CHAPTER 3

MATERIALS AND METHODS
Materials and Methods

The experiments undertaken in the present investigation were carried out in the laboratory and fields of the Department of Biotechnology, Gauhati University. Details of the materials used and methodologies followed in conducting these experiments are:

3.1. Geographical location

The experimental site is located at 26° 10'45" latitude, 91° 45'0"E longitude and at 55m above mean sea level.

3.2. Climate and weather

The city of Guwahati in Kamrup district of Assam experiences sub-tropical humid climate, with hot summer from May to August, cold winter from November to January and mild winter during September, October as well as February to April. The rainfall intensity is highest during the monsoon season, which normally commences from the first week of June. However, pre-monsoon showers normally begin in the month of April and the rainy season extends from April to October. The dry period with scanty rainfall prevails from January to March.

3.3. Source of the pathogen

Brinjal plants showing typical symptoms of bacterial wilt were collected from the brinjal cultivated fields of Singimari in Kamrup district (Plate 1, Plate 2 and Plate 3) during winter (December 2006 – January 2007) and brought immediately to the laboratory. For subsequent experiments, the infected seedlings were collected from the experimental plots of Deptt. of Biotechnology, Gauhati University.
3.4. Isolation of the pathogen

The pathogen *R. solanacearum* was detected in the infected plants by ooze test. Stems of infected brinjal plants were cut obliquely at the base and placed in sterile distilled water taken in a test tube. The stem pieces showing milky white ooze in water were selected for isolation of the pathogen.

The pathogen was isolated on triphenyl tetrazolium chloride (TTC) Agar medium (Kelman 1954). The infected stems showing milky white bacterial ooze were cut into small pieces of about 0.5 cm length and transferred to a test tube containing 10 mL sterile distilled water. The bacteria were allowed to diffuse into the water by shaking the pieces for 15 mins. By following the serial dilution plate technique (Waksman 1922), a serial dilution of the bacterial suspension stock was made upto $10^{-8}$ dilution. 0.1 mL of the final dilution ($10^{-8}$) was pipetted aseptically into petriplates, each containing 10 mL of TTC medium. The petriplates were then incubated at 28±1°C for 24 hrs. The virulent colonies in the medium characterized by dull white colour, fluidal, irregularly round with light pink centres were further streaked on TTC medium to get pure colonies of the bacterium.

3.5. Preservation of the pathogen inoculum

The pathogen inoculum was stored in water (Kelman and Person 1961). Two loopfuls of bacterium from 48 hr old colonies grown on Kelman’s TTC Agar were transferred to 5 mL of sterile double distilled water in screw capped vials. These were stored under refrigeration at 20°C for maintenance of virulence (Overbreek et al. 2004). To revive an isolate, the stored bacterium was streaked on TTC Agar medium and well separated fluidal colonies were selected.
Plate 1: Brinjal plantation at the harvesting stage at Bonsor in Singimari, Kamrup District, Assam
Plate 2: Harvesting of brinjal crops for the local markets of Assam
Plate 3: Wilt infected brinjal seen in the plantations at Singimari, Kamrup District, Assam
3.6. Preparation of the pathogen inoculum

Pure cultures of the pathogenic bacterium were made on Nutrient Agar slants. A bacterial suspension was prepared by pouring sterile distilled water over 24 hr old bacterial growths on Nutrient agar slants and allowed to stand for 1 hr to let the bacterial cells to diffuse into the water. The suspension so obtained was adjusted to optical density (O.D) 0.5 in Spectrophotometer (Spectronic 20) in blue filter (425nm) to obtain a bacterial population of $1 \times 10^8$ cfu/mL.

3.7. Pathogenicity test

Seeds of brinjal (*Solanum melongena* L. cv. Pusa Kranti) were obtained from National Seeds Corporation, Pusa. These were sown on earthen pots (26cm x 22cm x 32cm) simulating nursery beds. The pots were filled with sand and potting medium in the ratio 1:3 respectively. The potting medium was composed of humus, clay and peat in the proportion 15:35:50 respectively. On germination, the seedlings were transplanted into pots filled with the same potting substrate and sand. For the pathogenicity test, 30 days old brinjal plants were inoculated with pathogen inoculum @ $10^8$ cfu/mL by root inoculation technique (Winstead and Kelman 1952). (Plate-4) A set of three seedlings were inoculated with the bacterial suspension at concentration $10^8$ cfu/mL and another set of three seedlings were inoculated with sterile distilled water to serve as control (Plate- 5 and Plate- 7). The plants were observed and the symptoms produced were classified and graded following Kempe and Sequeira (1983) with certain modifications. Pathogenicity test was confirmed after Koch’s postulation. The pathogen was reisolated from the inoculated plants and cultured on TTC media and cultural characteristics observed. It was reinoculated on healthy brinjal seedlings and observed for the same symptoms.
Plate 4: Root inoculation of brinjal (Winstead and Kelman)
Plate 5: Pots inoculated with the pathogen showing wilting symptoms
Plate 6: View of a single pot inoculated with pathogen showing symptoms of wilt
Plate 7: Control plants inoculated with distilled water
Plate 8: View of a single control pot showing no signs of wilt
3.8. Quantitative determination of pathogen population in soil

Quantitative determination of pathogen population in soil was done by adopting serial dilution plate technique of Waksman (1922). Three soil samples were collected at random in polypropylene bags from the soil used for filling the pots i.e. sand and potting medium in the ratio 1:3. Potting medium consisted of humus, clay and peat in 15:35:50 proportion. Similarly, from the experimental field, soil samples were collected from three random sites in separate polypropylene sleeves. The soil samples were first air dried at room temperature and sieved separately through 400 mesh size sieve. 5g of soil from each sample was mixed with 50 mL sterile distilled water in an Erlenmeyer’s flask and was stirred in a rotary shaker for 20 mins. The suspensions were filtered through sterile Whatman No.1 filter paper into sterilized flasks. Six sterile plugged test tubes were taken, three for soil samples from experimental field and three for samples from soil for filling pots. Each of the six tubes was filled with 10 mL of respective soil suspensions which serve as stock solution. From each of these stock soil suspensions, six fold (10⁻⁶ level) dilutions were made by serially transferring 1 mL from the stock soil suspensions to 9 mL of sterile distilled water taken in test tubes labeled with 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions. In this way, serial dilutions from each of the six stock soil suspensions were made up to 10⁻⁶ dilution level. Finally, 0.1 mL suspension from each of 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions was pipetted aseptically on petriplates containing TTC media. Triplicate plates were made for each dilution and incubated at 28±1°C. After 48 hrs of incubation, colony forming units (cfu) R. solanacearum were counted with the help of colony counter.
3.9. Biovar determination of the pathogen

Biovar determination of the isolate was done by testing the ability of the bacterium to oxidize sugar and sugar alcohols by standard procedure (Hayward 1964). The following basal medium was used for biovar determination: NH$_4$H$_2$PO$_4$ 1g; KCl 0.2g; MgSO$_4$.7H$_2$O 0.2g; Peptone 1g; 1% (w/v) aq. solution of bromothymol blue 0.3 mL; Agar 1.5g; Distilled water 1L. The pH of the medium was adjusted to 7.1 with 40% (w/v) NaOH solution before addition of the agar.

Lactose, Maltose, Cellobiose solutions were filter sterilized, while mannitol and Sorbitol were autoclaved for 20 minutes as 10% (w/v) solutions (Hayward 1964). Dulcitol was added directly to the basal medium, which was then autoclaved for 20 mins. 5mL of each sugar and sugar alcohol solutions were added to 45 mL of molten cooled Hayward’s basal medium and 10 mL volumes of the resulting amended medium were dispensed into sterile plugged test tubes (Hayward 1964). Hayward’s medium without sugar or sugar alcohol carbon source served as control.

A suspension of the bacterium grown on Nutrient Agar for 48 hrs at 28±1°C was prepared by inoculating 300µL of sterile distilled water with a wire loopful of cells (Williamson et al. 2002). The test tubes of Hayward’s medium were inoculated with 30µL of the prepared suspensions and incubated at 28±1°C and checked for acid production (yellow colour) at various intervals for up to 5 weeks (Hayward 1964; He et al. 1983). The Biovar was determined from the following table according to Hayward (1964).
Table 3.1: Tests for biovar determination in *Ralstonia solanacearum*

<table>
<thead>
<tr>
<th>Utilization of:</th>
<th>Biovar</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
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<td>Cellobiose</td>
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<td>Mannitol</td>
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<tr>
<td>Sorbitol</td>
<td>-</td>
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<tr>
<td>Dulcitol</td>
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</tbody>
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+: Utilization of corresponding sugar / sugar alcohol

- : No utilization of corresponding sugar / sugar alcohol

**3.10. Morphological, physiological, cultural and biochemical characterization of the pathogen**

The pathogen *R. solanacearum* was characterized by following the guidelines described in the Bergey’s Manual of Systematic Bacteriology (Garrity G. 2001). Pure culture of the pathogenic bacterial culture was further characterized in Institute of Microbial Technology, Chandigarh, India.

**3.10.1. For morphological characterization** the following staining procedures were adopted.

**3.10.1.1. Gram’s staining:** Heat fixed smears of the bacterium were subjected to four different reagents in the order: Crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and safranin (counter stain). The stained slides were air dried and examined microscopically using oil immersion objective.
3.10.1.2. **Acid fast staining:** Heat fixed smears of the bacterium was stained with carbol fuchsin and then added acid alcohol (3% HCl + 95% alcohol) and methylene blue (counter stain) successively. The smears were observed under oil immersion objective of the compound microscope.

3.10.1.3. **Flagella staining:** Thin film of the bacterial suspension on the slide was covered with flagella mordant and then flooded with carbol fuchsin after washing with distilled water. Air dried slides were observed under oil immersion objective.

3.10.1.4. **Capsule staining:** Smear of the bacterium was flooded with 1% (w/v) aq. Crystal violet and then washed off with 20% copper sulphate. The dried smear was observed under oil immersion objective of compound microscope.

3.10.1.5. **Bacterial spore (endospore) staining:** Smear of the bacterium was prepared, air dried and heat fixed. The smears were then flooded with malachite green and then steamed for 5 mins, adding more stain from time to time. The slides were then washed with water and counter stained with safranin for 30 mins. The smear was then washed with distilled water, dried and observed under microscope.

3.10.2. The **cultural characteristics** were observed by culturing the pathogen in following culture media:

3.10.2.1. **Nutrient Agar Media:** Composition of the nutrient agar media is: Peptone 5g; Beef extract 3g; sodium chloride 5g; Agar 20g; Distilled water 1L; pH 7. The pathogenic bacterium isolated from stems of wilted brinjal plants in sterile distilled water serially diluted. 0.1mL of the final dilution was pipetted aseptically on Nutrient Agar plates and spread evenly on the surface of the media. The plates were observed after 48 hours of incubation at 28 ± 1°C and colony character recorded.
3.10.2.2. **TTC Agar media (Kelman 1954):** The media is specific for *R. solanacearum* and was used for the isolation of the pathogen from soil and wilted plants. The composition of the basal medium is: Dextrose 10g; Peptone 10g; Caesin hydrolysate 1g; Agar 20g; Distilled water 1L.

To each of the sterilized, cooled and melted basal medium, 5 mL of 1% (2,3,5-triphenyl tetrazolium chloride) solution was added to give a final concentration of 0.005%.

3.10.2.3. **King's medium B (King et al. 1954):** Fluorescence pigmentation was studied on KMB Medium which consisted of: Proteose peptone 20g; K$_2$HPO$_4$,3H$_2$O 1.5g; MgSO$_4$,7H$_2$O 1.5g; Glycerol 15 mL; Agar 20g; Distilled water 1L. pH 7.2. The pathogenic bacterium was incubated on this medium at 28°C for 48 hrs and after 48 hrs colonies were observed in the dark for fluorescens with a U.V lamp (366 nm) according to Sands (1990).

3.10.3. The **Physiological tests** performed for the bacterial pathogen are given below:

3.10.3.1. **Growth at different temperature:** The bacterial inoculum was streaked on Nutrient Agar plates labeled with different temperatures. The inoculated plates were incubated at variable temperatures viz. 0°C, 4°C, 22°C, 28°C, 30°C, 37°C and 42°C in ice box, refrigerator and electric incubator. The cultures were examined after 2 days of incubation at 22°C, 28°C, 30°C, 37°C and 42°C and after 10-14 days of incubation at 0°C and 4°C for the presence or absence of growth and the degree of growth i.e minimal, moderate and heavy.

3.10.3.2. **Growth at different pH:** Nutrient broth was prepared and taken in tubes. pH of the medium was adjusted at 4,5,6,7,8,9 and 10 in different tubes using a pH
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0.1 mL of the bacterial culture was inoculated in the media of variable pH and observed for growth.

3.10.3.3. Growth on NaCl (%): For this test NaCl broth of Hayward (1964) was used which contained Peptone 5g; yeast extract 3g; Glucose 5g; Distilled water 1L and either 5, 10, 15 or 20g of NaCl. pH 7-7-2. The broth was sterilized at 121°C for 15 mins and dispensed into sterile 100 mL flasks. The bacterium was inoculated into the flasks and incubated at 28±1°C. The flasks were observed for growth even 2 days.

3.10.3.4. Growth under anaerobic conditions: Nutrient agar deep tubes were taken and the medium melted in hot water bath at 100°C. The molten agar was cooled to 45-50°C and then inoculated by adding one loopful of the inoculum. This was followed by rotating the tube thoroughly mix the inculum with the molten medium. The medium was solidified rapidly by placing the tube in an upright position into ice water bath. The tubes were then incubated at 28±1°C for 48 hrs.

3.10.4. The biochemical tests conducted are listed below:

3.10.4.1. Oxidase test: The oxidase activity was detected by the method of Kovacs (1956). Freshly grown cultures (24 to 48 hrs) from nutrient agar with 1% glucose were patched onto a filter paper moistened with a fresh oxidase reagent (1% w/v aq. Solution of tetramethyl- paraphenylene diamine dihydrochloride) using a wooden stick. A purple reaction in 30s was recorded as positive (Sands 1990).

3.10.4.2. Catalase test: Catalase test was performed according to the method described by He et al. (1983). 1mL of 3% hydrogen peroxide solution was added to a petridish and a loop of fresh culture grown on Nutrient Agar medium was added into the solution. Release of bubble from the culture was recorded as Catalase positive (Sands 1990).
3.10.4.3. Carbohydrate Catabolism: The ability to utilize carbohydrate viz. glucose sucrose was tested for the pathogenic bacterium by Hugh Leifson's OF test (Aneja K.R. 2005). The medium was prepared and added into tubes and flasks. Composition of the medium: Peptone 2g; NaCl 5g; K$_2$HPO$_4$ 0.3g; Bromothymol blue solution 15mL; agar 3g; Distilled water 1000mL; pH 7.1. After sterilization, at 121°C for 15 minutes, 1 mL of sterile glucose and sucrose solution (10%) were added separately to produce final conc. of 1%. 10% (w/v) solution glucose was sterilize for 10 minute at 110°C while 10% (w/v) sucrose solution was filter sterilized. When the media solidified, the tubes were inoculated with the bacterium by stabbing with a straight wire. Two inoculated tubes were taken for each carbohydrate. One uninoculated tube for each served as control. In one inoculated tube for each carbohydrate, liquid paraffin was poured over the medium about 1 cm depth. The tubes were incubated at 28 ±1°C and observed for change in colour (yellow) for upto 7 days.

3.10.4.4. Nitrate reduction test: This test was accomplished as suggested by Hayward (1964) using the medium of Fahy and Hyward (1983). It was a semi soft agar medium containing (g/l) Peptone 10g, Potassium nitrate (KNO$_3$) 2g and Agar 3g which was boiled to dissolve the agar. The pH was adjusted to 7 with conc. NaoOH, and the medium dispensed into test tubes and autoclaved. The tubes were stab inoculated with a loop of the test bacterium and then filled with sterile melted 3% water agar. Control test tubes were not inoculated with the bacterium. Good growth in 5 days at 30°C was taken as indicative of nitrate reduction into nitrite (Sands 1990).

3.10.4.5. KOH solubility test: This test was performed according to Fahy and Hayward (1983) using 48 hr culture. Two to three drops of 3% KOH were put onto glass slide and the colony of test bacterium was stirred into the solution with a clean
loop for 5 to 10 sec. When the solution was viscous enough to stick to the loop causing a thin strand of slime, then the test is recorded as positive (KOH Soluble).

3.10.4.6. Tween 80 hydrolysis: Fatty acid esterase activity was tested by streaking the bacteria onto a nutrient agar medium containing Calcium chloride and Tween 80, a polymer consisting of polyoxy-ethylene-sorbitanmonooleate (Sands 1990). The medium contains Peptone 10g; CaCl₂ dihydrochloride 0.1g; Sodium chloride 5g; agar 15g; Distilled water 1L; with the pH adjusted to 7.4. Tween 80 was autoclaved separately and 10mL was added to 1L media and mixed before platting. Incubation was done at 28±1°C for upto 7 days (Fahy and Hayward 1983). An opaque zone of crystals around a colony was recorded as positive reaction for hydrolysis of Tween 80 (Sands 1990).

3.10.4.7. Hydrogen sulphide production test: Stab inoculation of the bacterium was done in SIM (Hydrogen sulphide, Indole and motility detection) Agar medium and incubated at 28±1°C for 48 hrs. composition of the SIM Agar medium is : Peptone 30g ; Beef extract 3g ; Ferrous ammonium sulphate 0.2g ; Sodium thiosulphate 0.025g ; Agar 3g ; Distilled water 1L pH 7.3. Presence of black coloration along the line of stab inoculation was taken as positive for H₂S production.

3.10.4.8. Starch hydrolysis test: The starch Agar medium was streaked with the test bacterium. After incubation for 48 hours at 28±1°C when heavy growth occurred, the plates were flooded with IKI solution (iodine 1g; Potassium iodide 2g; Distilled water 100mL). A clear zone around a colony was recorded as positive reaction (Sands 1990). Composition of the Starch Agar medium: Starch soluble 20g; Peptone 5g; Beef extract 3g, agar 20g; Distilled water 1L ; pH 7.
3.10.4.9. **Gelatin hydrolysis test:** Nutrient Agar was 0.4% (w/v) gelatin was poured onto petridishes, cooled and dried overnight. Nutrient gelatin deep tubes were also prepared in test tubes. The test bacterium was streaked on the plates and the nutrient gelatin deep tubes were inoculated by stab inoculation. The tubes and plates were incubated at 28±1°C for 4 to 7 days. After incubation the tubes were put in the refrigerator at 4°C for 15 mins. The plates were flooded with 5 mL mercuric chloride solution (HgCl₂ 12g; dist. Water 80mL; conc. HCL 16 mL), (Sands 1990). The refrigerated tubes were examined whether the medium was solid or liquid and the flooded plates for any clearing around the line of growth (Dickey and Kelman 1988).

3.10.4.10. **IMViC Tests:**

3.10.4.10.1. **Indole production test:** 1% tryptone broth was prepared by dissolving 10g peptone in 1L distilled water and sterilized at 121°C for 15 mins. The broth was inoculated with the bacterial culture and incubated for 48 hrs. at 28±1°C. After 48 hrs of incubation, 1mL Kovac’s reagent was added and shaken gently for 10-15 mins. The tubes were then allowed to stand to permit the reagent to come to the top.

3.10.4.10.2. **Methyl red and Voges Proskauer tests:** 5 mL of MRVP broth was taken in three test tubes and two of them inoculated with loopfulse of Nutrient broth culture of the bacterium. An uninoculated tube served as control. The tubes were incubated for 48 hrs at 28±1°C. 5 drops of methyl red indicator were added to one tube for MR test and observed for change of colour of Methyl red. 12 drops of VP reagent I (napthol solution) and 2-3 drops of VP reagent II (40% potassium hydroxide) were added successively to the other inoculated tube for VP test. The tube was shaken gently for 30 sec. with the caps off to expose the media to oxygen. The reaction was allowed 15-30 mins to complete. The tubes were then observed for
change in colour for the VP test. Composition of MRVP broth (pH 6.9) is: Peptone 7g; Dextrose/Glucose 5g; Potassium phosphate 5g; Distilled water 1L.

3.10.4.10.2. Citrate utilization test: Simmon’s citrate agar medium was sterilized and poured into culture tubes for slant preparation. Streak inoculation of the nutrient broth culture of the bacterium was done on the slants. Tubes were then incubated at 28±1°C for 48 hours. The slants were then observed for growth and colouration of the medium. Composition of Simmon’s Citrate Agar (pH 6.9) medium is: Ammonium dihydrogen phosphate 1g; Dipotassium phosphate 1g; Sodium Chloride 5g; Sodium Citrate 2g; Magnesium Sulphate 0.2g; Agar 15g; Bromothymol blue 0.8g; Dist. Water 1L. The phosphates were dissolved separately in 100 ml of water and then added to the rest of the constituents and volume was made up to 1L.

3.11. Isolation of *Pseudomonas fluorescens*, the potential biocontrol agent for the management of *Ralstonia solanacearum*

*Pseudomonas fluorescens*, the potential biocontrol agent for the management of *Ralstonia solanacearum*, was isolated from the rhizosphere rhizoplane of healthy brinjal plan. The healthy brinjal plants were uprooted from the brinjal cultivated fields of Singimari in Kamrup district and root system along with the surrounding soils were placed in the polypropylene sleeves and carried to the laboratory. Soil particles loosely adhering to roots were gently removed. The roots were severed from the plant and cut into small pieces of about 1 cm length. 5g of root pieces with the soil particles tightly adhered was weighted out and dipped in 500mL of sterile dist. Water taken in 1L conical flask. The suspension was shaken in a rotary shaker for 20 mins of release the rhizoplane bacteria. 0.1 mL of the suspension was then taken inoculated in King’s Medium B (KMB) Agar plates (King *et al.* 1954; Vidhyasekaran and Muthamilan
1995). The plates were incubated at 28±1°C for 48 hours and then observed under U.V. transilluminator at 366 nm for colonies with green fluorescence.

3.12. Preservation of antagonist culture

The bacterial antagonist was grown on KMB agar for 48 hrs at 28±1°C. Colonies showing green fluorescence under UV-transilluminator (366nm) were picked up and enriched in nutrient broth. Thereafter these were streaked in KMB plates and preserved in KMB slants. The slants were covered with mineral oil and preserved in the refrigerator at 4°C for further use.


The population of *P. fluorescens* in the rhizosphere-rhizoplane of healthy brinjal plant was enumerated by the serial dilution plate technique of Waksman (1922). The excised roots were vigorously shaken to remove all but the tightly adhering soil particles. After cutting the roots into small segments of about 1cm, 5g was weighted out and shaken in 50 mL sterile distilled water. The suspension was then filtered through Whatman no.1 filter paper to get the stock solution. Eight fold (10^-8) dilutions were made from the stock solution by following dilution plate method of Waksman (1922). 1mL from the stock solution was pipetted out aseptically and added to 9mL of sterile distilled water to get 10^-1 dilution. From this dilution 1mL was pipetted out and added to another tube with 8mL sterile distilled water to get 10^-2 dilution. In this way 10^-3, 10^-4, 10^-5, 10^-6, 10^-7 and 10^-8 dilutions were prepared in the following six test tubes. During inoculation, 0.1ml of bacterial suspension was pipette aseptically into plates containing 10 mL of King's Medium B Agar and spread uniformly on the surface of the medium. Triplicate plates were maintained for each
dilution and uninoculated KMB plates were used as control. The plates were incubated in an inverted position at Flagella staining. After 48 hrs., the colony forming units (cfu) of *Pseudomonas fluorescens* showing typical blue green fluorescence were observed under U.V. Transilluminator at 366 nm and enumerated. No. of colony forming units (cfu) /g of rhizosphere soil was calculated as follows –

\[
\text{No. of cfu/g soil} = \frac{\text{Av. no. of colonies in a dilution} \times \text{Dilution factor}}{\text{Dry wt. of soil}}
\]

3.14. **Characterization of the isolated potential biocontrol agent,**

*P. fluorescens*

Morphological, cultural, physiological and biochemical characterization of the isolated potential biocontrol agent was carried out in the laboratory by following the guidelines described in the *Bergey’s Manual of Systematic Bacteriology* (Garrity G. 2001) and *Experiments in Microbiology, Plant pathology and Biotechnology* (Aneja 2005). For further confirmation, the bacterial culture was characterized in Institute of Microbial Technology, Chandigarh.

The different tests conducted were as follows:-

3.14.1. For **morphological characterization** of the potential bacterial biocontrol agent, the following staining tests were performed.

a) Gram’s staining  
   b) Flagella staining

c) Acid fast Flagella staining  
   d) Capsule Flagella staining

e) Endospore Flagella staining

3.14.2. The **physiological tests** conducted were as follows:

a) Effect of temperature on growth
b) Effect of pH on growth

c) Effect of NaCL (%) on growth

d) Effect on anaerobic conditions on growth

3.14.3. The cultural characterization was done by the bacterium in the following culture media.

3.14.3.1. Nutrient Agar Media: The bacterial suspension in sterile distributed water was inoculated in NA media by spread plate method and incubated at 28±1°C for 48 hrs. The colonies formed were observed and the characters recorded. The composition of the media was same as already described in Section 3.10.2.1

3.14.3.2. King’s Medium B Agar: The bacterial suspension in sterile distilled water was serially diluted and 0.1 mL from 10th dilution was inoculated in King’s Medium B Agar by pour plate method and incubated at 28±1°C for 48 hrs. The colonies formed were observed under U.V. light (366nm) for detection of fluorescence. The colony characters were recorded. King’s Medium B Agar (King et al. 1954) was used for multiplication and preservation of fluorescent Pseudomonades. The medium was specific for typical fluorescent blue green pigment production by fluorescent Pseudomonads. The composition of the medium was same as described in Section 3.10.2.3.

3.14.4. The biochemical tests performed were as follows:

a) Oxidase test

b) Starch hydrolysis test

c) Hydrogen sulphide production test

d) Gelatin hydrolysis test
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e) Indole production test

f) Methyl red and Voges Proskauer tests

g) Citrate utilization test

h) Oxidation of carbohydrates

i) KOH Solubility test

3.15. Evaluation of *Pseudomonas fluorescens* as potential antagonist against the pathogen *Ralstonia solanacearum* in vitro

*In vitro* test for evaluation of *P. fluorescens* as potential biocontrol agent against the pathogen *R. solanacearum* was conducted by following the paper disc plate method of Blair *et al.* (1971).

48 hr Nutrient broth culture of both the pathogen *R. solanacearum* and the potential antagonist *P. fluorescens* were taken. 10mL of sterilized, cooled and molter nutrient agar media was poured into petriplates and allowed to solidify. The pathogen inoculum was adjusted to a concentration of $10^8$ cfu/mL (O.D. 0.5) at 425nm (blue filter) in Spectronic-20 Spectrophotometer. 1mL of the pathogen inoculum was pipetted aseptically into NA plates and spread uniformly with a bent glass rod. Paper discs (1mm diameter) were sterilized and dipped in *P. fluorescens* suspension in nutrient broth. The discs were then placed on the surface of the inoculated NA plates and incubated in an inverted position at 28±1°C for 48 hrs. The plates were observed every 24 hours for 5–7 days (Plate 9 and Plate-10).
3.17. **Efficacy of the antagonist used as suspension / broth culture against wilt pathogen *R. solanacearum***

To evaluate the efficacy of *P. fluorescens* in controlling bacterial wilt of brinjal, an experiment was carried out in earthen pots during December 2006–April 2007 (Plate 11). The experiment was conducted in the Dept. farm by following Completely Randomized Block Design (CRBD) where each treatment was replicated thrice with two plants per replication. All the recommended agronomic practices were followed during the crop period. The earthen pots taken were of size 26cm x 22cm x 32cm and the potting medium consisted of sand and potting substrate in the ratio 1:3. The potting medium used was sterilized in an autoclave at 121°C for 30 min. and filled in the pots. The potting substrate was composed of humus, clay and peat in the proportion 15:35:50 respectively. The cultivar of brinjal (*Solanum melongena* L.) taken for the experiment was Pusa Kranti. The seeds of *S. melongena* cv. Pusa Kranti were obtained from National Seeds Corporation, Pusa.

3.18. **Preparation of the pathogen and antagonist cell suspension**

48 hrs nutrient broth culture of *R. solanacearum* and King's B Broth culture of *P. fluorescens* were taken. Both the cultures were respectively harvested in 10 mL sterile distilled water. The cell suspensions of the pathogen and antagonist were serially diluted from 10^{-1} to 10^{-5} in 9 mL distilled water taken in test tubes. The cell concentration in the final dilution was adjusted to 10^5 cfu/mL in Spectronic-20 Spectrophotometer (O.D O.5 at 425nm blue filter).
Plate 9 and 10: Inhibition zones observed around *P. fluorescens* discs in *R. solanacearum* plates

Plate 11: Evaluation of the efficacy of broth culture of the isolated strain of *P. fluorescens* in management of bacterial wilt of brinjal in pot experiment
3.19. Application of *P. fluorescens* cell suspension by different methods to control bacterial wilt of brinjal caused by *R. solanacearum*

The *P. fluorescens* cell suspension was applied by different methods viz. seed treatment, root treatment, soil treatment and their combinations viz. seed + root treatment, root + soil treatment, seed + soil treatment and seed + root + soil treatment to evaluate the efficacy of the antagonist in controlling bacterial wilt of brinjal.

For seed treatment, *P. fluorescens* cell suspension @10^8 cfu/mL was applied to surface sterilized seeds. The seeds were surface sterilized by immersing in 1% sodium hypochlorite solution for 2 – 3 mins. and then rinsed with sterile distilled water and dried overnight in a sterile blotting paper. The treated seeds were sown in nursery beds and the seedling transplanted onto pots.

For soil treatment, 25mL of *P. fluorescens* cell suspension @10^8 cfu/mL was mixed with 500g sterilized potting medium (1:20 V/W) (Sivakumar and Narayanswamy 1998) for each pot one day before transplanting of seedlings. Seedlings raised from seeds treated with *P. fluorescens* cell suspension were transplanted in treated soil to achieve seed + soil treatment.

For root treatment, brinjal seedlings raised from treated as well as untreated seeds were uprooted and the soil particles adhering to it were removed. The roots were then dipped in *P. fluorescens* cell suspension (@ 10^8 cfu/mL) for 30min. and dried for 1 hr. These were then transplanted onto pots filled with the potting medium.
For root + soil treatment, the seedlings treated at the roots were transplanted onto pots filled with potting medium amended with the *P. fluorescens* cell suspension @ $10^8$ cfu/mL.

For root + seed treatment, the seedling raised from treated seeds were also treated with *P. fluorescens* cell suspension @ $10^8$ cfu/mL at the roots and then transplanted onto sterilized potting medium.

For seed + root + soil treatment, the seedlings raised from treated seeds were uprooted and after gently removing the soil particles, the roots were dipped in *P. fluorescens*, cell suspension @ $10^8$ cfu/ml for 30 min. The treated roots were allowed to dry for 1 hr and then transplanted onto pots containing potting medium which was sterilized and amended with *P. fluorescens* cell suspension (@ $10^8$ cfu/mL) one day before transplanting.

Each of the above treatments was replicated thrice with 2 plants / replication.

15DAT, the brinjal plants receiving treatment by different methods were challenged with *R. solanacearum* cell suspension @ $10^8$ cfu/mL by following root inoculation technique (Winstead and Kelman 1952) except uninoculated control. For control treatment a set of 3 seedlings untreated at the seed, root or soil were challenged with the pathogen cell suspensions at 15 DAT (inoculated control) whereas another set of 3 seedlings were left uninoculated by the pathogen (uninoculated control).

**Disease record:** The wilt incidence was recorded upto 90 DAT.

The % wilt incidence (PWI) was calculated as follows:

\[
\frac{\text{No. of plants wilted in a treatment}}{\text{Total no of plants receiving that treatment}} \times 100
\]
3.20. Quantitative determination of pathogen and antagonist population in the rhizosphere soil

30, 60 and 90 days after transplanting of brinjal seedlings onto the pots, the pathogen and antagonist population in the rhizosphere soil were enumerated. 1g of rhizosphere soil was added to 10mL sterile distilled water blank and shaken thoroughly for 10 min in a rotary shaker. Serial dilutions $10^{-2}$ to $10^{-6}$ were prepared by adding 1ml of soil suspension from the previous dilution onto 9 mL of sterile dist. water taken in test tubes. Finally, 0.1 mL from $10^{-4}$ to $10^{-6}$ dilutions were inoculated into TTC agar and KMB agar plates. Thus replicates were maintained for each of the dilutions. The cfu from each plate were counted out and population of $P. \text{fluorescens}$ and $R. \text{solanacearum}$ / g rhizosphere soil was calculated as follows:

$$\text{No. of org/g rhizosphere soil} = \frac{\text{Av. No. of colonies in a dilution \times Dilution factor}}{\text{Dry wt. of soil}}$$

The correlation studies between percentage wilt incidence (PWI) and population of $P. \text{fluorescens}$ and also between $P. \text{fluorescens}$ and the pathogen $R. \text{solanacearum}$ were also carried out.

3.21. Preparation of substrate based bioformulation of $\text{Pseudomonas fluorescens}$

For mass production of the bacterial agents, solid state fermentation was done. The solid fermentation media consists of inert carriers with food bases for the mass production and amendment of biocontrol agents. The enriched organic manure with biocontrol value could be used for the management of pathogens and plant growth promotion (Lewis 1991; Nakkeeran et al. 2005)
3.21.1. Collection of solid substrates: The substrates were collected from different sources depending on their availability. Rice bran, wheat bran and decomposed mustard oil cake were collected from the local markets of Guwahati city. Farmyard manure, vermicompost, and rice straw were collected from the local farmers of Greater Guwahati and banana leaf was collected from the Department farm.

3.21.2. Preparation of substrates: The substrates were air dried for 14 days and then grinded properly. Banana leaves were chopped into small pieces and then dried before grinding. The powder of different substrates thus prepared was sieved through 350 meshes separately. Powder of substrates each weighing 100g were filled into polypropylene sleeves (12cm x 15cm) to which 10mL of sterile distilled water was added. Three packets of 100g each were prepared for each organic substrate. The packets were then heat sealed and sterilized at 121°C for 30 mins for 3 consecutive days.

3.21.3. Growth and multiplication of *Pseudomonas fluorescens* in different organic substrates:

The sterilized organic substrates viz. rice bran, wheat bran, vermicompost, decomposed mustard oil cake, rice straw, banana leaf and farmyard manure were taken. Three adhesive viz. polyvinyl alcohol (1.5% aq.), carboxymethyl cellulose (1% aq.), and white flour gum (50%) were used for comparative study. Mannitol (3% aq.) was used as an osmoticant. The biocontrol agent *Pseudomonas fluorescens* was grown on KMB Agar slant for 48 hrs. The slant culture of the biocontrol agent was then washed with sterile distilled water and allowed to stand for 1 hr to allow the bacterial cells to diffuse into the water. The cell suspension in distilled water was then adjusted
to optical density 0.5 in the Spectrophotometer to obtain a cell concentration of $10^8$ cfu/mL.

The modified method of Kloepper and Schroth (1981) was followed for the preparation of substrate based bioformulation of *P. fluorescens* which is as follows:-

The adhesive solutions polyvinyl alcohol (1.5% aq.), carboxymethyl cellulose (1% aq.), and white flour gum (50%) were taken and 10 mL of each was mixed with 100g substrate carrier contained in polypropylene bags (1:10 v/w). The pH was adjusted to 7 by adding suitable amount of CaCO$_3$, which varied with the type of substrate carrier adhesive mixture. The polypropylene bags were heat sealed and sterilized at 121°C for 30 mins.

The mixture was then spread in a sterilized non sticky disposable plate under sterile conditions with the help of a sterilized spoon. 8.5 mL of 3% mannitol was added as osmoticant to 100g of above mixture aseptically. Subsequently 10 mL of *P. fluorescens* cell suspension @ $10^8$ cfu/mL was immediately pipetted into the mixture (1:10 v/w) and thoroughly mixed with the help of sterilized spoon. The substrate carrier-adhesive-bioagent mixture was thinly spread on the non sticky plates (Plate 12), covered with another sterilized plate and incubated and allowed to dry for 3 days at room temperature (Kloepper and Schroth 1981). For ease of storage of the bioformulations and for subsequent studies, the 100g mixtures were divided into 3 parts (33.3g each) and packed in separate polypropylene sleeves (8cm x 6.5cm) and heat sealed (Plate 13). Another set of bioformulations was prepared with 3 packets for each substrate carrier and stored at 4°C for comparative study.
3.22. Population dynamics of *P. fluorescens* in the different substrate carrier based formulations

The population dynamics of the bioagent *P. fluorescens* was determined at different days after storage of the substrate carrier-adhesive- *P. fluorescens* formulations at room temperature and at 4°C. The different formulations prepared are listed below:

**Table 3.2:** List of substrate carrier – adhesive based bioformulations prepared and their abbreviations used in the present study.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Abbreviations</th>
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<tbody>
<tr>
<td>F1 Carboxymethyl cellulose + Rice bran + <em>P. fluorescens</em></td>
<td>CRbPf</td>
</tr>
<tr>
<td>F2 Carboxymethyl cellulose + Wheat bran + <em>P. fluorescens</em></td>
<td>CWPf</td>
</tr>
<tr>
<td>F3 Carboxymethyl cellulose + Rice straw + <em>P. fluorescens</em></td>
<td>CRPf</td>
</tr>
<tr>
<td>F4 Carboxymethyl cellulose + Vermicompost + <em>P. fluorescens</em></td>
<td>CVPf</td>
</tr>
<tr>
<td>F5 Carboxymethyl cellulose + Banana Leaf + <em>P. fluorescens</em></td>
<td>CBPf</td>
</tr>
<tr>
<td>F6 Carboxymethyl cellulose + Farmyard manure + <em>P. fluorescens</em></td>
<td>CFPf</td>
</tr>
<tr>
<td>F7 Carboxymethyl cellulose + Mustard oil cake + <em>P. fluorescens</em></td>
<td>CDPf</td>
</tr>
<tr>
<td>F8-F14 Polyvinyl alcohol + Substrate carrier + <em>P. fluorescens</em></td>
<td>CRbPf – PDPf</td>
</tr>
<tr>
<td>F15-F2 Whiteflour gum + Substrate carrier + <em>P. fluorescens</em></td>
<td>WRbPf – WDPf</td>
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</table>

The experiment was conducted by following the Completely Randomized design (CRD) with three replications for each treatment.

Viable population of *P. fluorescens* in the powder formulations was determined at 7, 15, 30, 60, 90 and 120 days after storage (DAS) at room temperature. The formulations were prepared by following the modified method of Kloepper and Schroth (1981) as already described in Section 3.21.3. 150g of each organic substrate-adhesive-*P. fluorescens* formulation was prepared. For this, 150g of substrate carrier
was mixed with 15mL of adhesive solutions (1:10 v/w) and pH adjusted to 7. After sterilization at 121°C for 30 mins, 12.75 mL of 3% mannitol (@ 8.5 mL of 3% mannitol for 100g formulation) was added aseptically followed by mixing with 15 mL of *P. fluorescens* cell suspension @ 10^8 cfu/mL (1:10 v/w). The carrier adhesive bioagent mixture was incubated for 72 hrs. at room temperature. These were packed separately in polypropylene bags, heat sealed and tested for the population of *P. fluorescens* after 7, 15, 30, 60, 90 and 120 days after storage (DAS) at room temperature.

For determining the number of colony forming units of *P. fluorescens* at different days after storage, the serial dilution plate technique of Waksman (1922) was followed. 1g of the adhesive-substrate carrier – *P. fluorescens* mixture was aseptically added to 10 mL of sterile distilled water and mixed thoroughly for 20 mins in a rotary shaker. Serial dilutions (8 fold) were prepared by pipetting 1 mL successively from the previous dilution to 9 mL sterile distilled water. 0.1 mL aliquots from each of 10^-5 to 10^-8 dilutions were then spread on KMB plates. The plates were incubated at 28±1°C for 48 hrs and then the cfu/g formulations were counted out. On the basis of highest recovery of cfu/g of *P. fluorescens*, the five best formulations were selected and evaluated for management of bacterial wilt of brinjal in pot experiment and in field.

### 3.23. Population dynamics of *P. fluorescens* in the different substrate carrier adhesive formulations at 4°C

The same experiment with 15 packets for each formulation including three replicates for each of the five treatments viz. 15, 30, 60, 90 and 120 days after storage (DAS) was conducted following Completely Randomized Design by storing the
powder formulations at 4°C for comparative analysis of the shelf life of the bioagent at room temperature and 4°C.

The method for preparation of different substrate carrier based formulations adopted to evaluate the population dynamics of *P. fluorescens* at 4°C is similar to that described in Section 3.21.3. The different formulations prepared for this experiment are similar to those listed in Table 3.2. However, for this experiment, the formulations were first stored at room temperature for 15 days to increase the initial population of *P. fluorescens*. Initial determination of population of *P. fluorescens* was made at 15 days after storage at room temperature and later samples were made at 30, 60, 90 and 120 days after storage (DAS) at 4°C.

Population dynamics was examined by mixing 1g of formulations aseptically with 10 mL sterile distilled water for 20 mins in a rotary shaker. Serial dilutions were prepared and 0.1 mL aliquot from $10^5$ to $10^8$ dilutions were spread on KMB plates. After incubating the plates at 28±1°C for 48 hrs the cfu/g formulations were counted out.

3.24. Evaluation of five best *P. fluorescens* based bioformulation against bacterial wilt of brinjal in pot experiment

On the basis of shelf life of *P. fluorescens* in the different carrier substrates, five best were selected out (Plate 14 and Plate 15). These were applied by different methods to evaluate their effectiveness in the control of bacterial wilt of brinjal in pot experiment. The experiment was conducted during August 2007 – January 2008 and repeated during February 2008 – July 2008.
Plates 12: Preparation of substrate carrier-adhesive-\textit{P. fluorescens} based formulation for application in the management of bacterial wilt of brinjal

Plate 13: Prepared substrate carrier-adhesive-\textit{P. fluorescens} based formulation

The cultivar of brinjal chosen for the experiment was Pusa Kranti, a bacterial wilt susceptible cultivar of brinjal (*Solanum melongena* L.) (Bhattacharya 1993). The brinjal seeds cv. Pusa Kranti were obtained from National Seeds Corporation, Pusa. The earthen pots taken were of size 26cm x 22cm x 32cm. A potting medium consisting of sand and potting substrate in the ratio 1:3 was sterilized at 121°C for 30 mins. and filled in the pots. The potting medium was composed of humus, clay and peat in the proportion 15:35:50 respectively (*Plate 16* and *Plate 17*).

The seeds of *Solanum melongena* L. cv. Pusa Kranti were surface sterilized by immersing in 1% sodium hypochlorite solution for 2-3 mins, rinsed with sterile distilled water and dried overnight in a sterile blotting paper covered with sterile petriplate.

The experiment was conducted in the Department Farm by following the Completely Randomized Block Design (CRBD), where each treatment was replicated thrice with two plants per replication.

For **seed treatment (S)**, 0.15g of each formulation containing *P. fluorescens* @ $10^8$ cfu/g was taken and mixed with 0.15 mL of rice gruel to form fine slurry (30g formulation mixed with 30 mL rice gruel to treat 1 kg seed) and kept for 30 mins to coat the seeds. The coated seeds were spread in a petriplate and dried overnight. Care was taken to avoid clumping of the seeds. Another set of seeds (5g) were treated with the slurry prepared by mixing 0.15 mL rice gruel with 0.15 mL sterile distilled water to serve as untreated seeds. The treated as well as untreated seeds were sown on earthen pots simulating nursery beds.

For **root dip treatment (R)**, 30 days old brinjal seedlings raised from bioformulation untreated seeds were uprooted. The soil particles loosely adhered to roots was gently removed. The roots of these seedlings were dipped into the
respective formulation rice gruel slurry for 30 mins and dried for 1 hr in shade. These were then transplanted into pots filled with the potting medium.

For soil treatment (So), 1.5g of each formulation was mixed with 30g sand (1.20 w/w) (Sivakumar and Narayanaswamy 1998) and 5.25g formulation sand mixture was incorporated into each pot one day before transplanting of seedlings. Formulation sand mixture amounting 31.5g was applied in six pots required for each formulation replicated thrice with two plants per replication. For control treatment, 5.25g sand was mixed with soil in each pot.

For seed + root treatment, roots of the seedlings raised from treated seeds were dipped for 30 minutes in each of formation-rice gruel slurry and dried for 1 hr in shade before transplanting into the pots filled with soil.

For the combination treatment seed + soil, seedlings raised from treated seeds, but untreated at the roots were transplanted into pots containing the respective formulation talc mixture, each formulation with three replications and two plants per replication.

For root + soil treatment, seedlings raised from untreated seeds and treated with particular bioformulation at the roots were taken and transplanted onto a set of six pots containing the respective formulation talc mixture. Same procedure was followed for rest of the four P. fluorescens based formulation.

For seed + soil + root treatment, seedlings raised from formulation treated seeds were uprooted and the corresponding formulation was applied to roots for 30 mins and dried for 1 hr. These seedlings treated at the roots were then transplanted onto 6 pots containing that particular formulation in soil. Same procedure was followed for rest of the four P. fluorescens based formulations.
For control treatments, the roots of seedlings raised from untreated seeds were dipped in the rice gruel and sterile distilled water slurry for 30 mins, dried for 1 hr in shade before transplanting onto pots.

At 15 days after transplanting, all brinjal plants were challenged with cell suspension of *R. solanacearum* (@ $10^8$cfu/mL) by following root inoculation technique (Winstead and Kelman 1952) except in uninoculated control.

The different treatments in the pot experiment along with methods of application are listed in the Table 3.3.

**Table 3.3:** Treatment combinations comprising of formulations and methods of applications

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Seed (S)</th>
<th>Soil (So)</th>
<th>Root (R)</th>
<th>Seed+Soil (S+So)</th>
<th>Soil+Root (So+R)</th>
<th>Seed+Root (S+R)</th>
<th>Seed+Soil+Root (S+So+R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC + Vermicompost +</td>
<td>CVPf (S)</td>
<td>CVPf (So)</td>
<td>CVPf (R)</td>
<td>CVPf (S+So)</td>
<td>CVPf (So+R)</td>
<td>CVPf (S+R)</td>
<td>CVPf (S+So+R)</td>
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<tr>
<td><em>P. fluorescens</em></td>
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<tr>
<td>CMC+Farmyard manure +</td>
<td>CFPf (S)</td>
<td>CFPf (So)</td>
<td>CFPf (R)</td>
<td>CFPf (S+So)</td>
<td>CFPf (So+R)</td>
<td>CFPf (S+R)</td>
<td>CFPf (S+So+R)</td>
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<td><em>P. fluorescens</em></td>
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<tr>
<td>CMC+ Decomposed Mustard oilcake +</td>
<td>CDPf (S)</td>
<td>CDPf (So)</td>
<td>CDPf (R)</td>
<td>CDPf (S+So)</td>
<td>CDPf (So+R)</td>
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<td>CDPf (S+So+R)</td>
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<td><em>P. fluorescens</em></td>
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<tr>
<td>PVA+Vermicompost + <em>P. fluorescens</em></td>
<td>PVPf (S)</td>
<td>PVPf (So)</td>
<td>PVPf (R)</td>
<td>PVPf (S+So)</td>
<td>PVPf (So+R)</td>
<td>PVPf (S+R)</td>
<td>PVPf (S+So+R)</td>
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<tr>
<td>PVA+Farmyard Manure +</td>
<td>PFPf (S)</td>
<td>PFPf (So)</td>
<td>PFPf (R)</td>
<td>PFPf (S+So)</td>
<td>PFPf (So+R)</td>
<td>PFPf (S+R)</td>
<td>PFPf (S+So+R)</td>
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<tr>
<td><em>P. fluorescens</em></td>
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<tr>
<td>Untreated seedlings/seed (only pathogen)</td>
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<tr>
<td>Untreated seedlings/seed (no pathogen)</td>
<td>uninoculated control</td>
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3.25. Disease Record

The number of wilted plants in each treatment was continuously recorded upto 90 days after inoculation with pathogen. The number of completely wilted plants was tabulated for each formulation applied by different methods. The % wilt incidence was calculated out by using the formula given below –

\[
\text{% wilt incidence} = \frac{\text{No. of plants wilted in each treatment-formulation}}{\text{Total no. of plants receiving that treatment}} \times 100
\]

For statistical analysis, the percentage values were converted to angular values.

3.26. Quantitative estimation of antagonist and pathogen population in rhizosphere soil

At 30, 60 and 90 days after transplanting, the population dynamics of antagonist \( P. \text{fluorescens} \) as well as of the pathogen \( R. \text{solanacearum} \) in the rhizosphere soil in the respective pots were estimated by the serial dilution plate technique of Waksman (1922). For each formulation treatment combination, the brinjal plant was uprooted and the root system was shaken to remove the soil loosely adhered to it. 1g of rhizosphere soil was weighed out and put in 10 mL sterile distilled water. This soil suspension was shaken thoroughly in a rotary shaker for 15 mins and then filtered through Whatman no. 1 filter paper. The filtrate was treated as stock solution and serially diluted upto \( 10^{-4} \) dilution in sterile distilled water. KMB plates and TTC plates were made and 0.1 mL aliquot from each of \( 10^{-5} - 10^{-8} \) dilutions were pipetted aseptically into KMB plates and TTC plates for enumeration of \( P. \text{fluorescens} \) and \( R. \text{solanacearum} \) colony forming units/g of rhizosphere soil.
respectively. Since populations of bacteria approximate a log normal distribution (Loper et al. 1985), values were log transformed before analysis to normalize variance.

\[
\frac{\text{No. of organism}}{\text{g}} = \frac{\text{Av. No. of colonies in a dilution} \times \text{dilution factor}}{\text{Wt. of soil taken}}
\]

3.27. Evaluation of five best *P. fluorescens* based bioformulation against bacterial wilt of brinjal in field experiment

The five best *P. fluorescens* based bioformulations selected on the basis of shelf life of *P. fluorescens* were evaluated for their efficiency in controlling the incidence of bacterial wilt of brinjal in field experiment (Plate-18, Plate-19 and Plate 20). The field experiment was done in the Department farm during October 2008 – March 2009 and repeated during August 2009 – January 2010.

The field area in which the experiment was conducted measured 12m x 8m. Each block measured 0.6 x 8m\(^2\) and space between the blocks was 0.25 x 8m\(^2\). In each block the space between two plants measured 0.25 x 3m\(^2\). The space between two rows of a block was 3m while the space between two columns was 0.2m.

The experiment was laid out in Completely Randomized Block Design (CRBD) and the *P. fluorescens* based formulations were applied in the same method as in pot experiment. The treatments for each of the five formulations were Seed treatment (S), Soil treatment (So), root treatment (R) and their combinations *viz.* S+So, So+R, S+R and S+So+R. The controls included.
Plates 16 and 17: Evaluation of five best substrate carrier-adhesive- 
*P. fluorescens* based formulations in the management of bacterial 
wilt of brinjal in pot experiment.

Plates 18, 19 and 20: Evaluation of five best substrate carrier-adhesive- 
*P. fluorescens* based formulations in the management of bacterial 
wilt of brinjal in field experiment.
Materials & Methods

a) Only the pathogen inoculated treatment with no bioformulation applied (inoculated control).

b) No pathogen or bioformulation applied (uninoculated control)

The CRBD used for carrying out the field experiment is depicted in Fig. 3.1

For each formulation, three plants were treated with a single method of application i.e. formulation treatment combination was replicated thrice. 15 days after transplanting all brinjal plants were inoculated with *R. solanacearum* cell suspension containing $10^8$ cfu/mL following root inoculation technique except in uninoculated control treatment.

The formulations were prepared and applied by the same method as followed for the pot experiment.

**Disease record**: The observations on wilt symptoms was made up to 90 days after transplanting and the % **wilt incidence** was recorded by the same method as described in Section 3.25.
### Materials & Methods

Field area: 12mx8m = 96 sqm

Total No. of bioformulations: 5

Total no. of treatments for each bioformulation: 9 (including control)

No. of replications for each treatment: 3

Total no. of blocks for each bioformulation: 3

Total no. of blocks in the field area: 5 x 3 = 15

Area of each block: 0.6 m x 8m

No. of plots in each block: 9

Space between two plants within a block: 0.25 m x 3 m

![Diagram of block design](image)

**Fig. 3.1:** Completely randomized Block Design for field experiment with 9 treatments, each replicated thrice for a single bioformulation.

<table>
<thead>
<tr>
<th>1 bioformulation</th>
<th>Other bioformulations</th>
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Treatment A: Seed; B: Root; C : Soil; D: Seed + Root; E: Root+Soil; F: Seed + Soil; G: Seed + Root + Soil; H: Inoculated control; I: Uninoculated control
Materials & Methods

3.28. Quantitative estimation of antagonist and pathogen population in rhizosphere soil

From each of the plots, root samples were collected from the plant receiving a formulation treatment combination. The root samples were made by severing at 2.5 cm away from the main root. The roots along with the surrounding soils were collected from each plant and carried to the laboratory in polypropylene bags separately. Soils loosely adhering to the roots were removed and the roots and soil tightly adhering to roots were designated as the rhizosphere soil samples for determination of microbial populations. The soil samples collected from each of the treatments were air dried separately at room temperature.

The population dynamics of antagonist *P. fluorescens* as well as the pathogen *R. solanacearum* in the rhizosphere soils in the respective plots were estimated at 30, 60 and 90 days after transplanting by following the serial dilution technique. The no. of colony forming units of both the pathogen and the antagonistic bacterium/g of rhizosphere soil was calculated out by the following formula.

\[
\text{No. of org/g soil} = \frac{\text{Av. No. of colonies in a dilution} \times \text{dilution factor}}{\text{Wt. of soil taken}} \times 100
\]

3.29. Physiological and biochemical analysis of bioformulation treated crops to evaluate the efficacy of the biocontrol agent as plant growth promoting rhizobacteria (PGPR)

To evaluate the efficacy of the biocontrol agent *P. fluorescens* as a PGPR applied to brinjal with different substrate carriers and to make a comparative study of its effect
upon % wilt incidence, the following biochemical parameters of brinjal plants were tested out 90 days after application of the bioformulations.

3.29.1. Determination of total carbohydrate by Anthrone method

100mg of sample (i.e. fruits taken from bioformulation treated brinjal) was weighed out and hydrolysed in a boiling water bath for 3 hrs with 5 mL of 2.5 N HCl and cooled to room temperature. Sodium carbonate was added for neutralization. When the effervescence ceased, the volume was made up to 100mL and centrifuged. The supernatant was collected and 0.5 mL and 1 mL aliquots were taken for analysis. 100 mg of glucose dissolved in 100 mL water served as stock. 10 mL of stock solution diluted to 100 mL with distilled water served as working standard. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard. 0 serves as blank. The volume was made up to 1 mL in all the tubes including the sample tubes by adding distilled water. Anthrone reagent was prepared by dissolving 200mg Anthrone in 100 mL of ice cold 95% sulphuric acid. 4 mL of anthrone reagent was then added and heated for 8 mins in a boiling water bath. It was cooled rapidly and the green to dark green colour of the test sample and standards were read at 630nm in a Spectrophotometer. There is a linear relationship between the absorbance and the amount of sugar that is present in the original sample. A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph the amount of carbohydrate present in the sample tube was calculated.

\[
\text{Amt. of carbohydrate present in 100mg sample} = \frac{\text{mg of glucose}}{\text{Vol. of test sample}} \times 100
\]

Amount of carbohydrate was presented in mg/g.
3.29.2. Estimation of protein Lowry’s method:

**Protein extraction:** 500 mg sample fruit (brinjal) was crushed thoroughly with some distilled water and volume was made upto 100 mL with distilled water. The mixture was filtered after homogenization. The filtrate was centrifuged to remove extra particles. To the supernatant 10% TCA was added to form precipitate. This was followed by centrifugation. To the precipitate 2N NaOH was added. The precipitate dissolved in 2N NaOH was used for protein estimation.

**Standard protein solutions:** 50 mL of 1% sodium dodesyl sulphate (SDS) was prepared by adding 0.5g SDS in 50 mL dist. water. To prepare stock solution, 50 mg of BSA (Fraction, sigma) was dissolved in 50 mL of 1% SDS.

**Working standard:** 5 mL of stock standard was diluted to 50 mL with distilled water to get a concentration of 100 µg/mL or 0.1 mg/mL.

**Protein estimation:** 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard was pipetted into test tubes. Volume was made upto 1 mL in all tubes with distilled water. 1 mL distilled water tube served as blank.

To each tube including test sample tube was added 1 mL of alkaline copper reagent, mixed and allowed to stand undisturbed for 10 min. Then 2 mL of phenol regent was added forcibly and rapidly to each tube. It was mixed well and incubated at room temperature in the dark for 30 min. Blue colour was developed. The absorbance of samples and standards was recorded at 660 mm in spectro photometer against blank. The protein content of samples was calculated by comparing with standard.
3.29.3. Estimation of chlorophyll by Arnon method:

The leaves of brinjal receiving different formulation treatment combinations in the field experiment were collected and 1g for each sample was weighed into a clean mortar. The tissue was ground to a fine pulp with the addition of 20 mL of 80% acetone. It was centrifuged (5000 rpm for 5 mins) and the supernatant was transferred to a 100 mL volumetric flask. The residue was ground with 20 mL of 80% acetone, centrifuged and the supernatant transferred to the same volumetric flask. This procedure was repeated until the residue was colourless. The mortar and pestle was washed thoroughly with 80% acetone and then finally the supernatant after centrifugation was added to the volumetric flask. The volume was made upto 100 mL with 80% acetone (10 mg plant material extracted in 1 mL acetone). The absorbance of the solution was read at 645, 652 and 663 nm against the solvent (80% acetone) blank.

\[
\text{Mg of chl a/g tissue} = 12.7 (A_{663}) - 2.69 (A_{645}) \times \frac{V}{1000 \times W}
\]

\[
\text{Mg of chl b/g tissue} = 22.9 (A_{645}) - 4.68 (A_{663}) \times \frac{V}{1000 \times W}
\]

\[
\text{Mg of total chl/g tissue} = 20.2 (A_{645}) + 8.02 (A_{663}) \times \frac{V}{1000 \times W}
\]

Where \(A\) = absorbance at specific wavelength

\(V\) = final volume of chlorophyll extract in 80% acetone

\(W\) = fresh weight of tissue extracted
3.30. Analysis of yield and yield attributes of bioformulation treated crops to evaluate the efficacy of the biocontrol agent as PGPR

Data were recorded on the following characters of bioformulation treated brinjal plants to evaluate the efficacy of *P. fluorescens* as PGPR.

3.30.1. Plant height (cm): The length of the main stem in cm from the base to the growing tip of the plant was measured at maturity. The height of each plant receiving a particular substrate carrier adhesive based *P. fluorescens* formulation was measured and the data averaged out.

3.30.2. No. of branches/plant: The total no. of primary branches/plant was recorded at maturity. The data of plants receiving a particular bioformulation was collected and the average calculated out.

3.30.3. No. of fruits/plant: The total no. of effective fruits from each plant in a replication was counted and averaged out.

3.30.4. Yield/plant (kg): The total weight of fruit harvested at different dates from each plant/replication was calculated and averaged out to have yield/plant.

3.30.5. Average fruit weight (g)/plant: The average fruit weight was calculated from the total fruit weight per plant divided by the total no. of fruits/plant.

3.30.6. Mean leaf area (cm$^2$): The area of lease on the primary branches of a plant for each replication was calculated and average found out and recorded.

3.31. Soil Profile and Meteorological Parameters

The soil profile of the experimental site was done in the Soil Testing Laboratory of the Deptt. of Agriculture, Govt. of Assam and is recorded below in Table 3.4. The
meteorological parameters during the period of experiment collected from the Regional Meteorological Centre, Borjhar, Guwahati, are presented in Section 3.31.2.

3.31.1. Soil Sample collected from: Farm, Department of Bio-Technology, Gauhati University, Guwahati

Table 3.4.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>OBSERVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.80 (Acidic)</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>0.74 (Medium)</td>
</tr>
<tr>
<td>P\textsubscript{2}O\textsubscript{5} (kg/ Acre)</td>
<td>2.68 (low)</td>
</tr>
<tr>
<td>K\textsubscript{2}O (kg/ Acre)</td>
<td>76.42 (Medium)</td>
</tr>
<tr>
<td>Texture</td>
<td>Sandy Clay Loam</td>
</tr>
</tbody>
</table>

Source: Soil testing laboratory (Static), Ulubari, Department of Agriculture, Govt of Assam.

3.31.2. Meteorological Parameters recorded during the period of pot and field experiments

Source: India Meteorological Department, Regional Meteorological Centre, Govt of India
Materials & Methods

### Average Mean Maximum Temperature (Degree Celsius) Recorded During Period of Study

<table>
<thead>
<tr>
<th>Period</th>
<th>Mean max. temp. (Deg. C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug 07-Jan 08</td>
<td>29.28</td>
</tr>
<tr>
<td>Feb 08-Jul 08</td>
<td>31.15</td>
</tr>
<tr>
<td>Oct 08-Mar 09</td>
<td>21.75</td>
</tr>
<tr>
<td>Aug 09-Jan 10</td>
<td>29.85</td>
</tr>
</tbody>
</table>

Average mean maximum temperature (Degree Celsius) recorded during period of study.

### Average Mean Minimum Temperature (Degree Celsius) Recorded During Period of Study

<table>
<thead>
<tr>
<th>Period</th>
<th>Mean min. temp. (Deg. C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug 07-Jan 08</td>
<td>19.67</td>
</tr>
<tr>
<td>Feb 08-Jul 08</td>
<td>20.82</td>
</tr>
<tr>
<td>Oct 08-Mar 09</td>
<td>16.15</td>
</tr>
<tr>
<td>Aug 09-Jan 10</td>
<td>19.53</td>
</tr>
</tbody>
</table>
Materials & Methods

Average relative humidity (%) at 0830 hrs during period of study

<table>
<thead>
<tr>
<th>Period</th>
<th>Rel. humidity (%) at 0830 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug 07-Jan08</td>
<td>84</td>
</tr>
<tr>
<td>Feb 08-Jul 08</td>
<td>79.33</td>
</tr>
<tr>
<td>Oct 08-Mar 09</td>
<td>83.33</td>
</tr>
<tr>
<td>Aug 09-Jan 10</td>
<td>83.33</td>
</tr>
</tbody>
</table>

Average relative humidity (%) at 1730 hrs during period of study

<table>
<thead>
<tr>
<th>Period</th>
<th>Rel. humidity (%) at 1730 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug 07-Jan08</td>
<td>75.67</td>
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<tr>
<td>Feb 08-Jul 08</td>
<td>66.5</td>
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<tr>
<td>Oct 08-Mar 09</td>
<td>71.5</td>
</tr>
<tr>
<td>Aug 09-Jan 10</td>
<td>77.67</td>
</tr>
</tbody>
</table>
3.32. Statistical analysis

The recorded data was subjected to Statistical analysis. The experimental design was completely randomized. Data on PWI was analyzed using one way Analysis of Variance (ANOVA). The data on population dynamics of pathogen and biocontrol agent in rhizosphere of crops at different days after transplanting and after receiving different treatments applied by different methods were analysed using two-way ANOVA. Duncan’s Multiple Range Test (DMRT) was then applied.

In the present investigation, the variability of experimental data on percent wilt incidence (PWI), number of colony forming units (cfu) and yields are very high and heterogenous. In order to stabilize heterogeneity, the data on PWI and cfu were transformed to corresponding angular and logarithmic values respectively, before analysis of variance. Subsequently, Fisher’s metod (1954) of Analysis of Variance (ANOVA) was made on the transformed data in the replicated treatments. Data were analysed using appropriate and Standard Computerized Analysis Programme for the experiments. Significance of Variance among data was interpreted by calculating the F value based on computer programming and comparing with appropriate table value of F at \( P = 0.05 \).

**Critical difference (CD):** The analysis of variance gives only a broad indication of performance of the treatments. But in order to get clear appraisal of the specific phenomenon, calculation of critical difference was considered necessary.

**Test of significance:** The method of calculating the probability of obtaining an observed result from some hypothesis and regarding the hypothesis to be rejected or not, is known as test of significance. If the calculated value is greater than tabulated value, the observed result is said to be statistically significant at a chosen...
level of probability. If the calculated value is more than the tabulated value at 1% level of probability, then it is said to be highly significant and if the calculated value is lower than the tabulated value at 1% level of probability but higher than tabular value at 5% probability, it is said to be significant.

**Standard error of difference (SEd)** was calculated out using the following formulae-

\[
SEd = \sqrt{\frac{2 \times \text{Error Mean Square}}{\text{Number of replications}}}
\]

SEd for formulations = \( \sqrt{\frac{2 \times \text{Error Mean Square}}{\text{Number of replications} \times \text{levels of formulations}}} \)

SEd for methods of application = \( \sqrt{\frac{2 \times \text{Error Mean Square}}{\text{No. of replications} \times \text{levels of methods}}} \)

SEd for interaction effect = \( \sqrt{\frac{2 \times \text{Error Mean Square}}{\text{No. of replications}}} \)

For comparison of treatment means in different experiments, the Critical Difference (CD) values have been computed as follows:

CD (0.05) = SEd \times t_{0.05} \text{ for error d.f}

The significant and non significant differences among the treatment means were interpreted by comparing the difference in mean values with the corresponding CD (0.05) value.

**Correlation studies:** To establish the relationship between different dependant and independent variables, the mean transformed data were subjected to correlation studies. The correlation coefficient (r) value was calculated using the formula given below:
Correlation coefficient ($r_{xy}$) = \[ \sqrt{\frac{\text{Cov.}(X, Y)}{(\text{Var.}(X))(\text{Var.}(Y))}} \]

\[ = \frac{\sum XY \sum X \sum Y/n}{(\sum X^2 - \sum X^2/n)(\sum Y^2 - \sum Y^2/n)} \]

Where,

X = Independent variable
Y = Dependent variable
n = No of pairs of variables

$r_{xy}$ = Correlation coefficient between variables X and Y

The test of significance of r value was tested by comparing the calculated the t value with table t value at (n-2) d.f.

The t test is given as follows:

\[ t = \frac{r\sqrt{n-2}}{\sqrt{1 - r^2}}, \text{with } \sqrt{n-2} \text{ d.f} \]

Where, n = no of pairs of variables under test

Regression coefficient of Y on X is given by:

\[ b_{xy} = \frac{\text{Cov.}(X, Y)}{\text{Var.}(X)} \]

\[ = \frac{\sum XY - \sum X \cdot \sum Y/n}{\sum X^2 - (\sum X)^2/n} \]

Where,

X = Independent variable
Y = Dependent variable
n = No of pairs of variables
$b_{xy}$ = Regression coefficient between variables X and Y
Test of significance for regression coefficient:
\[ T_b = \frac{b}{SE_b} \]
Where,
\[ SE_b = \left[ \frac{S^2}{\sum x^2} \right]^{1/2} \]

\[ S^2 = \frac{\sum y^2 - (\sum xy)^2}{n-2} / \sum x^2 \]

The calculated value of \( t_b \) was compared with table value of \( t \) at \((n-2)\) d.f at \( P_{0.05} \) and \( P_{0.01} \)

Regression line was drawn using linear formula as:
\[ Y = \alpha + \beta x \]

Where, \( Y = \) Dependant Character (estimated)
\( \alpha = \) Interception, \( \beta = \) Slope of regression line, \( X = \) Independent character