3 MATERIALS AND METHODS

3.1 GENERAL

3.1.1 Glass Ware

Glass ware were soaked in cleaning solution (100 g of potassium dichromate dissolved in 100 mL distilled water followed by addition of 500 mL of concentrated sulphuric acid) for 12 h, boiled in soap water for few minutes and washed in tap water. They were thoroughly rinsed in distilled water and dried.

Glass ware used for culture work were sterilized in a hot air oven at 180°C for 4 h.

3.1.2 Chemicals

All the chemicals were of reagent grade quality unless otherwise stated and glass distilled water was used throughout the study.

3.1.3 Maintenance of *Azospirillum*, *Pseudomonas* and *Methylobacterium* reference strains

The reference strains of *Azospirillum* viz., *Azospirillum brasilense* (ATCC-29145), *Azospirillum lipoferum* (ATCC-29707), *Azospirillum halopraeferens* (ATCC-51182) and *Azospirillum amazonense* (ATCC-35119), *Pseudomonas* viz., *Pseudomonas fluorescens* (ATCC-7283), *Pseudomonas putida* (ATCC-12633), *Pseudomonas aeruginosa* (ATCC-10145) and *Pseudomonas stutzeri* (ATCC-14405) and *Methylobacterium* viz., *Methylobacterium phyllosphaerae* (CBMB-27), *Methylobacterium oryzae* (CBMB-20), *Methylobacterium suomiense* (NCIMB-1377) and *Methylobacterium fujisawaense* (NCIMB-12417) were obtained from Microbial Type Culture Collection (MTCC), Gene Bank, Institute of Microbial Technology (IM Tech) Chandigarh, India and used as reference strains.
throughout the study. They were maintained in Nutrient agar slant, King’s “B” agar and Methanol mineral salts (MMS) agar slants, respectively at 35 °C with monthly transfer and used throughout the study.

*Xanthomonas oryzae pv oryzae* (AU 099), obtained from Department of Plant Pathology, Faculty of Agriculture, Annamalai University, was used as reference strain for the biocontrol study and the same was maintained in Peptone sucrose agar (PSA) slants and examined periodically for its virulence.

**3.1.4 Composition of media**

**3.1.4.1 Composition of Nutrient Agar medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

**3.1.4.2 Composition of King’s medium B (KMB) (King’s et al., 1954)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>Protease peptone</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>
3.1.4.3 Composition of Methanol mineral salts medium (MMS) (Green and Bousfield, 1983)

- \( \text{K}_2\text{HPO}_4 \) - 1.20 g
- \( \text{KH}_2\text{PO}_4 \) - 0.62 g
- \( \text{CaCl}_2\cdot6\text{H}_2\text{O} \) - 0.05 g
- \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \) - 0.20 g
- \( \text{NaCl} \) - 0.10 g
- \( \text{FeCl}_3\cdot6\text{H}_2\text{O} \) - 1 mg
- \( (\text{NH}_4)_2\text{SO}_4 \) - 0.5 \( \mu \)g
- \( \text{CuSO}_4\cdot5\text{H}_2\text{O} \) - 5.0 \( \mu \)g
- \( \text{MnSO}_4\cdot5\text{H}_2 \) - 10.0 \( \mu \)g
- \( \text{Na}_2\text{MoO}_4\cdot5\text{H}_2\text{O} \) - 10.0 \( \mu \)g
- \( \text{H}_3\text{BO}_3 \) - 10.0 \( \mu \)g
- \( \text{ZnSO}_4\cdot7\text{H}_2\text{O} \) - 70.0 \( \mu \)g
- \( \text{CoCl}_2\cdot6\text{H}_2\text{O} \) - 5.0 \( \mu \)g
- Methanol - 0.5 per cent
- Agar - 15.0 g
- Distilled water - 1000 mL
- pH - 7.0

3.1.4.4 Composition of Peptone sucrose agar (PSA) medium (Schaad, 1998)

- Peptone - 10 g
- Sucrose - 10 g
- Sodium glutamate - 1.0 g
- Agar - 18 g
Distilled water - 1000 mL
pH - 7.2

3.2 SURVEY FOR THE OCCURRENCE OF AZOSPIRILLUM PSEUDOMONAS AND METHYLOBACTERIUM FROM THE RHIZOSPHERE AND PHYLLOSHERE OF LOWLAND RICE AND GRADING THE ISOLATES ON THE BASIS OF THEIR DINITROGEN FIXING PHYTOHORMONE PRODUCTION AND BIOCONTROL ABILITY

3.2.1 Survey for Azospirillum, Pseudomonas and Methylobacterium occurrence in rice rhizosphere and phyllosphere

The survey was conducted at ten locations in Chidambaram and Kurinjipadi taluks of Cuddalore district, Tamil Nadu, India where rice is a predominant cereal food crop grown under wetland conditions. Random selection of locations was made so that each and every sector of the experimental area would get a representation in the survey. Five locations, from each of two taluks of Cuddalore District, were selected for the survey of Azospirillum, Pseudomonas and Methylobacterium occurrence from the rhizosphere and phyllosphere of lowland rice.

3.2.1.1 Details of locations

The name of the ten locations which were selected for the survey of Azospirillum, Pseudomonas and Methylobacterium occurrence are given in Table - 1 with their respective taluk name.

3.2.1.2 Collection of rice plant sample from each location

In each and every location of the surveyed area, a field which has been under the long rice cultivation was selected for the collection of rice plant
Materials and Methods

3.3 **Enumeration of *Azospirillum* Population from the Rhizosphere of BPT-5804 Rice**

The adhered soil of rice roots, collected from 5 rice plants of a particular location, were pooled and 10 g of soil sample was transferred to 90 ml of sterile distilled water in a 250 mL Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension of each soil sample was subjected to ten fold serial dilution, ranging from $10^{-1}$ to $10^{-9}$ in mineral salts solution of Day and Dobereiner (1976), without malate, as detailed elsewhere in the text. One ml of each dilution was inoculated in a set of five tubes containing 9ml of nitrogen-free semisolid malate medium (Day and Dobereiner, 1976). Atleast three consecutive dilutions were inoculated and were incubated for three days at 30 ± 2°C. Tubes showing sub-surface, thin pellicle were identified as positive tubes for dinitrogen fixing spirilla and were subjected to acetylene reduction Assay (ARA) (> 10 n moles / h / tube of C₂H₄).

The MPN counts of *Azospirillum* were calculated on the basis of positive tubes using the table provided by Cochran (1950) (Annexure – 1).

**3.3.1 Enumeration of *Pseudomonas* Population from the Rhizosphere of BPT-5804 Rice**

The adhered soil of rice roots, collected from five rice plants of a particular location, were pooled and 1 g of soil sample was transferred to
9 mL of sterile water in a 250 mL Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 min. at ambient temperature. The well mixed suspension was then diluted appropriately up to $10^{-7}$ dilution. One mL of suspension from $10^6$ and $10^7$ dilution was aseptically transferred to sterile petriplates and 15-20 mL of selective King’s “B” medium was added. The plates were rotated in clockwise and anticlockwise direction for uniform distribution and incubated at 28 ± 2 °C for 48 hr. Three replications were maintained for each dilution. The colonies which produced fluorescent pigment colonies on King’s B agar plates were counted in a coulter counter. The total number of colonies, in the original sample was expressed as CFU g$^{-1}$ of oven dry soil.

3.3.2 Enumeration of *Methylobacterium* population from the Phyllosphere of BPT-5804 Rice

The representative rice plant samples of a particular location were taken and the entire leaves of the plant were clipped off, cut into 1 cm pieces, pooled and 10 g of the pooled materials were placed into a 100 mL conical flask containing 50 mL of phosphate buffer (pH 6.8). The materials in the conical flask were sonicated for 7 min and then vortexed for 20 s. Serial dilutions of the leaf washing were made using sterile distilled water. One ml sample from these diluted suspensions were removed and transferred aseptically to sterile petridishes and the melted MMS medium was added. The methanol mineral salts (MMS) medium was amended with cycloheximide (100 µg mL$^{-1}$) in order to prevent any fungal growth. The suspension was mixed well with the MMS medium by rotating the petridishes in clockwise and anticlockwise direction and then allowed to solidify. Then, the plates were incubated for 7 days at
28 ± 2 °C and the bacterial colonies were then counted in Coulter counter. The total number of pink colonies in the original sample was expressed as CFU g⁻¹ fresh weight of leaf.

3.3.3 Enumeration of total heterotrophic population from the Rhizosphere and Phyllosphere of BPT-5804 Rice

The enumeration of total heterotrophic population from the rhizosphere and phyllosphere of rice cv. BPT-5804 was carried out on nutrient agar medium as described by Malik et al. (1997).

3.4 Isolation of Azospirillum, Pseudomonas and Methylobacterium from the rhizosphere and phyllosphere of lowland rice

3.4.1 Isolation of Azospirillum from the rhizosphere soil sample of rice

Ten gram of air dried soil sample of rice rhizosphere was transferred to 90 ml sterile distilled water in a 250 ml Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension was then diluted appropriately and 0.1 ml of the suspension from 10⁻⁶ dilution was aseptically transferred into test tubes containing 10 ml of semisolid malate medium (Nfb) (Day and Dobereiner, 1976) and incubated at 35°C for 36 to 58 h to allow sub-surface pellicle formation. After the incubation period, the cotton plugs were replaced with suba seals and 10 per cent of the air was replaced with acetylene gas. Nitrogenase assay (C₂H₂ reduction) was checked and quantified by gas chromatograph (Chemito, India) fitted with Flame Ionisation Detector (FID), as detailed elsewhere in the text. Azospirillum cultures were isolated and enriched from the tubes which showed characteristic pellicle formation and nitrogenase activity on N-free malate agar plates as described by Day and Dobereiner (1976).
3.4.2 Isolation of *Pseudomonas* from the rhizosphere soil sample of rice

Ten gram of air dried rhizosphere soil sample of rice was transferred to 90 mL of distilled water in a 250 mL Erlenmeyer flask and incubated on a rotary shaker (100 rpm) for 30 min at ambient temperature. The well mixed suspension was then diluted appropriately up to $10^{-6}$ dilution. One mL of the suspension from $10^{-6}$ dilution was aseptically transferred to sterile petriplates and 15-20 mL of selective King’s B medium was added. The plates were rotated clockwise and anticlockwise direction and allowed to set. Then, the plates were incubated at $28 \pm 2 ^{\circ}C$ for 48 h. After the incubation period, the colonies which exhibited fluorescent pigment production were selected and transferred to King’s B agar slants and maintained at 4 °C for further study.

3.4.3 Isolation of *Methylobacterium* from the phyllosphere of rice 
 *(Holland et al., 2000)*

Leaf samples of rice, collected from each location of the surveyed area, were washed with sterile water to remove dirt and soil that may adhere to the surface of leaf. A total of four leaf imprints were done on MMS agar plates under aseptic conditions. Then, the plates were incubated at $28 \pm 2 ^{\circ}C$ for 7 days and the appearance of pink colonies on the plates were then picked out and re-streaked on fresh MMS medium for maintaining pure culture.

3.5 Purification for PGPB isolates

3.5.1 Purification of *Azospirillum* Isolates

A loopful of the enriched culture was streaked on RC medium 
*(Rodriguez-Caceres, 1982)*. Light pink colonies which became scarlet upon storage were picked out and streaked on solidified potato infusion agar *(BMS)*
plates (Baldani and Dobereiner, 1980). Typical pink, often wrinkled colonies were picked out and maintained in nutrient agar slants for further study.

3.5.2 Composition of Media

Nitrogen Free Malate Medium (NFB) (Day and Dobereiner, 1976)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L – Malic acid</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen orthophosphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Fe EDTA (1.64% W/V), aqueous</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>(0.5% aqueous solution (dissolve in 0.2 N KOH)</td>
<td></td>
</tr>
<tr>
<td>Vitamin solution</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

For semisolid and solid medium 1.75 g and 15.0 g agar per litre were added, respectively. The pH was adjusted to 6.8 with 1 N NaOH.

Trace element solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium molybdate</td>
<td>0.200g</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.234 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.280 g</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>0.008 g</td>
</tr>
</tbody>
</table>
Materials and Methods

Zinc sulphate  -  0.024 g
Distilled water  -  1000 mL

**Vitamin solution**

Biotin  -  0.01 g
Pyridoxin  -  0.02 g
Distilled water  -  1000 mL

**RC medium (Rodriguez – Caceres, 1982)**

L – Malic acid  -  5.0 g
Dipotassium hydrogen orthophosphate  -  0.5 g
Magnesium sulphate  -  0.2 g
Sodium chloride  -  0.1 g
Yeast extract  -  0.05 g
Ferric chloride  -  0.015 g
Potassium hydroxide  -  4.0 g
Agar  -  15.0 g
Distilled water  -  1000 mL
pH  -  7.0

After autoclaving, 15 mL of 1: 400 aqueous solution of sterilized Congo red was added, aseptically.

**Potato infusion agar (BMS) (Baldani and Dobereiner, 1980)**

Washed, peeled, sliced potato  -  200.0 g (g L⁻¹)
L – Malic acid - 2.5 g
Potassium hydroxide - 2.0 g
Sucrose - 2.5 g
Vitamin solution* - 1.0 mL
Agar - 15.0 m

*(Biotin, 0.01 g; Pyridoxin, 0.2 g; Distilled water, 1000 mL)

Washed potatoes were boiled for 30 min and the solution was filtered through layers of cheese cloth. Malic acid was dissolved in 50 mL of water, 2 drops of bromothymol blue (0.5 per cent solution in ethanol) added and pH adjusted to 7.0 as indicated by the solution turning green with KOH. This solution together with sucrose, agar and vitamins were added to the filtrate, made up to 1000 mL with distilled water and sterilized by autoclaving.

3.5.3 Purification of Pseudomonas Isolates

All the ten Pseudomonas isolates were purified by streak plate method using PAF medium, frequently.

3.5.3.1 Composition of PAF medium

Peptone - 10.0g
Caesin hydrolysate - 10.0g
Anhydrous Magnesium Sulphate - 1.5g
Dipotassium hydrogen phosphate - 1.5g
Glycerol - 10.0 mL
Agar - 15g
Distilled water - 1000mL
3.5.3.2 Purification of *Methylobacterium* isolates

All the ten *Methylobacterium* isolates were purified by streak plate method using MMS medium frequently.

3.6 *Azospirillum* genus characterization

In the N-free semisolid malate medium (Nfb), the genus *Azospirillum* was accomplished by the presence of white, dense, fine, undulating pellicles (Dobereiner and Day, 1976).

Phase contrast microscopic observations of 72 h old culture revealed plump, slightly curved and straight rods about 1.0 μm in diameter and 2.1 – 3.8 μm in length. Intracellular granules of poly-β-hydroxy butyrate (PHB) were present (Tarrand et al., 1978) and the isolates exhibited a very characteristic vibrioid movement in broth cultures and gram staining was performed as per Vincent (1970).

3.7 *Pseudomonas* - genus characterization (schaad et al., 1998)

The genus *Pseudomonas* produces fluorescent diffusible pigment on nutrient agar, short rod shaped, gram negative motile and non-spore formers. Physiological and biochemical tests revealed the existence of oxidase, proteolytic and lipolytic activities. Positive utilization of carbon sources viz., mannitol, glucose, galactose and sorbitol recorded no growth with ethanol was observed. Retarded growth was also observed at higher temperature viz., 40 °C.

3.8 *Methylobacterium* - genus characterization (Green, 2006)

The genus *Methylobacterium* are gram negative, non-endospore forming, aerobic, motile rods (0.63-0.63 x 1.8-2.7 μm) produces non-flourescent pink pigment on Methanol mineral salts medium (MMS) (Green
Materials and Methods

The colony morphology of Methylobacterium sp. are pink to red, convex, translucent with regular edges, slow-grower on MMS medium. In static liquid methanol mineral salts medium (Green and Bousfield, 1983) the isolates form a pink surface ring or pellicle.

3.9 Designation of PGPB isolates

After the genus characterization, all the ten Azospirillum isolates were designated as AZ series, Pseudomonas isolates were designated as PF series while the ten Methylobacterium isolates were designated as MB series and numbered randomly.

3.9.1 Screening the Azospirillum isolates for their dinitrogen fixing efficiency under in vitro condition

3.9.1.1 Microkjeldahl assay (Bremner, 1960)

Hundred ml of the nitrogen free malate medium (Nfb) was taken into 250 ml Erlenmeyer flasks and sterilized by autoclaving. The flasks were separately inoculated with 1 ml of (1 x 10⁷ CFU mL⁻¹) 48 h old cultures of Azospirillum, viz., AZ-1 to AZ-10, aseptically. The flasks were incubated at 30 ± 2 °C for one week under stationary condition. After the incubation period, 1 mL of the broth was transferred to 50 mL pyrex microkjeldhal flask. A quarter teaspoonful of the digestion mixture (10 g of reagent grade potassium sulphate, 1 g of cupric sulphate and 0.1 g of selenium metal powder) and 4 ml salicylic-sulphuric acid mixture (0.1g of salicylic acid, 1.0 g of sodium thiosulphate and 30 mL of concentrated sulphuric acid) were introduced into it. The contents were slowly heated till frothing ceased and then heated strongly. Completion of the digestion was indicated by the solution turning into bluish green. After cooling, about 15 mL of distilled water was added to the flask,
swirled and cooled. The contents were transferred into the distillation unit and 25 mL of 40 per cent sodium hydroxide was added. The ammonia was steam distilled for 15 min into an excess of 0.1 N sulphuric acid (10 mL) containing 2 drops of methyl red. The contents were back titrated with 0.1 N potassium hydroxide till the appearance of golden yellow colour. The nitrogen content of the sample was calculated using the following factor

1 mL of 0.1 N H$_2$SO$_4$ = 0.0014 g of N

3.9.2 Screening the *Pseudomonas* and *Methylobacterium* isolates for phytohormone production and biocontrol ability against *Xanthomonas oryzae pv oryzae*

All the ten *Pseudomonas* and *Methylobacterium* isolates, obtained from the rhizosphere and phyllosphere of rice, respectively, were screened for their phytohormone viz., indole acetic acid production and the biocontrol ability against *Xanthomonas oryzae pv oryzae* under *in vitro* condition. The preparation of inoculum for conducting these studies was as stated below, and three replications were maintained for each study.

3.9.2.1 Preparation of inoculum

All the ten *Pseudomonas* and *Methylobacterium* isolates were individually grown on King’s “B” broth and methanol mineral salts broth (MMS) in a shaking bath at 28 ± 2 ºC for 7 days. After the incubation period, the medium was centrifuged, separately, at 5000 x g for 10 min to harvest the log growth phase of cells and the pellets were washed three times with 0.1 M phosphate buffer (pH 6.8). Finally, the cells were resuspended in the same buffer to a cell concentration of 1 x 10$^7$ CFU mL$^{-1}$ by measuring the absorbency at 600 nm and used as inoculum.
3.9.2.2 Indole acetic acid (IAA) production

Hundred mL volumes of King’s “B” broth and synthetic methanol mineral salts broth supplemented with 100 mg L⁻¹ of DL-Tryptophan was dispensed individually into 250 mL Erlenmeyer flasks under sterilized condition. One mL of each Pseudomonas and Methylobacterium isolate was added aseptically to the respective broth. The flasks were incubated for 7 days in an incubator at 28 ± 2 °C in dark and under static condition.

3.9.2.3 Extraction and estimation of IAA from the medium

After the incubation period, the medium was centrifuged separately at 7000 × g for 30 min and the supernatant was reduced to 50 mL volume by flash evaporation under vacuum and IAA extracted into ethyl acetate and n-butanol as per the procedure of Tien et al. (1979) as stated below.

Concentrated cell free culture broth

\[ \text{Adjusted to pH 2.8 with 1 N HCl extracted with ethyl acetate (3 x)} \]

Ethyl acetate fraction

\[ \text{Evaporation under vacuum} \]

Aqueous fraction

\[ \text{Adjusted to pH 7.0 with 1 N NaOH extracted with water saturated n-butanol (3x)} \]

Re-dissolve in absolute methanol

n-butanol fraction

aqueous fraction

Colorimetric assay
To one mL of methanol residue in a test tube, 4 mL of salper reagent (Gordon and Paleg, 1957) and 1.5 mL of distilled water were added. The reagent was added in a dropwise manner but rapidly with continuous agitation. The sample was incubated in dark for 1 h, and the absorbance of pink colour was measured at 535 nm against a solvent reagent blank. The quantity of IAA was estimated from a standard curve drawn from known concentrations of IAA (1 Div. = 0.307 µg of IAA).

3.9.2.4 Zone of inhibition of *Xanthomonas oryzae pv oryzae*

Filter paper discs (4 cm dia.), impregnated in each cultures of *Pseudomonas* and *Methylobacterium*, were transferred separately to the centre of PSA plates which have been preinoculated with *Xanthomonas oryzae pv oryzae*. Then, the plates were incubated at 28 ± 2 ºC for 7 days. After the incubation period, the diameter of zone of inhibition against *Xanthomonas oryzae pv oryzae* was measured. Three replications were maintained for each isolate.

3.10 Grading *Azospirillum* on the basis of the Dinitrogen Fixing efficiency

All the ten isolates of *Azospirillum* were graded into three categories on the basis of their dinitrogen fixing efficiency determined by Microkjeldahl assay (Bremner, 1960).

I Category - ‘N’ fixation above 15.00 mg g⁻¹ of malate.

II Category - ‘N’ fixation between 14.00 and 14.99 mg g⁻¹ of malate.

III Category - ‘N’ fixation below 14.00 mg g⁻¹ of malate.
3.10.1 Grading the *Pseudomonas* isolates on the basis of their phytohormone production and biocontrol ability against *Xanthomonas oryzae pv oryzae*

All the ten isolates of *Pseudomonas* were graded into three categories on the basis of their phytohormone production *viz.* IAA and the biocontrol activity against *Xanthomonas oryzae pv oryzae*.

**I category** - IAA production above 4.80 µg mL\(^{-1}\), zone of inhibition > 10.00 mm, dia.

**II category** - IAA production between 4.00 µg mL\(^{-1}\) and 4.79 µg mL\(^{-1}\), zone of inhibition between 9.00 and 9.99 mm, dia.

**III category** - IAA production below 4.00 µg mL\(^{-1}\), zone of inhibition < 9.00 mm, dia.

3.10.2 Grading the *Methylobacterium* isolates on the basis of their phytohormone production and biocontrol activity against *Xanthomonas oryzae pv oryzae*

All the ten isolates of *Methylobacterium* were graded into three categories on the basis of their phytohormone production *viz.* IAA and the biocontrol activity against *Xanthomonas oryzae pv oryzae*.

**I category** - IAA production above 2.75 µg mL\(^{-1}\) and zone of inhibition > 8.70 mm, dia.

**II category** - IAA production between 1.50 µg mL\(^{-1}\) to 2.70 µg mL\(^{-1}\) and zone of inhibition 2.74 µg mL\(^{-1}\) and 8.0 mm - 8.69 mm, dia.

**III category** - IAA production ≤ 1.50 µg mL\(^{-1}\) and zone of inhibition below 8.0 mm, dia.
3.11 Studies on the inter strain differences of *Azospirillum*, *Pseudomonas* and *Methylobacterium* under *in vitro* condition

The following parameters were studied with selected six efficient PGPB isolates *vìz.*, AZ-3, AZ-6, PF-3, PF-6, MB-3 and MB-6, from the previous experiments to explore the possibility of any interstrain differences among the PGPB isolates. The preparation of inoculum and plant materials for conducting these studies was as stated else were in the text, unless otherwise specifically mentioned. Three replicates were maintained for each study.

3.11.1 Surface sterilization and germination of rice seeds

The association of *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates with rice (*Oryza sativa* L.) was studied with cultivar BPT-5804. The surface sterilization and germination of the BPT-5804 rice seeds were done as detailed below.

BPT-5804 rice seeds were surface sterilized by immersing in 95% ethanol for 1 min, followed by 20 min in 1 per cent NaOCl. After rinsing three times with sterile distilled water, the sterilized seeds were placed on the surface of 1 per cent water agar in petriplates (9 cm dia. at the rate of five seeds per plate). Then, they were incubated in an inverted position for 3 days at room temperature to allow germination. The plates were sealed with paraffin wax to avoid agar dryness during germination.

3.11.2 Interstrain Difference of *Azospirillum* *Pseudomonas* and *Methylobacterium* on generation time (GT) under N-free and N-supplemented conditions

Hundred ml volumes of synthetic malate broth (Day and Dobereiner, 1976), with and without supplementation of 0.05% yeast extract was dispensed
into 250 mL Erlenmeyer flasks individually under sterilized condition. In the same way, hundred ml volumes of King’s “B” broth (King’s et al., 1954) synthetic MMS broth (Green and Bousfield, 1983) were dispensed into 250 mL Erlenmeyer flasks under sterilized condition, separately. One mL culture (1 $\times$ 10$^7$ CFU mL$^{-1}$) of each Azospirillum Pseudomonas and Methylobacterium isolates was added separately and aseptically to the respective medium. (Nfb medium for Azospirillum, King’s “B” for Pseudomonas and MMS medium for Methylobacterium) and incubated under static condition at 30 ± 2 °C for one week. The optical density of each broth was determined at 30 min interval by reading the absorbance at 420 nm for Azospirillum 450 nm for Pseudomonas and 500 nm for Methylobacterium in Spectronic-20 colorimeter. Suitable control without inoculum was also maintained. A linear growth curve was plotted employing different optical density values against their appropriate time interval. By employing the growth curve, the Generation time (g) of the individual Azospirillum, Pseudomonas and Methylobacterium isolate was obtained.

3.11.3 Interstrain difference of Azospirillum on Acetylene Reduction Assay (ARA) under free living condition (Hardy et al., 1973)

Nitrogen free semisolid malate medium (Nfb) supplemented with 0.005 per cent (w/v) yeast extract was prepared as detailed earlier, dispensed in 30 ml quantities in 100 ml vials and sterilized by autoclaving. one ml log phase culture of each Azospirillum isolate (1$\times$10$^7$ CFU/mL) was added to each vial individually and incubated for 72 h at 30 ± 2°C. Then, the cotton plugs were replaced with needle puncture rubber stopper, aseptically. Using a syringe, 10 per cent volume of the vial was replaced with acetylene gas and incubated for
12 h. The control flask which received no *Azospirillum* culture was also injected with acetylene gas. After the incubation period, 0.5 ml of the gas sample was withdrawn from the vial and fed into a gas chromatograph with the following features:

- **Gas chromatograph**: Chemito, 3800 model
- **Column**: Porapak T (80-100 mesh) 3 M x 3.1 mm stainless steel
- **Detector**: Flame Ionization Detector
- **Carrier gas**: Nitrogen, Iolar, 40 mL min$^{-1}$

**Temperature**

- **Injection port**: 110°C
- **Detector port**: 110°C
- **Oven**: 100°C (Internal)

**Flame**

- **Hydrogen**: 30 mL min$^{-1}$
- **Oxygen**: 30 mL min$^{-1}$
- **Sample value**: 500 microlitre
- **Retention time**: Ethylene 52 sec., Acetylene 83 sec.

The ethylene peak was recorded for each culture of *Azospirillum*. The protein content of the *Azospirillum* culture from each vial was estimated by
modified Lowry’s method (Lowry et al., 1957). The acetylene reduction activity was calculated using the following formula.

\[ \text{Nitrogenase activity} = \frac{n \text{ mole of } C_2H_4}{mg \text{ of protein} / \text{h}}. \]

**Calculations**

- **Peak height (in mm) of** $C_2H_4$ = ‘a’ mm in injection volume
- **Peak height (in mm) in one** = ‘b’ mm injection volume
- **Nanomoles of** $C_2H_4$ corresponding = ‘c’ nm, moles to ‘b;’ mm peak height from standard curve

Volume of the vial = ‘d’ ml

Volume of medium ‘e’ ml = $d-e = F \text{ mL}$

Volume of gas phase in vial

n mole of $C_2H_4$ / vial = $F \times C$ for 12 h incubation

Calculate the protein content of = mg of protein ‘e’ ml *Azospirillum* in vial

\[ \frac{F \times C \times 60}{12 \times X} \]

n mole of $C_2H_4$ / mg protein / h

3.11.4 **Interstrain difference of Azospirillum on nitrogen fixation in Spermsphere model** (Thomas – Bauzon et al., 1982)

Rice (*Oryza sativa* L.) cv.BPT-5804 seeds were surface sterilized and germinated as described in 3.11.1. Germinated seeds were placed in the glass tubes of “Spermsphere model” which contained 100 ml of modified Weaver’s medium (Weaver et al., 1975).
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Each glass tube of Spermosphere model was covered with sterile rubber stoppers and the plants were grown for 7 days in an environmentally controlled growth chamber as described above. On eighth day, 1ml of 48 h old culture of each *Azospirillum* isolate was pipette out in sterile conditions on the surface of the Weaver’s medium in the “Spermosphere model” apparatus (Thomas – bauzon *et al.*, 1982).

The model was incubated for another 7 days in growth chamber and then screened for nitrogenase activity under 10% C\textsubscript{2}H\textsubscript{2}. After 12 h incubation, the ARA was measured as described earlier. The model without rice plant served as the control.

**Weaver’s medium (Weaver *et al.*, 1975)**

**Solution A**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc sulphate</td>
<td>0.43 g</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>1.30 g</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>2.80 g</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>0.026 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 mL</td>
</tr>
</tbody>
</table>

**Solution B**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulphate</td>
<td>100 mg</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>100 mg</td>
</tr>
<tr>
<td>Ferric sulphate</td>
<td>22 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>20 mg</td>
</tr>
</tbody>
</table>
Solution A  
-  20 mL

Distilled water  
-  1000.0 mL

Solution C  

$K_2HPO_4$  
-  0.6 g

$KH_2PO_4$  
-  0.9 g

Distilled water  
-  1000.0 mL

Add 50 mL of B and 15 mL of C to 1 litre of distilled water.

### 3.11.5 Interstrain difference of *Azospirillum*, *Pseudomonas* and *Methylobacterium* on siderophore production

#### 3.11.5.1 Preparation of inoculum

All the six efficient *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates were grown separately in synthetic malate broth, King’s B broth, methanol mineral salts (MMS) broth respectively, maintained separately in 250 mL Erlenmeyer flasks as detailed earlier in 3.9.2.1 except the omission of FeEDTA. To reduce iron contamination, deionized water was used in all the medium components and glass ware were cleaned with deionized water.

#### 3.11.5.2 Determination of Siderophore production by *Azospirillum* isolates

Hundred ml volumes of synthetic malate broth supplemented with 0.05 per cent (w/v) yeast extract was maintained in 250 mL Erlenmeyer flasks under sterilized condition. While preparing the medium, FeEDTA was omitted and the experiment was conducted with iron-deficient medium and glass ware. One ml of each *Azospirillum* isolate (1×10$^7$ CFU mL$^{-1}$) was added separately and
aseptically to the medium. Then the flasks were incubated for 72 h at 30 ± 2°C under static condition. After the incubation period, the growth of *Azospirillum* isolates cultured in iron deficient, synthetic malate mineral salts broth was determined by measuring the absorbance at 420 nm in Spectronic-20 colorimeter.

### 3.11.5.3 Determination of Siderophore production by *Pseudomonas* isolates

Hundred ml volumes of synthetic King’s B broth was maintained in 250 mL Erlenmeyer flasks under sterilized condition. While preparing the medium, FeEDTA was omitted and the experiment was conducted with iron-deficient medium and glass wares. One mL of each *Pseudomonas* isolate (1×10⁷ CFU mL⁻¹) was added aseptically to the medium. Then, the flasks were incubated at 28 ± 2ºC under static condition for 72 hrs. After the incubation period, the growth of a *Pseudomonas* isolates cultured in iron deficient King’s broth was determined by measuring the absorbance at 450 nm in Spectronic-20 colorimeter.

### 3.11.5.4 Determination of siderophore production by *Methylobacterium* isolates

Hundred ml volumes of synthetic methanol mineral salts (MMS) broth was maintained in 250 mL Erlenmeyer flasks under sterilized condition. While preparing the medium, FeEDTA was omitted and the experiment was conducted with iron-deficient medium and glass wares. One mL of each *Methylobacterium* isolate (1×10⁷ CFU mL⁻¹) was added aseptically to the medium. Then the flasks were incubated for 7 days at 28 ± 2ºC under static condition. After the incubation period, the growth of a *Methylobacterium*
isolates cultured in iron deficient methanol mineral salts broth was determined by measuring the absorbance at 500 nm in Spectronic-20 colorimeter.

3.11.5.5 Extraction of Siderophore from the medium (Modi et al., 1985)

After the incubation period, the spent culture fluid was separated from cells by centrifugation at 7000×g for 15 min. The supernatant was concentrated to one-fifth of the original volume by flash evaporation at 45 °C.

Catechol type phenolates were extracted on ethyl acetate extract of the culture supernatant twice with an equal volume of solvent at pH 2.0. The ethyl acetate layer was removed and evaporated to dryness and the residues were dissolved in a minimum quantity of distilled water while hydroxamate types were measured from the untreated culture supernatant.

3.12 Estimation of Siderophore Production

3.12.1 Preparation of Hathway’s reagent (Reeves et al., 1983)

One mL of 0.1 M Ferric chloride in 0.1 N HCl was added in 100 mL of distilled water and to this 1 mL of 0.1 M Potassium ferric cyanide was added.

3.12.2 Estimation of Phenolate siderophores

One volume of Hathway’s reagent was added to one volume of the sample and the development of wine colour showed the presence of phenolate like siderophores. The absorbance was determined at 560 nm for salicylates with sodium salicylate as standard and 700 nm for dihydroxyphenols with 2,3-dihydroxy benzonic acid (DHBA), as standard 1.0 µmol of sodium salicylate gave an absorbance of 0.5 whereas 2,3 DHBA gave an absorbance of 0.75.
3.12.3 Estimation of Hydroxamate siderophores (Gibson and Magrath, 1969)

To 0.5 mL of culture supernatant, 0.5 mL of 6 M \( H_2SO_4 \) was added and the mixture was autoclaved in a glass tube. One mL of sulphanilic acid (1%v/v) in 30 per cent acetic acid (v/v) and 0.5 mL of 1.3 per cent iodine in 30 per cent acetic acid (w/v) were added to autoclaved chemicals. The excess of iodine was destroyed by the addition of 1 mL of 2 per cent (w/v) sodium arsenate solution. 1 mL of \( \alpha \)-naphthylamine solution (0.3% in 30% acetic acid) was then added and the total volume was made up to 10 mL with distilled water. After 30 min, the absorbance at 526 nm was measured. Hydroxylamine hydrochloride was used as standard and 1.0 µmole of compound gave the absorbance of 0.1.

3.12.4 Interstrain difference of *Azospirillum* and *Methylobacterium* on Exopolysaccharide production

3.12.4.1 Exopolysaccharide (EPS) Production

The exopolysaccharide (EPS) production of efficient *Azospirillum* and *Methylobacterium* isolates were evaluated by growing the isolates separately in N-free synthetic malate broth and N-free methanol mineral salts (MMS) broth, respectively, dispensed in 250 mL Erlenmeyer flasks under sterilized condition. One mL each culture of *Azospirillum* and *Methylobacterium* isolates (1 x 10\(^7\) CFU mL\(^{-1}\)) were added to the medium and incubated at 28 ± 2 °C for one week. The broth was kept in a rotary shaker at 250 rpm for 15 min every day.

After the incubation period, the cells were harvested by centrifugation (7000 × g) and used for the analysis of alkali stable polysaccharide. The supernatant was used for the analysis of water soluble polysaccharide.
3.12.4.2 Estimation of Water soluble polysaccharide

To 20 mL of the supernatant fraction, 60 mL of iso-propyl alcohol was added and incubated at 40 °C overnight to precipitate the water soluble polysaccharide. The precipitate was collected by filtering through pre weighed Whatman No.42 filter paper and dried in an oven at 70 °C for 24 h (Sutherland and Wilkinson, 1971).

3.12.4.3 Estimation of Alkali Stable Polysaccharide

The harvested cells were washed with distilled water and one mL of 30 per cent potassium hydroxide was added. The contents were heated for 1 h at 100 °C over a water bath. It was then cooled to room temperature and 20 mL of ethyl alcohol was added. The mixture was thoroughly shaken and the precipitated polysaccharide was collected by centrifugation.

3.12.4.4 Analysis of Alkali Stable Polysaccharide

To estimate the alkali stable polysaccharide in the sample, anthrone method (Dubois et al., 1951) was employed. Five mL of the anthrone reagent was pipetted out into the tubes and 100 mL of the sample was added and the mixture was shaken. The tubes were closed and heated exactly for 10 min on a boiling water bath. Then, the tubes were cooled rapidly under tap water. The absorbance was measured in a Bausch and Lomb spectronic-20 colorimeter at 620 nm. The quantity of polysaccharide present in the sample was determined by referring to the standard graph prepared with glucose and expressed as µg of glucose released g⁻¹ of cells (Nelson, 1944).
3.13 Interstrain difference of *Azospirillum*, *Pseudomonas* and *Methylobacterium* on adhesion to rice root

3.13.1 Plant Growth

Rice *cv.* BPT-5804 seeds were surface sterilized and germinated as detailed elsewhere in the text. After the germination, the three day old sterile seedlings were transferred to slopes of Fahraeu’s solution (Fahraeus, 1957) solidified with 1.5 per cent agar in test tubes. Sterile Fahraeu’s solution was added to fill the empty portion of the agar slopes and the tubes were incubated for three more days (24 °C day / 22 °C night). After the incubation period, the roots were collected from each tube separately, washed first with sterile water and later three times in 0.1 M phosphate buffer (pH 6.8), cut into 5 cm pieces and used in the adsorption experiments.

3.13.2 Adsorption Assay (Gafni et al., 1986)

Rice root bits (1g) collected as described earlier, were placed in a 10 mL sterile test tube containing 5 ml non-aggregated suspension of each *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates prepared as described in 3.11.2. The tubes were shaken at 35 °C for 1 h at 100 rpm. The initial number of *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates in the inoculum solution was determined by plate count method on nutrient agar plates before adding to the rice roots. After the adsorption was completed, the roots were removed aseptically and the number of bacteria remaining in the solution was redetermined. The number of adsorbed bacteria g⁻¹ dry weight of rice roots (Cb) was calculated as per the procedure of Gafni *et al.* (1986).
3.14 Interstrain difference of *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates on thermal and desiccation tolerance

3.14.1 Thermal tolerance experiment

One mL suspension of *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates were collected from 7 days old Nitrogen free malate medium, King’s ‘B’ and Methanol mineral salts medium respectively, taken in a vial and immersed in water bath adjusted to 50 °C temperature. After 20 min exposure, the tubes were removed and cooled rapidly. Then 100 µL of samples were taken from each tube and placed on respective agar plates for determining the viability of the cells.

3.14.2 Desiccation tolerance experiment

One mL suspension of each *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates were collected from 7 days old Nitrogen free malate medium, King’s ‘B’ and Methanol mineral salts medium, respectively, placed in a sterile 1.5 mL eppendorf microcapillary tubes and the tubes were in turn kept open in a sterile petri dish. The petri dish with the tubes was then placed in an incubator at 37 °C. After 7 days, the dried cells from the capillary tube were washed with 100 mL of sterile distilled water with vigorous agitation for the complete removal of PGPB cells and the viability determined by plating them on respective agar plates.

3.15 Phenotypic characteristics of the efficient isolate *Azospirillum* (AZ-3) with other *Azospirillum* sp.

On the basis of the results obtained from the previous experiments, the *Azospirillum* isolate *viz.*, AZ-3 was found to be the most efficient one and the
same was for their phenotypic characteristic compared with other *Azospirillum* sp. *viz.*, *Azospirillum brasilense* (ATCC 29145), *Azospirillum lipoferum* (ATCC 29707), *Azospirillum halopraeferens* (ATCC 51182) and *Azospirillum amazonense* (35119), according to Mehnaz *et al.* (2007). (Table-24)

### 3.16 Phenotypic characteristics of the efficient isolate *Pseudomonas* (PF-3) with other *Pseudomonas* sp.

On the basis of the results, obtained from the previous experiments the *Pseudomonas* isolate *viz.*, PF-3 was found to be most efficient one and the same was for their phenotypic characteristic compared with other *Pseudomonas* sp. *viz.*, *Pseudomonas fluorescens* (ATCC 7283), *Pseudomonas putida* (ATCC 12633), *Pseudomonas aeruginosa* (ATCC 10145), and *Pseudomonas stutzeri* (ATCC 14405), according to Schaad *et al.* (1998). (Table-25)

### 3.17 Phenotypic characteristics of the efficient isolate *Methylobacterium* (MB-3) with other *Methylobacterium* sp.

On the basis of the results, obtained from the previous experiments, the *Methylobacterium* isolate *viz.*, MB-3 was found to be the most efficient one and the same was compared with other *Methylobacterium* sp. *viz.*, *Methylobacterium phyllophaeae* CBMB-27, *Methylobacterium oryzae* CBMB -20, *Methylobacterium suomiense* and *Methylobacterium fujisawaense* according to Madhaiyan *et al.* (2009). (Table-26)

### 3.18 Studies on the multigenic co-aggregation mechanism of *Azospirillum, Pseudomonas* and *Methylobacterium* cells

#### 3.18.1 Preparation of inoculum

Unless otherwise specifically mentioned, the three efficient PGPB isolates, obtained from the previous interstrain experiments, were grown in
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synthetic malate broth, King’s “B” broth and methanol mineral salts broth respectively, duly supplemented with 0.05% yeast extract (w/v), and peptone 0.1% (w/v), respectively in a shaking bath at 30 ± 2 °C for 5 days to get stationary phase cultures of *Azospirillum, Pseudomonas* and *Methylobacterium*. Then, the medium was centrifuged separately, at 5000 x g for 10 min to harvest the stationary phase cells and the pellets washed three times with 0.1 M phosphate buffer (pH 6.8). Finally the cells were resuspended in the same buffer to a cell concentration of $1 \times 10^7$ CFU mL$^{-1}$ by measuring the absorbance at 420 nm for *Azospirillum*, 450 nm for *Pseudomonas* and 500 nm for *Methylobacterium* and used as inoculum.

3.18.2 Preparation of Co-AG Buffer (Grimaudo and Nesbitt, 1997)

The co-aggregation buffer was prepared as stated below:

1. 20 mM Tris-HCl buffer (pH 7.8)
2. 0.1 mM CaCl$_2$
3. 0.01 mM MgCl$_2$
4. 0.15 M NaCl and
5. 0.02% NaN$_3$

3.18.3 Preparation of Plant Seed Extract

The following plant seed extracts, namely, *Moringa oleifera, Strychnos potatorum, Allium cepa, Sappindus emaginatus* and *Asteracantha longifolia* were prepared as stated below. Matured seeds of the above said plant seed materials were collected, crushed and sieved (0.8 mm mesh). The seed powder is mixed with a small amount of sterile water to form a paste. Then, the paste is diluted to the required strength *viz.*, 5 per cent concentration before using it.
The insoluble materials are filtered out using either a fine mesh screen or muslin cloth. The clarified suspension of plant seed materials was used for co-aggregation studies.

3.18.4 Co-Aggregation Assay (Jabra-Rizk et al., 1999)

One mL aliquot of each PGPB culture viz., *Azospirillum, Pseudomonas* and *Methylobacterium* was mixed together in 10 mL Co-Ag buffer. Uninoculated buffer served as control. The mixtures were vortexed for 10 seconds, shaken on a rotary platform shaker for 3 min. and left undisturbed at room temperature for 24 h. All Co-Ag reactions were performed in triplicate.

3.18.5 Estimation of Co-Aggregation Percentage (Madi and Henis, 1989)

After the incubation period, the aggregates settled at the bottom of the tube while some of the free cells remained in suspension. The supernatant was sampled and its turbidity measured in spectronic-20 colorimeter at 420 nm. The flocs were then mechanically dispersed by treatments in a tissue homogenizer for 1 min and the total OD was measured and per cent co-aggregation was calculated as follows:

\[
\text{OD}_{t} - \text{OD}_{s} \times 100
\]

\[
\% \text{ Co-aggregation} = \frac{\text{OD}_{t} - \text{OD}_{s}}{\text{OD}_{t}} \times 100
\]

Where

\[
\text{OD}_{t} = \text{total optical density after mechanical dispersion and}
\]

\[
\text{OD}_{s} = \text{OD of supernatant after co-aggregate had settled}
\]
3.19 FACTORS AFFECTING THE NATURAL MULTIGENERIC CO-AGGREGATION OF PGPB CELLS

3.19.1 Effect of Cell age on Multigeneric Co-Aggregation of PGPB cells

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown individually in synthetic malate broth, King’s “B” broth and Methanol mineral salts broth, respectively, duly supplemented with 0.05% yeast extract (w/v), and peptone 0.1 (w/v), respectively in a shaking bath at 30 ± 2 °C. The lag, log and stationary phase cultures of the PGPB cells were harvested at 0, 24 and 120 h, respectively and the co-aggregation percentage was estimated according to Madi and Henis (1989) in coaggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 6.0 level.

3.19.2 Effect of culture media on Multigeneric Co-Aggregation of PGPB cells

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown N free and N supplemented broth *viz.*, Nitrogen free synthetic malate broth and Nutrient broth, DFMM and King’s “B” broth and Nitrogen free MMS broth and MMS broth, respectively, maintained in a shaking bath at 35 °C for 120 h. After the incubation period, the cells of each PGPB isolate from the respective media were harvested and the co-aggregation percentage was estimated as stated earlier in co-aggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 6.0 level.

3.19.3 Effect of pH on Multigeneric Co-Aggregation of PGPB cells

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown individually for 120 h as per the
conditions mentioned in chapter 3.18.1. After 120 h incubation, the cells of each PGPR isolate were harvested individually and the multigeneric co-aggregation percentage was estimated according to Madi and Henis, (1989) in co-aggregation buffer (Grimaudo and Nesbitt, 1997) maintained at different pH levels, namely 6.0, 6.5, 7.0 and 7.5.

3.19.4 Effect of Temperature on Multigeneric Co-Aggregation of PGPB isolates

The PGPB cells viz., Azospirillum (AZ-3), Pseudomonas (PF-3) and Methylobacterium (MB-3) were grown individually for five days as per the conditions mentioned in chapter 3.18.1. and the temperature was maintained at different levels, namely, 25, 30, 35, 40 and 45 °C for the growth of PGPB cells. After 120 h incubation, the cells of each PGPB isolate were harvested individually and the multigeneric co-aggregation percentage was estimated according to Madi and Henis, (1989) in co-aggregation buffer (Grimaudo and Nesbitt, 1997) maintained at pH 6.0 level.

3.19.5 Effect of divalent cations on multigeneric co-aggregation of PGPB cells

The PGPB cells viz., Azospirillum (AZ-3), Pseudomonas (PF-3) and Methylobacterium (MB-3) were grown individually in synthetic malate broth, King’s “B” broth and methanol mineral salts broth, respectively, at 35°C for 120 h as per the conditions mentioned in 3.18.1. Then, the cells were harvested separately and the multigeneric co-aggregation percentage was estimated as stated elsewhere in the text in co-aggregation buffer (Grimaudo and Nesbitt, 1997) supplemented with different divalent cations viz., Ca$^{2+}$, Mg$^{2+}$ and Ba$^{2+}$,
with a view to test their efficacy on the induction of co-aggregation at 0.1 mM level.

### 3.19.6 Effect of chelating agents on Multigeneric Co-Aggregation of PGPB cells

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown individually as per the specifications mentioned in chapter 3.18.1 and the co-aggregation percentage was estimated in co-aggregation buffer (Grimaudo and Nesbit, 1997) at 6.0 pH level together with the addition of EDTA (Ethylene diamine tetra acetic acid) or EGTA (Ethylene glycol-bis-(β-amino ethyl ether) N-N’ tetra acetic acid) at 1 mM level.

### 3.19.7 Effect of Cell Number on Multigeneric Co-Aggregation of PGPB cells

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown individually for 120 hr in different culture media mentioned in chapter 3.18.2. After the incubation period, the cells of each PGPB isolate were harvested separately and the co-aggregation percentage was estimated at different levels of inoculum number, *viz.*, $10^4 : 10^4$, $10^5 : 10^5 : 10^6$, $10^6 : 10^6 : 10^7$, $10^7 : 10^7 : 10^8$ and $10^8 : 10^8 : 10^8$ as detailed in the same chapter.

### 3.20 Induction of Artificial Multigeneric Co-aggregation among PGPB cells

#### 3.20.1 Effect of Different Plant seed Materials on Induction of Multigeneric Co-Aggregation among PGPB Cells under log phase

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were grown in respective broth and the cells were
harvested at log phase as stated in chapter 3.18.1. One mL aliquot of each PGPB isolate (1 x 10^7 cells mL^-1) was mixed together in 10 mL of Co-Ag buffer (Grimaudo and Nesbitt, 1997) together with the addition of one ml of individual plant seed extract viz., *Moringa oleifera, Strychnos potatorum, Allium cepa, Sappindus emaginatus* and *Asteracantha longifolia*. The mixture was vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for 1h. The co-aggregation percentage was estimated according to Madi and Henis (1989).

**3.20.2 Comparative performance of Natural and Artificial Multigeneric Co-Aggregates of *Azospirillum Pseudomonas* and *Methylobacterium* with respect to thermal tolerance**

The PGPB cells *viz., Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were subjected to natural and artificial Multigeneric co-aggregation as per the details mentioned in 3.17.4 and 3.18.8.1. After the incubation period of 24 h the natural and artificial multigeneric co-aggregates were collected separately mechanically dispersed with tissue homogenizer and adjusted to a final concentration of 1 x 10^7 CFU mL^-1 with 0.1 M phosphate buffer. Then, one mL of natural and artificial co-aggregates was taken, separately, in a vial and immersed in a water bath adjusted to 50 °C temperature. After 20 min exposure, the tubes were removed and cooled rapidly. Then, 100 μL of samples were taken from each tube and plated on nutrient agar plates for determining the viability of the co-aggregated cells.
3.20.3 Comparative performance of Natural and artificial Multigeneric co-aggregates of *Azospirillum* *Pseudomonas* and *Methylobacterium* with respect to desiccation tolerance

The PGPB cells *viz.*, *Azospirillum* (AZ-3), *Pseudomonas* (PF-3) and *Methylobacterium* (MB-3) were subjected to natural and artificial multigeneric co-aggregation as per the details mentioned in 3.17.4 and 3.18.8.1. After the incubation period of 24 h the natural and artificial multigeneric co-aggregates were collected, mechanically dispersed with tissue homogenizer and adjusted to a final concentration of $1 \times 10^7$ CFU mL$^{-1}$ with 0.1 M phosphate buffer. One ml of the natural and artificial multigeneric co-aggregates was collected separately, placed in sterile 1.5 mL Eppendorf microcapillary tubes and the tubes were in turn kept open in a sterile petridish. The petridishes with the tubes were then placed in an incubator at 37 °C. After 1 week incubation period, the dried cells from the capillary tubes were washed with 100 µL sterile distilled water with vigorous agitation for the complete removal of PGPB cells and the viability was determined by plating them on nutrient agar plates.

3.21 Bioinoculation effect of different bioformulations of PGPB cells on the enhancement of seed vigour index of rice *cv.* BPT-5804

Rice (*Oryza sativa* L.) *cv.* BPT-5804 seeds were surface sterilized following the procedure given in 3.11.2. The *Azospirillum*, *Pseudomonas* and *Methylobacterium* isolates were grown and harvested as described in 3.14.1 and the different bioformulations of PGPB cells were prepared as described in 3.14.4 and 3.15.8.1. Surface sterilized healthy rice seeds (10 seeds / set) were treated with the following treatments.

1. Control (No inoculation)
2. $1 \times 10^7$ CFU mL$^{-1}$ of Azospirillum alone (AZ-3).
3. $1 \times 10^7$ CFU mL$^{-1}$ of Pseudomonas alone (PF-3).
4. $1 \times 10^7$ CFU mL$^{-1}$ of Methylobacterium alone (MB-3).
5. $1 \times 10^7$ CFU mL$^{-1}$ of co-inoculation of Azospirillum, Pseudomonas and Methylobacterium.
6. $1 \times 10^7$ CFU mL$^{-1}$ of mechanically dispersed natural co-aggregates of Azospirillum, Pseudomonas and Methylobacterium.
7. $1 \times 10^7$ CFU mL$^{-1}$ of mechanically dispersed artificial co-aggregates of Azospirillum, Pseudomonas and Methylobacterium.

The rice seeds were subjected to the above treatments, dried in shade for 30 min. Then, the inoculated rice seeds were arranged in two rows on a sheet of blotting paper dipped in sterile water. Then, they were covered with another blotting paper dipped in sterile water, rolled and placed vertically in a moist chamber at 20 °C. Uninoculated seeds with distilled water treatment served as control. After the incubation for 5 days, each roll was opened and the vigour indices of germinated rice seeds were calculated by the method of Abdul-Baki and Anderson, (1973).

Vigour index = Germination % x Total length of seedling (mm)  
(Root and shoot length)

3.22 Bioinoculation effect of different bioformulations of PGPB cells on the enhancement of adhesion with rice roots cv. BPT-5804

The Azospirillum, Pseudomonas and Methylobacterium cells were grown and harvested as mentioned in 3.17.1 and the different bioformulations of PGPB cells were prepared as detailed in 3.17.4 and 3.13.1. The growth of rice plants and the collection of rice roots were done according to 3.13.1. The
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Adsorption assay of *Azospirillum, Pseudomonas* and *Methylobacterium* cells under different bioformulations *viz.*, 1. Single strain inoculation of *Azospirillum, Pseudomonas* and *Methylobacterium*, 2. Co-inoculation of *Azospirillum Pseudomonas* and *Methylobacterium* cells 3. Multigeneric PGPB co-aggregates (natural and artificial) was carried out as per 3.11.8.2.

**3.22.1 Effect of foliar application of ISR inducing chemicals**

Rectangular cement pots of size 18”x12”x12” were filled with 45 kg of paddy field soil flooded with water for 2 days and brought to fine puddle conditions. Seeds of the rice variety BPT-5804 were loosely packed separately in small gunny bag and soaked in water for 12 h. Then the bags were subsequently kept in dark place after covering with wet gunny bags to ensure optimum condition for germination. The seeds germinated within about 24 h. after soaking. The pre-germinated seeds of BPT-5804 rice was sown in rows in pots separately. On the 5th day of sowing the seedlings were thinned to get 50 numbers per pot. The seedlings were raised under wet conditions and the age was counted from the time of sowing.

After the sowing of rice seeds, four resistance inducing chemicals, *viz.*, Acibenzolar, Naphthalene acetic acid, Jasmonic acid and salicylic acid (Central Drug House) at 0.075 and 0.1 per cent level, were sprayed individually on 15th DAS prior one day to the challenge inoculation of *Xanthomonas oryzae pv oryzae* to rice plant. The observations were recorded one week after the challenge inoculation of *Xanthomonas oryzae pv oryzae* and expressed as percentage disease incidence as detailed elsewhere in the text.
3.22.2 Effect of different bioformulation with salicylic acid on plant height and disease incidence

The surface sterilization, germination, preparation of growth chamber, placement of germinated rice seeds in growth chamber and maintenance of the same were done as specified in 3.11.1 and 3.18.15.1. The effect of different bioformulations viz., single strain inoculation, coinoculation and coaggregates application (natural and artificial) along with the salicylic acid application at 0.1% concentration was tested for the incidence of bacterial leaf blight disease in rice. The leaf blight disease incidence was evaluated by score-chart method (IRRI, 1980). All the following treatments were tested.

T₁ - Control
T₂ - Azospirillum alone
T₃ - Pseudomonas alone
T₄ - Methylobacterium alone
T₅ - Azospirillum + SA
T₆ - Pseudomonas + SA
T₇ - Methylobacterium + SA
T₈ - Azospirillum + Pseudomonas + Methylobacterium CoI
T₉ - Azospirillum + Pseudomonas + Methylobacterium CoI + SA
T₁₀ - Azospirillum + Pseudomonas + Methylobacterium Co-AG (N)
T₁₁ - Azospirillum + Pseudomonas + Methylobacterium Co-AG (N) + SA
T₁₂ - Azospirillum + Pseudomonas + Methylobacterium Co-AG (A)
T₁₃ - Azospirillum + Pseudomonas + Methylobacterium Co-AG (A) + SA
3.23 Bioinoculation effect of different bioformulation of PGPB cells along with salicylic acid supplementation and challenge inoculation of *Xanthomonas oryzae pv oryzae* on ISR mediated biocontrol against *Xanthomonas oryzae pv oryzae* in rice cv. BPT-5804

Rice (*Oryza sativa* L.) cv. BPT-5804 seeds were surface sterilized, germinated as detailed in 3.11.2 and transferred on to a steel wire mesh (3 mm dia) in a growth chamber filled with 100 mL weaver's medium (Weaver's *et al.*, 1975). One ml of inoculum of the PGPB isolates under different bioformulations viz., 1. Control, 2. *Azospirillum* alone, 3. *Pseudomonas* alone, 4. *Methylobacterium* alone, 5. *Azospirillum* with Salicylic acid, 6. *Pseudomonas* with Salicylic acid, 7. *Methylobacterium* with Salicylic acid, 8. *Azospirillum Pseudomonas* and *Methylobacterium* Co-inoculation, 9. *Azospirillum Pseudomonas* and *Methylobacterium* Co-inoculation with Salicylic acid, 10. *Azospirillum, Pseudomonas* and *Methylobacterium* Co-aggregates natural (N), 11. *Azospirillum, Pseudomonas* and *Methylobacterium* Co-aggregates natural (N) with Salicylic acid was prepared and maintained to evaluate the biocontrol response of PGPB isolates, against *Xanthomonas oryzae pv oryzae* and the following treatments were tested.

T<sub>1</sub> - Control

T<sub>2</sub> - *Azospirillum* alone

T<sub>3</sub> - *Pseudomonas* alone

T<sub>4</sub> - *Methylobacterium* alone

T<sub>5</sub> - *Azospirillum* + SA
T₆ - *Pseudomonas* + SA

T₇ - *Methylobacterium* + SA

T₈ - *Azospirillum* + *Pseudomonas* + *Methylobacterium* CoI

T₉ - *Azospirillum* + *Pseudomonas* + *Methylobacterium* CoI + SA

T₁₀ - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Co-AG (N)

T₁₁ - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Co-AG (N) + SA

### 3.23.1 Preparation of growth chamber

The growth chamber was a desiccator (12 x 10 cm) consisting of two parts. The lower part was filled with 200 mL weaver’s medium (Weaver’s *et al.*, 1975) and upper part contained stainless steel wire mesh (mesh size 3 mm) supports. The lid was placed over the cotton and the chamber was closed before sterilization. The growth chamber was sterilized by autoclaving.

Fifty germinated rice seeds with coleoptile (2 cm high) were transferred aseptically onto the stainless steel wire mesh, incubated for 10d in the growth chamber with 14 h day and 10 h night cycle and temperature ranging from 24°C at night to 32 °C around noon. By this time, the rice roots yielded many lateral roots, well spread in the weaver’s medium maintained at the lower part of the growth chamber.

After 10 d of incubation, the rice plants were challenge inoculated with *Xanthomonas oryzae pv oryzae* by spraying the cell suspension at $1 \times 10^7$ CFU mL⁻¹ inoculum level on 14th DAS with an atomizer whereas the control plant was sprayed with distilled water only. High humidity was created by sprinkling the water frequently in the polyhouse.
3.23.1.1 Composition of Yeast extract dextrose calcium carbonate
medium (YDC) (Schaad, 1998)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

After one week of challenge inoculation, three plants from each treatment were carefully removed and rinsed with sterile distilled water. The leaf blight disease incidence was enumerated with a score chart of 0 to 9 grades devised by International Rice Research Institute (1980).

3.23.1.2 SES (Standard Evaluated System)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description of the disease condition on the plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No lesion</td>
</tr>
<tr>
<td>1</td>
<td>Small water soaked stripes from the tips</td>
</tr>
<tr>
<td>2</td>
<td>Water pores are rapidly enlarged</td>
</tr>
<tr>
<td>3</td>
<td>Yellow lesion formed a wavy margin</td>
</tr>
<tr>
<td>4</td>
<td>Typical leaf blight lesions, elliptical, 1-2 cm long, usually confined to the area of the two main veins, infecting less than 2% of the leaf area.</td>
</tr>
</tbody>
</table>
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5 Typical leaf blight lesions infecting less than 10% of the leaf area

6 Typical leaf blight lesions infecting 10-25% of the leaf area

7 Typical leaf blight lesions infecting 25-50% of the leaf area

8 Typical leaf blight lesions infecting 51-75% of the leaf area and many leaves dead

9 Later on disease areas turn white to gray

10 Infected leaves wilt, roll up, finally entire plant die.

From the above score chart, the percentage of disease index (PDI) was worked out with the following formula.

Percentage of disease index =

\[ \frac{\text{Total ratings}}{\text{Total number of leaves graded} \times \text{Maximum grade in the score chart}} \times 100 \]

3.24 ESTIMATION OF BIO-CHEMICAL CONSTITUENTS

3.24.1 Methods of sampling

Plant sample material from each treatment was taken at 0, 7, 14 and 21 days after challenge inoculation of *Xanthomonas oryzae pv. oryzae* for estimating the changes in the biochemical constituents *viz.*, reducing sugars, non-reducing sugars and starch content, ortho dihydroxy phenol (OD Phenol), total phenolic content and enzymes like peroxidase (PO) and polyphenol oxidase (PPO).
3.24.2 Preparation of ethanol extract

Plant samples were collected, pooled and 4 g of pooled samples were taken for extraction. They were chopped and then extracted in boiling 80 percent ethanol (Mahadevan and Sridhar, 1986) and the extract was used for the estimation of sugars, phenol and enzymatic activity.

3.25 Quantitative estimation of sugars

3.25.1 Reducing sugar

Reducing sugar present in the extract was estimated according to Nelson (1944) method.

3.25.2 Non reducing sugars

Non-reducing sugars with ethanol extract were hydrolyzed and the total sugars were estimated by employing Nelson’s method. The total reducing sugars were calculated as glucose equivalents. The final concentration was calculated by detecting the reducing sugar present in the un hydrolyzed original sample from the reducing sugar present in the hydrolyzed sample. Hydrolysis of non-reducing sugars was carried out according to InMan (1965).

3.25.3 Quantitative estimation of phenols

a. Total phenols

Total phenols were estimated by employing Folin-ciocalteau reagent (Bray and Thorpe, 1954).

b. Ortho-dihydroxy (OD) phenol

Arnow’s reagent (Johnson and Schaal, 1957), specific for ortho groups was used for estimation of OD phenol.
3.25.4 Enzyme assay

3.25.4.1 Enzyme extraction

One g of the leaf material was cut into small bits, crushed in chilled 0.1 M sodium phosphate buffer pH 7.1 and the volume was made upto 5 mL with the buffer, centrifuged at 2,100 rpm for 30 min and the supernatant was used as the enzyme source and all the enzyme assays viz., polyphenol oxidase and peroxidase performed in a UV Spectrophotometer at 28 ± 1°C (Sridhar et al., 1969).

The activity of polyphenol oxidase was estimated by the method of Matta and Dimond (1963), Peroxidase by Hampton (1968). The enzymes activity in the sample was expressed in terms of unit/minute/mg of protein

3.26 Effect of different bioformulations of PGPB cells together with supplementation of salicylic acid and challenge inoculation of \textit{Xanthomonas oryzae pv oryzae} on the enhancement of growth and yield parameters in lowland rice cv. BPT-5804

A pot culture experiment was conducted to study the effect of different bioformulations viz., single strain inoculation, co-inoculation and coaggregates application of PGPB cells \textit{viz.}, \textit{Azospirillum}, \textit{Pseudomonas} and \textit{Methylbacterium} together with salicylic acid supplementation and challenge inoculation of \textit{Xanthomonas oryzae pv. oryzae} at 75% recommended ‘N’ level on the enhancement of growth and yield in lowland rice. The study was conducted during Rabi season (Sep to Jan, 2011-12).

Rectangular cement pots of size 18”x12”x12” were filled with 45 kg of paddy field soil, flooded with water for 2 days and brought in to fine puddle
condition. Seeds of the rice variety BPT-5804 were loosely packed separately in small gunny bag and soaked in water for 12 hr. Then, the seeds were bioinoculated as per the detail in the treatments and subsequently kept in dark place after covering with wet gunny bags to ensure optimum condition for germination. The seeds germinated within 24 hr after soaking. The pre-germinated seeds of rice (cv. BPT-5804) was sown in rows in pots separately. On the 5th day of sowing, the seedlings were thinned to get 50 numbers per pot. The age of the seedlings were counted from the time of sowing.

**Treatments**

- **T<sub>1</sub>** - Control (100% N without bioinoculation)
- **T<sub>2</sub>** - *Azospirillum* alone + 75% N
- **T<sub>3</sub>** - *Pseudomonas* alone + 75% N
- **T<sub>4</sub>** - *Methylobacterium* alone + 75% N
- **T<sub>5</sub>** - *Azospirillum* + SA + 75% N
- **T<sub>5</sub>** - *Pseudomonas* + SA + 75% N
- **T<sub>6</sub>** - *Methylobacterium* + SA + 75% N
- **T<sub>8</sub>** - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Co-I + 75% N
- **T<sub>9</sub>** - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Co-I + SA + 75% N
- **T<sub>10</sub>** - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Coaggregates (N) +75%N
- **T<sub>11</sub>** - *Azospirillum* + *Pseudomonas* + *Methylobacterium* Coaggregates (N) + SA+75% N
During the experimental period the annual mean minimum and maximum temperature of the experimental area was 25 °C and 39 °C, respectively, and the mean highest and lowest humidity were 96 and 78 per cent, respectively. The mean annual rainfall of the area was 1500 mm.

A fertilizer schedule of 100% N ha\(^{-1}\) was followed for control plots while all other treatments followed 75% of recommended dose of nitrogen respectively.

The crop was given a hand weeding on 30\(^{th}\) DAS and well protected against pests and diseases. The experiment was maintained under limited water supply as per the conditions prevailing in lowland rice ecosystem. Five representative samples of plant hills in each pot were pegmarked for periodical observation.

3.26.1 Challenge inoculation of rice plants with *Xanthomonas oryzae pv. oryzae*

The inoculum of the pathogen which was maintained in Yeast extract – Dextrose – Calcium carbonate agar (YDC) medium was used for the inoculation purpose. Thick cell suspension was prepared with sterile distilled water from 10 d old culture maintained in YDC medium.

Then, the cell suspension \(1 \times 10^7\) CFU mL\(^{-1}\) inoculum level was added with few drops of Tween-80 which increased the adherence capacity of the cells and acts as a sticker. Before spaying, the plants were pre-incubated for sometime by covering with thick, water sprayed polyethylene sheets to preserve moisture and increase the leaf cells more prone to the pathogen. Then, sheets were removed and spraying of cell suspension was done late in the evening. Control plants were also sprayed with sterile distilled water. After spraying, the
plants were covered with polyethylene bags again for about 72 h to maintain the humidity.

3.26.1.1 Sampling

For biochemical analysis, the samples were collected at 0, 7, 14 and 24 d after challenge inoculation of *Xanthomonas oryzae pv oryzae*.

3.26.1.2 Evaluation of disease incidence

Assessment of the disease incidence was done with a score chart of 0 to 9 grades devised by International Rice Research Institute (1980).

3.27 GROWTH PARAMETERS

3.27.1 Effect on plant growth

The height of the plant from each treatment was measured at 30<sup>th</sup> and 45<sup>th</sup> days after sowing (DAS). The mean value of plants from 3 replications was recorded.

3.27.2 Effect on dry weight of root and shoot

The dry weight of the root and shoot was taken at 30<sup>th</sup> and 45<sup>th</sup> days after sowing (DAS). Three plant samples were drawn, washed, air dried and later dried to a constant weight in an oven at 60 °C. The oven dried weight of the root and shoot samples were recorded.

3.27.3 N content of plant

The plant samples were collected at 30<sup>th</sup> and 45<sup>th</sup> day after sowing (DAS), washed in water, air dried and later dried to a constant weight in an oven at 60 °C. Then, they were powdered, sieved and 100 mg of sample was taken for analysis. The total nitrogen content was estimated by Microkjeldahl
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method (Bremner, 1960). Phosphorus content was estimated by triple acid digestion method (Jackson, 1973).

3.27.4 Organic carbon content

The rhizosphere soil (loosely adhered soil with rice roots after shaking – off) was collected and air dried for 2 days. After air drying, 5 g of the soil was analyzed for organic carbon content following the procedure of Walkley and Black (1947).

3.27.5 Extraction and estimation of IAA from rice (Tien et al., 1979)

One g of rice roots were collected from each treatment, washed thoroughly with water and freeze dried. The frozen tissues were cut into small pieces of 1-2 cm and homogenized to a fine paste in a mortar with pestle. The homogenates were extracted with 200 mL of peroxide free ethyl ether by thoroughly shaking in a separatory funnel at 4 °C. The extraction was repeated with 3 x 100 mL ether. After the extraction, the volume of ether was reduced to represent 2 mL of extract for 5g tissue by evaporation at 40 to 50 °C on a water bath. This fraction was shaken with 40 mL of 5% NaHCO₃ in a separatory funnel to separate neutral and acidic auxins. The bicarbonate fraction was acidified to pH 3.0 with 6 N HCl and again extracted with 4 x 100 mL of ether. Then, the extracts were pooled and ether was evaporated to dryness. The residue was dissolved in minimum quantities of methanol and used for IAA estimation as described earlier in chapter 3.12.1(1).

3.27.6 Total chlorophyll content of rice leaves

One g of the fresh leaves was cut into small pieces, homogenized with excess acetone in a clean mortar with pestle and the extract was decanted by
using Whatmann No.42 filter paper. The extraction was repeated again with 80 per cent acetone. The contents decanted and the brie was washed with acetone until colourless. The filtrates were pooled, volume made upto 100 mL in a volumetric flask. Then, 5 mL of the extract was diluted to 50 mL with acetone (80%) as per the procedure of Mahadevan and Sridhar, (1986).

The absorbance of this extract was measured at 645 nm and 663 nm and the total chlorophyll content of rice leaves determined by using the following formula.

\[
\text{Total chlorophyll (mg/g)} = \frac{A_{645} + 8.02 A_{663} \times V}{a \times 1000 \times w} 
\]

where:
- \( A_{645} \) and \( A_{663} \) - Absorbance at 645 nm and 663 nm, respectively.
- \( V \) - Volume of the extract in mL.
- \( w \) - Fresh weight of the sample in g.
- \( a \) - Length of light path in the cell.

3.28 YIELD PARAMETERS

3.28.1 Grain and Straw Yield of Rice

The matured crop was harvested from each pot, hand threshed, winnowed and sun dried. The dried grains from each treatment were weighed and recorded. After threshing, the rice straw was sun dried and the weight was recorded.

3.29 STATISTICAL ANALYSIS

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan’s multiple range test (DMRT) as per the procedure described by Gomez and Gomez (1984).