fresh (FW) and dry weight (DW) were recorded. Data were statistically analyzed using analysis of variance (ANOVA) and the means were compared with Duncan’s Multiple Range Test (DMRT) at $P=0.05$ level using SPSS.

Potent isolates were selected based on the above screening strategies and further studies were concentrated on those isolates.

3.3 Identification of efficient PGPR based on biochemical and molecular techniques

PGPR were identified by conventional methods like morphological, physiological and biochemical methods (Cappuccino and Sherman, 1992) according to Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994), BIOLOG GEN-III microbial identification system (BIOLOG, Hayward, CA, USA) and 16S rRNA sequencing. Their phenotypic and RFLP patterns were also generated.

3.3.1 Identification by conventional methods

3.3.1.1 Morphological methods

Bacterial isolates were examined for colony morphology (on NA/KB agar), cell shape, ability to fluorescence in UV, motility and Gram’s reaction (Gram staining kit, Himedia) as per the standard procedures.

3.3.1.2 Biochemical methods

Biochemical tests conducted for the identification of selected isolates includes:

- Catalase test – Fresh culture of isolates grown in KBA/NA medium were transferred to a glass slide using a sterile loop and 100 µl of 3% H$_2$O$_2$ was
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added. The occurrence of gas bubbles was scored positive for catalase production.

- Voges-Proskauer test - Ten drops of α-naphthol solution was added to the test culture grown in peptone broth and gently shaked. Ten drops of 10% KOH were added and the development of red colour in the broth was taken as positive for the test.

- Gelatin hydrolysis - The gelatin liquefaction ability of the isolates was examined using nutrient-gelatin agar. The test cultures were inoculated in nutrient gelatin tubes and incubated at 30°C for 24 hours. After incubation the test tubes were kept in a refrigerator at 4°C for 30 minutes. The tubes with cultures that remained liquefied were taken as positive.

- Casein hydrolysis - Skim milk agar plates were inoculated with the test culture and incubated at 30°C for 24 to 48 hours. Clear zone around the colony was taken as positive.

- Arginine dihydrolase test - Test tubes with semisolid medium of arginine dihydrolase were stabbed with overnight cultures of the test isolates incubated at 30°C for 24 to 48 hours. Development of a deep purple colour was taken as positive reaction.

- H₂S production - Test cultures were inoculated in peptone water and H₂S test strip was placed between the plug and inner wall of the tube, above the inoculated medium. Cultures were incubated at 30°C for 24 hours. Blackening of the strip was considered as positive reaction.

- Citrate utilization - Simmon’s citrate agar slants were streaked with fresh cultures of the test organisms and incubated at 30°C for 48 hours. The colour change from green to blue was taken as positive reaction.

- Starch hydrolysis - The test cultures were streaked on the starch agar plates. After incubation, the plates were flooded with iodine solution. Formation of clear zone around the colony was taken as positive reaction.

- Nitrate reduction test - Five ml of nitrate broth was inoculated with test culture and incubated at 30°C for 48 hours. After incubation, 10 drops of
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Sulfanilic acid and alpha naphthylamine were added. Development of red color indicated that nitrate had been reduced to nitrite.

- Utilization of carbon sources - The ability of the isolates to utilize different carbon sources (0.5%) *viz.*, glucose, sucrose, sorbitol, mannitol, trehalose, arabinose, xylose, glycerol and meso-inositol were tested in phenol-red broth. The colour change from red to yellow was taken as positive.

3.3.1.3 Physiological tests

- Growth at 4°C - The test organisms were streaked on the King’s B agar and incubated for 24 to 48 hours at 4°C. Observation on growth was recorded at the end of incubation period.

- Growth at 41°C - The test organisms were streaked on the King’s B agar and incubated for 24 to 48 hours at 41°C. Observation on growth was recorded at the end of incubation period.

- Anaerobic growth - Isolates were streaked on nutrient agar and incubated in Anaerobic jar for 48 hours. Observation on growth was recorded at the end of incubation period.

3.3.2 Phenotypic fingerprinting using BIOLOG GEN III microbial identification system

Identification of the isolates was validated and metabolic profile was generated using GEN-III microplates of the BIOLOG system. Cells grown for 24 hours on Biolog universal growth (BUG) agar were collected and processed according to the manufacturer’s instructions (http://www.biolog.com). Cultures were transferred to Inoculating fluid A (IF A/IFB) and inoculum density was adjusted to 98% T using BIOLOG turbidimeter. Using multi channel pipette, cell suspension was inoculated into BIOLOG GEN III Microplates (100 µl/well) containing 96 wells that provides 94 phenotypic tests. Plates were incubated at 33°C for 24 hours. The optical density at 590 nm produced from the reduction of tetrazolium violet in each well was read after 24 hours using a BIOLOG Microplate reader (version 5.1.1). Dendrograms were constructed using the digitized data of C-utilization using NTSYS to determine the percent similarity among isolates.
3.3.3 Identification and characterization by molecular methods

Partial sequencing of the 16S rRNA gene was used for the identification of bacterial isolates. For this, the genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma, USA). 16S rRNA gene was amplified using the universal 16S rRNA gene primers F27/R1492. PCR reaction mixture contained 10X PCR buffer (2.5µl), 2.5mM dNTPs (2 µl), 25mM MgCl2 (1.5 µl), 5U/µl Taq DNA polymerase (0.125 µl) and 20 µM primers F27 and R1492 (1.25 µl each). The PCR reaction conditions included an initial denaturation of 3 minutes at 94°C followed by 30 cycles of 1 minute at 94°C, 30 seconds at 55°C and 45 seconds at 72°C with a final extension of 10 minutes at 72°C. Amplified products were resolved by electrophoresis in 0.8% agarose gels and the expected band of 1.5 kb was observed. PCR products were purified and sequenced (3730 xl 96 Capillary Analyzer, Applied Biosystems). These 16s rRNA gene sequences were used to carry out BLAST with the nr database of NCBI genbank database.

3.3.3.1 Restriction digestion of the amplified 16S rDNA

10 µl of the PCR products were digested with 20 U of each enzyme at 37°C for 6 hours according to the condition recommended by the manufacturer (Fermentas). Restriction endonucleases (Fermentas) used in this study were Bsu (HaeIII),MspI,EcoRI,HinfI and HhaI. The reaction mixture contained 10 µl of the PCR product,18 µl Nuclease free water,2 µl 10 X buffer and 2 µl restriction enzyme. Digested PCR products were electrophoresed through 2% agarose gel containing ethidium bromide for ≈ 2 hours at 100 V using 0.5 X TBE buffer.

3.4 Abiotic stress tolerance studies and quantitative estimation of growth promotion metabolites of selected PGPR

3.4.1 Stress tolerance studies

3.4.1.1 Tolerance to pH

The test isolates were inoculated to King’s B/nutrient broth and incubated at 30°C for 24-48 hours. Trypticase soya broth was (TSB) prepared using citrate buffer, phosphate buffer and Tris-HCl buffer for maintaining pH of 4.2, 5.2, 6.2, 7.2, 8.2 and 9.0 respectively. Trypticase soya broths having different pH were inoculated with
test culture (10⁸ cfu ml⁻¹) and incubated for 3 days. After the incubation time, 1 ml of the culture was centrifuged at 15,000 rpm at 4°C for 15 minutes (Himac CR22G, Hitachi, Japan). Pellet was dissolved in 3 ml of the sterile distilled water and OD was detected at 600 nm (Shimadzu UV 1601).

3.4.1.2 Tolerance to temperature

Fresh culture of the test organisms in their respective medium (KB/NB) were spot inoculated on Trypticase soya agar (TSA) plates and incubated at different temperatures 4°C, 15°C, 30°C, 50°C, 55°C and 60°C. Observation on growth was recorded up to 7 days.

3.4.1.3 Tolerance to different NaCl concentrations

Twenty four hour old cultures of the test organisms were streaked on Trypticase soya agar (TSA) plates having different NaCl concentration (2%, 4%, 6%, 8%, 10% and 12%). Plates were incubated at 30°C and observation on growth was recorded up to 7 days.

3.4.2 Quantitative estimation of growth promotion metabolites of selected PGPR

3.4.2.1 Quantification of IAA

Inoculum was prepared by growing the PGPRs in KB/NB at 30°C for 24 hours. Bacterial suspension (1x10⁸ cfu ml⁻¹) was added to 50 ml of Luria Broth supplemented with 5 mM L⁻¹ Tryptophan and incubated at 30°C in dark for one week. The cultures were centrifuged at 15,000 rpm for 15 minutes at 4°C and the pH of the supernatant was adjusted to 2.8 to 3.0 using 1N HCl in a 250 ml conical flask. Extraction of IAA was done with equal volume of pre-cooled diethyl ether in a separatory funnel at 4°C for two hours with intermittent shaking. The process was repeated twice with 50 ml of diethyl ether. The solvent phase was collected and evaporated to dryness in a rotary vacuum evaporator (Equitron, Evator Rotary Evaporator). 2 ml of methanol was added to the dried material, and kept at -22°C until analysis. The IAA present in the methanol extract was determined by the method of Gorden and Webber (1951). Two ml of Salkowski reagent was added to 1 ml of methanol extract, and incubated in dark for one hour. The intensity of pink
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colour developed was read at 535 nm in Shimadzu UV 1601 spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity of IAA in the culture filtrate was determined and expressed as μg/ml of the broth medium.

3.4.2.2 Estimation of soluble phosphorus

Bacterial suspension having 2 x10⁸ cfu ml⁻¹ was inoculated to 30 ml of Pikovskaya's liquid medium (Pikovskaya, 1948) containing insoluble tri-calcium phosphate (Ca₃(PO₄)₂). All cultures were incubated for a period varying from 24 to 144 hours at 30°C. Three replications were kept for each culture and un-inoculated flasks were kept as control. After each incubation period, the culture was centrifuged at 15,000 rpm for 10 minutes and the supernatant was filtered through sterile 0.2μm membrane (Millipore). Water soluble P in the culture supernatant was estimated by the chlorostannous reduced molybdophosphoric acid blue method as described by Jackson (1973). To 1ml of supernatant, 10 ml of chloromolybdic acid reagent and 1 ml of chlorostannous acid reagent was added and made up to 50 ml as quickly as possible. The blue color intensity of the solution was measured at 600 nm. From a standard curve prepared with known concentrations of KH₂PO₄, the quantity of phosphate in the culture supernatant was determined and expressed as μg per ml of broth medium. The pH of the culture supernatant was measured by pH meter (Eutech, Singapore).

3.4.2.3 β-1, 3 Glucanase activity

β-1, 3 Glucanase activity was estimated as described by Lim et al. (1991). Cultures were inoculated in 10 ml of Laminarin-peptide broth and incubated at 180 rpm at 30°C for 96 hours in a refrigerated incubator shaker (Innova, 4335). The cultures were then centrifuged using a High speed refrigerated centrifuge (Himac CR22G, Hitachi, Japan) at 15,000 rpm at 4 °C for 15 minutes. 250 µl of the supernatant was taken as enzyme source. To the 0.25 ml of enzyme solution, 0.3 ml of 0.1M phosphate buffer (pH 5.5) and 0.5 ml of laminarin (Sigma) (0.2%) was added and the reaction mixture was incubated at 40°C in a water bath for 2 hours. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid (DNS) reagent and the mixture was heated in a boiling water-bath for 10 min, allowed to cool and made up to 10 ml. Reducing sugar equivalents were measured in the solutions by the
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spectrophotometric method of Miller (1959), with glucose as standard at 540 nm in Shimadzu UV 1601. From a standard curve prepared with known concentrations of glucose, and β-1, 3- glucanase activity was determined as µg glucose released per minute per mg of protein.

3.4.2.3.1 Estimation of protein in the culture

Protein content in the same samples was determined as described by Bradford (1976). To 250 µl of 96 hours grown culture, 250 µl of 0.1 N NaOH was added for cell lysis. The sample was kept in a floating boat in boiling water for 10 minutes and transferred to a fresh boiling tube and the volume was made up to 1 ml using 1 N NaOH. Protein content in the sample was estimated by adding 5 ml of Bradford reagent, with bovine serum albumin (BSA) as the standard at 540 nm in Shimadzu UV 1601.

3.4.2.4 Chitinase activity

Chitinase activity was determined according to Lim et al. (1991). Test isolates were inoculated in 30 ml of chitin–peptone medium and incubated at 30°C for 96 hours in a refrigerated incubator shaker (Innova, 4335). The cultures were centrifuged at 15,000 rpm for 20 minutes at 4°C and the supernatant was used as the enzyme source. To 0.25 ml of enzyme solution, 0.3 ml of 1M sodium acetate buffer (pH 5.3) and 0.5 ml of colloidal chitin (0.1%) was added and the reaction mixture was incubated at 50°C for 4 hours in a water bath. The reaction was stopped by adding 1 ml of 3, 5 dinitrosalicylic acid (DNS) reagent and the mixture was heated in a boiling water-bath for 10 minutes. After proper cooling the solution was made up to 10 ml. Chitinase activity was determined by measuring reducing sugar equivalents by the method of Miller (1959) with NAG as standard at 540 nm in Shimadzu UV 1601. One unit (U) of chitinase activity was defined as the amount required for releasing one µg N-acetyl glucosamine from chitin per hour per mg protein. Protein content in all the samples was determined (see section 3.4.2.3.1) as described by Bradford (1976) using bovine serum albumin as the standard at 540 nm in Shimadzu UV 1601.

3.4.2.5 Salicylic acid production

The quantity of salicylic acid (SA) in the culture filtrate was estimated as given by Meyer and Abdallah, (1978). Test isolates were inoculated to 100 ml conical
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Flasks containing 30 ml of succinate broth and incubated at 30°C for 48 hours in refrigerated incubator shaker (Innova, 4335). After incubation the cultures were centrifuged at 10,000 for 5 minutes. Four ml of cell free culture filtrate was acidified with 1 N HCl to pH 2.0 and SA was extracted in CHCl₃ (Merck) (2x2 ml). To the pooled CHCl₃ phases, 4 ml of distilled water and 5 µl of 2M FeCl₃ were added. The absorbance of the purple iron-SA complex, which was developed in the aqueous phase, was read at 527 nm in a Spectrophotometer (Shimadzu UV 1601). From a standard curve prepared with known concentrations of SA, the quantity of SA in the culture filtrate was determined and expressed as µg /ml of broth medium.

3.5 Effects of single and dual inoculations of selected PGPR on cocoa seedlings.

Bacterial isolates were tested for evaluating their plant growth promotion effect on cocoa seedlings in polybags under field conditions. One to two month old healthy seedlings of cocoa (Forastero variety) obtained from CPCRI Regional Station, Vittal, Karnataka were used for the studies.

3.5.1 Polybag studies

3.5.1.1 Single inoculation

The treatments used in single inoculation studies are given below;

Trial No. 1: The experiment consisted of 12 treatments as detailed below;

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>P. putida</em> KDSF 23</td>
</tr>
<tr>
<td>2</td>
<td><em>P. aeruginosa</em> KDSF 7</td>
</tr>
<tr>
<td>3</td>
<td><em>P. putida</em> KZSF 6</td>
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<td><em>P. putida</em> KGSF 20</td>
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<td>5</td>
<td><em>B. subtilis</em> ASB 12</td>
</tr>
<tr>
<td>6</td>
<td><em>B. cereus</em> CSB17</td>
</tr>
<tr>
<td>7</td>
<td><em>B. cereus</em> TSB 15</td>
</tr>
<tr>
<td>8</td>
<td><em>B. megaterium</em> TSB 17</td>
</tr>
<tr>
<td>9</td>
<td><em>B. cereus</em> KDSB 3</td>
</tr>
<tr>
<td>10</td>
<td><em>B. subtilis</em> PEB 2</td>
</tr>
</tbody>
</table>
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11. *B. licheniformis* KGEB 16
12. Control

**Trial No. 2:** The experiment consisted of 11 treatments as detailed below:

<table>
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<th>Sl. No.</th>
<th>Treatments</th>
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<td>1</td>
<td><em>P. putida</em> KDSF 9</td>
</tr>
<tr>
<td>2</td>
<td><em>B. cereus</em> ASB 3</td>
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<tr>
<td>3</td>
<td><em>B. subtilis</em> CSB 8</td>
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<td><em>B. subtilis</em> VEB 4</td>
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<td>7</td>
<td><em>B. subtilis</em> CEB 9</td>
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<td>8</td>
<td><em>B. subtilis</em> PEB 4</td>
</tr>
<tr>
<td>9</td>
<td><em>B. subtilis</em> KGEB 10</td>
</tr>
<tr>
<td>10</td>
<td><em>B. megaterium</em> WEB 6</td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
</tr>
</tbody>
</table>

**3.5.1.2 Dual inoculation**

The experiment consisted of 10 treatments as detailed below:

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<th>Sl. No.</th>
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</tr>
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<tr>
<td>1</td>
<td><em>B. cereus</em> ASB3</td>
</tr>
<tr>
<td>2</td>
<td><em>B. cereus</em> ASB3+ <em>P. putida</em> KDSF23</td>
</tr>
<tr>
<td>3</td>
<td><em>B. cereus</em> ASB3+ <em>B. subtilis</em> VEB4</td>
</tr>
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<td>4</td>
<td><em>P. putida</em> KDSF23</td>
</tr>
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<td>5</td>
<td><em>P. putida</em> KDSF23+ <em>B. licheniformis</em> KGEB16</td>
</tr>
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<td>6</td>
<td><em>B. licheniformis</em> KGEB16</td>
</tr>
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<td>7</td>
<td><em>B. licheniformis</em> KGEB16+ <em>B. cereus</em> ASB3</td>
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<td>8</td>
<td><em>B. subtilis</em> VEB4</td>
</tr>
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<td>9</td>
<td><em>B. subtilis</em> VEB4+ <em>P. putida</em> KDSF23</td>
</tr>
<tr>
<td>10</td>
<td><em>B. subtilis</em> VEB4+ <em>B. licheniformis</em> KGEB16</td>
</tr>
</tbody>
</table>

Bacterial isolates were inoculated in King’s B/ Nutrient broth and incubated for 48-60 hours so as to reach $10^8$ cells ml$^{-1}$. This culture broth was used as inoculum for cocoa seedlings. The composite cultures of PGPRs having equal cell density ($10^8$
cells/ml) were used as inoculum for co-inoculation studies. Cocoa seedlings were planted in polybags containing potting mixture (soil, vermicompost and sand in the ratio 3:1:1 @ 10 Kg Polybag⁻¹). Each seedling was inoculated with 500 ml of culture broth. 500 ml of respective PGPRs were applied as booster dose after third month of planting. 20 replications were maintained for each treatment in a completely randomized design and seedlings with uninoculated broth served as control. All the routine cultural operations like weeding and irrigation (once in 2 days) were carried out. At the end of the experimental period (six months), the cocoa seedlings were uprooted and growth parameters such as number of leaves (NL), roots (NR), number of branches (NB), collar girth (CG), length of shoot (SL) and root (RL), fresh weight of shoot (FWS) and root (FWR) were recorded. Seedlings were dried in an oven at 60 °C till constant dry weight was obtained and then dry weight of shoot (DWS) and root (DWR) was recorded. All the data were subjected to analysis of variance (ANOVA) and the means were compared with Duncan’s Multiple Range Test (DMRT) at P=0.05 level using SPSS. Leaf samples were ground into powder and stored for further analytical use. The colony forming units (cfu) of beneficial microbes like N-fixers, phosphate solubilizers, fluorescent pseudomonads and Bacillus spp. in the rhizosphere soil and roots of cocoa seedlings were enumerated. Data were log transformed and expressed as log cfu ml⁻¹ before subjecting to ANOVA.

3.5.2 Nutrient analysis

Rhizosphere soil samples were taken for the estimation of available phosphorus, potassium and total nitrogen content. Plant samples (leaves) were oven dried and ground in a Wiley mill and used for the estimation of total nitrogen, phosphorus and potassium by standard procedures.

3.5.2.1 Available Phosphorus

Available phosphorus in soil samples were estimated by following Bray and Kurtz method (Bray and Kurtz, 1945). To prepare soil extract, two gram of the dried soil sample was taken in 250 ml conical flask and a small spatula of activated charcoal and 20 ml Bray No. 1 reagent was added, shaken for 1 minute and filtered. Two ml 0.8 M boric acid and 4 ml very freshly prepared mixed reagent was added to 1 ml of the soil extract and made up to 25 ml. Blue colour produced on reduction of the molybdate was determined at 660 nm in Shimadzu UV 1601. From a standard
curve prepared with known concentrations of KH$_2$PO$_4$, the quantity of phosphorus in the extract was determined and expressed as ppm.

\[
\text{Available P (ppm)} = R \times \text{Dilution factor} = R \times \frac{Ve}{Ws} \times \frac{Vm}{Vs}
\]

R- concentration of P (ppm), Ve- volume of extracting solution (ml), Vm-final make up volume (ml), Vs- volume of extract taken for estimation (ml) and Ws-weight of sample

**3.5.2.2 Available Potassium**

Available potassium was determined by using the method of Hanway and Heiden (Hanway and Heiden, 1952). Twenty five ml of 1N ammonium acetate was added to 5 g soil and shaken for 5 minutes. The extract was filtered through Whatman No. 1 filter paper. The potassium content of the filtrate was then determined in flame photometer (Systronics) using KCl as standard. Total ppm was calculated with known concentration of KCl.

\[
\text{Available K (ppm)} = R \times \frac{C1 \times Ve}{C2 \times W}
\]

R-concentration of K (ppm), C1- total concentration of standard (ppm), C2- total reading obtained for standard (ppm), Ve-volume of extract taken for estimation and W-weight of sample (g)

**3.5.2.3 Total Nitrogen**

**3.5.2.3.1 Digestion of sample**

The total nitrogen content in soil and leaf samples were estimated by following the Kjeldahl method (Kjeldhal, 1883). The analysis was done using 500 mg of oven dried finely ground leaf sample and 1 g soil sample. The samples were digested with 10 ml concentrated sulphuric acid in the presence of 1 g catalyst mixture in Kjeldatherm KB/KBL (Gerhardt) at 360°C till the samples became clear in digestion tubes. The tubes rack was carefully removed out of the block-digester and allowed to cool until it reaches room temperature. About 50 ml distilled water was
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added slowly through the sides of the tubes and allowed to cool. Reagent blanks were kept with each batch of samples for digestion (without sample).

3.5.2.3.2 Distillation

Distillation was carried out in an automatic distillation system (Vapodest 20, Gerhardt). N in the form of ammonia was collected in 30 ml of 1% boric acid solution containing mixed indicator having pH 4.5, placed underneath the condenser tip. The distillate was standardized by titrating against 0.02 N H$_2$SO$_4$. The percentage of nitrogen was calculated by recording the volume of acid run down.

\[
\text{Total N} \, (\%) = 0.00028 \times \frac{(V_s - V_b)}{W} \times 100
\]

$V_s$ - titre value of sample (ml), $V_b$ - titre value of blank (ml) and $W$ - weight of sample (g)

3.5.2.4 Total P and K estimation

3.5.2.4.1 Preparation of diacid extract

Total phosphorus and potassium content in soil and leaf samples were analyzed following the method of Jackson (Jackson, 1973). One gram soil/ 0.5 g of the dried plant sample was weighed and taken in a 100 ml standard flask. 9 ml of diacid (concentrated HNO$_3$:HClO$_4$) was added (2:1). Digestion was carried out at 200°C in a hot plate until the solution becomes clear. A blank digestion was run with the reagents added in same amount employed in the test samples. Residues were diluted with distilled water and made up to 100 ml in a volumetric flask and filtered. The filtrate is called diacid extract.

3.5.2.4.2 Total Phosphorous

One ml of diacid extract was pipetted out in a 25 ml volumetric flask. Two ml 0.8 M boric acid and 4 ml very freshly prepared mixed reagent was added and made up to 25 ml. Quantity of phosphorus in the extract was determined as given in section 3.5.2.1

\[
\text{Total P (ppm)} = R \times \text{Dilution factor} = R \times \frac{V_e \times V_m}{W \times V_s}
\]
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R- concentration of P (ppm), Ve- make up volume of diacid extract (ml), Vm-final make up volume (ml), Vs- volume of diacid extract taken for estimation (ml) and W-weight of sample (g)

3.5.2.4.3 Total Potassium

Total potassium content of the diacid extract was determined using flame photometer as given in section 3.5.2.2

\[
\text{Total K (ppm)} = R \times \frac{C_1 \times V_e}{C_2 \times W}
\]

R-concentration of K (ppm), C1- total concentration of standard (ppm), C2- total reading obtained for standard (ppm), Ve-make up volume of diacid extract (ml) and W-weight of sample (g)

3.5.3 Compatibility of efficient PGPR

The compatibility among the selected isolates from the single inoculation studies were tested in vitro according to the procedure given by Reginaldo et al. (2007). Bacterial isolates were inoculated in King’s B, Nutrient broth and incubated for 48-60 hours. Bacterial suspension having OD of 0.2000 at 540 nm was prepared in sterile saline water (0.85%). Sterile paper discs soaked in bacterial suspension and incubated for 10 minutes were allowed to dry and transferred to TSA plates lawn cultured with test isolate and incubated at 30°C for 24-48 hours. Compatibility among the isolates was noted. Presence of inhibition zone was recorded as incompatible.

3.6 Formulation of PGPR

3.6.1 Inoculum preparation

Test isolates were grown in their respective medium (King’B/ NB) for 24 hours in an incubator shaker (Innova 4335; USA) at 30°C and 180 rpm. After incubation, 1 ml of the broth was transferred to 6 x 100 ml of the KB/ NB broth and incubated for 48 hours under shaking condition.
3.6.2 Talc-based formulation of PGPR

The talc-based formulation of the individual bacterial strain was prepared according to Vidhyasekaran and Muthamilan (1995). Five hundred ml of bacterial inoculum was added to 1 kg of the sterile talc powder (Himedia) and mixed well under sterile conditions so as to get $\geq 10^8$ cells ml$^{-1}$. The moisture content was maintained at about 30%. The materials were packed in polypropylene bags, sealed and incubated at room temperature (27± 2°C) for 180 days. Three independent samples were analysed for each culture. Samples were drawn at monthly intervals and the bacterial population in carrier was determined by counting the cfu g$^{-1}$ of powder using the standard dilution plating technique.