MATERIAL AND METHODS
CHAPTER III
MATERIAL AND METHODS

3.1. General

3.1.1. Location

All the laboratory experiments were conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore which is located at an altitude of 920 meters above MSL, 12.98° N latitude and 77.58° E longitude.

3.1.2. Glasswares

Borosil made glassware were cleaned with soap solution and soaked in chromic acid solution for 4 – 6 h, then rinsed with distilled water for two to three times, dried and sterilized in hot air oven at 160°C for 3 h before use.

3.1.3. Chemicals

The analytical reagent (AR) grade chemicals of Hi-Media, Qualigens, BDH, E-Merck and Sigma were used for media preparation and biochemical studies.

3.1.4. Enzymes and markers

All enzymes and primers used in genetics studies were obtained from M/S. Bangalore Genei (P) Ltd, Bangalore.

3.1.5. Composition of media, reagents, buffers and solutions

The composition of reagents, buffers and different media used are given in Appendix I. The solutions and buffers used for DNA studies are given in Appendix II.
3.2. Sample collection

3.2.1. Sample site and crops used

The agricultural farm at the University of Agricultural Sciences, GKVK, Bangalore was selected as the site for collection of samples. The shoot and root samples were collected from the following crops:

Cereal crops: Rice (*Oryza sativa*) and ragi (*Eleusine corocana*)

Pulse crops: Cowpea (*Vigna unguiculata*) and soybean (*Glycine max*)

Oilseed crops: Groundnut (*Arachis hypogea*) and sunflower (*Helianthus annuus*)

Vegetables: Chilli (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*)

The samples of the crops were collected before flowering stage at 45 days of planting.

3.3. Isolation of endophytic bacteria from shoot regions

3.3.1. Preparation of media

The nutrient agar medium was used for the isolation of endophytic bacteria. The medium was sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. The pH of the medium was adjusted to pH 7.0. Solidified media was prepared by the addition of two per cent Oxoid purified agar before autoclaving. Glycerol stock was used for storing the cultures for a longer time at refrigerated conditions (-4°C).

3.3.2. Isolation of endophytes

The isolation of endophytes was done according to the procedure by Bacon *et al.* (2002). The randomly selected plants were uprooted manually and washed in running tap water. Stem sections of 2 cm length were excised using flame sterilized scalpel from 1 cm to 2 cm above the soil line. The root sections were similarly prepared. All the samples were blotted dry with filter paper and then weighed to have final sample of 0.5 gm. The surface sterilization of the shoot and root pieces was done with
the following immersion sequence: 70% ethanol for 1 min, 3% Sodium hypochlorite for 5 min in young plants and 10 min in case of older plants followed by 70% ethanol wash for 1 min. They were then rinsed four times with sterile water and dried in laminar flow. Surface disinfestations parameters like selection of disinfectant, its strength, duration of immersion in disinfectant were optimized prior to experimentation. The cut ends of surface sterilised segments were removed with flame sterilized scalpel and were placed in appropriate agar media with the cut surface touching the agar. The plates were incubated for four to eight days at 27°C.

For sterility check, the shoots were rolled on nutrient agar plates as well as 0.1 ml aliquot from the final wash was inoculated to 10 ml nutrient broth (NB) (Gyaneshwar et al., 2001). Samples were discarded if any growth was detected in the sterility check.

3.4. Enumeration of endophytic bacterial population

3.4.1. Standard Plate Count Method

The endophytic bacteria were enumerated by modifying the isolation procedure described by Sturz et al. (1999) and Gyaneshwar et al. (2001). One gram of shoot/root sample was macerated under aseptic conditions with a sterile mortar and pestle in 9 ml of sterile water. From this, 1.5 ml of aliquot was transferred to a sterile microfuge tube and centrifuged at 1,300 rpm at 4°C for 10 min. The supernatant was serially diluted up to 10^{-5} and each dilution was transferred (1 ml) to nutrient agar plates with three replications and incubated for four to eight days at 27°C. Nutrient agar medium was sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. The bacterial isolates were purified by streak plate method.
3.4.2. Selection of endophytic bacterial isolates

The endophytic bacterial isolates were selected based on distinct colony morphology and growth in broth media. All the isolates were purified by streaking on nutrient agar plates. Long-term storage of the purified isolates was at −80 °C in nutrient broth with 50 % (w/v) glycerol. Short-term storage for further characterisation was on nutrient agar plates at 4 °C.

3.5. Characterization of the endophytic isolates

3.5.1. Morphological tests

The following morphological tests viz., cell shape, Gram reaction, and motility were carried out to characterize the tentatively identified endophytes.

3.5.2. Cell shape (Aneja, 2006)

The purified cultures, at log phase were observed microscopically for the cell morphological characteristics.

3.5.3. Gram staining (Hucker and Conn, 1923)

Gram staining was carried out as per modified Hucker's method. The slides were viewed with the light microscope under oil-immersion. Gram-positive bacteria appear violet and gram-negative bacteria appear pinkish red.

3.5.4. Motility in liquid media (Aneja, 2006)

The 72 h grown endophytic cultures were observed microscopically using cavity slide for observing the bacterial motility.
3.6. Biochemical tests

The following biochemical tests were carried out.

3.6.1. Oxidase test (Cappuccino and Sherman, 1996)

The endophytic isolates were streaked on Trypticase soy agar medium and incubated at 30°C in an inverted position for 48h. After the incubation period, 2-3 drops of para-aminodimethyl aniline oxalate solution were added on the streaked area and the plates were observed for the color change from pink to maroon and finally to purple within 30 sec indicated a positive reaction.

3.6.2. Nitrate reduction test (Cappuccino and Sherman, 1996)

The endophytic isolates were inoculated into 10 ml of nitrate broth taken in test tubes and the tubes were inoculated at 30°C. After 14 days, 2 ml of the broth was tested by adding equal amounts of sulfanilic acid and alpha naphthylamine. Development of red color indicated that nitrate had been reduced to nitrite.

3.6.3. Hydrogen sulphide production (Seeley and Vandemark, 1981)

Sulfide indole motility (SIM) agar stabs were inoculated with the isolates of endophytic bacteria and incubated at 30°C for 48 hr. Black coloration along the line of stab inoculation indicated H₂S production.

3.6.4. Catalase activity (Aneja, 2006)

A loopful of 24h old culture of endophytic isolates maintained on nutrient agar slants were transferred to a glass test tube containing 0.5 ml distilled water and mixed thoroughly with 0.5 ml of 3 per cent hydrogen peroxide solution and observed for the presence of the effervescence.
3.6.5. **Indole production** (Seeley and Vandemark, 1981).

The endophytic isolates were inoculated into sterilized glucose tryptone broth taken in test tubes and the tubes were incubated at 30°C. After 48 h of incubation, 0.3 ml of Kovacs reagent was added and mixed well. The reddening of the alcohol layer within few minutes indicated indole production.

3.6.6. **Methyl Red and Voges Proskauer Test** (Seeley and Vandemark, 1981).

The Methyl red and Voges-Proskauer (MR-VP) broth prepared in two sets was inoculated with the endophytic isolates and incubated for 48 h at 30°C. To the first set of tubes, few drops of an alcoholic solution of methyl red were added. The development of distinct red color was indicative of positive reaction for MR test.

-naphthol solution (5 per cent solution in 70 per cent ethyl alcohol) was added to the second set of tubes and shaken gently for 15 min. The positive reaction of acetyl methyl carbinol production was indicated by development of red color. This indicates positive result for the VP test.

3.6.7. **Citrate Utilization Test** (Seeley and Vandemark, 1981).

The endophytic isolates were inoculated into test tubes having Simmons citrate agar medium and incubated for 48 h at 30°C. Simmons citrate agar contained citrate as its only carbon and energy source. The presence of growth and change of color from green to blue due to pH change indicated positive reaction.
3.7. Fermentation of carbohydrates by endophytic bacterial isolates

(Aneja, 2006)

Fermentative degradation of various carbohydrates such as glucose, sucrose and lactose by microbes was carried out in a fermentation tube that contains a Durham tube for the detection of gas production as an end product of metabolism. The following carbon compounds viz., glucose, sucrose and lactose were used at 0.5 percent level in the medium. Sterile fermentation tubes of glucose broth, sucrose broth and lactose broth containing phenol red indicator was inoculated with the endophytic isolates and incubated at 30°C for 48 hours. Uninoculated tubes were maintained as control. Change in colour due to production of acid and change in colour and appearance of bubbles due to production of acid and gas was compared with the control tubes and was recorded.

3.8. Enzymatic activity of the endophytic bacterial isolates

3.8.1. Gelatin hydrolysis (Aneja, 2006)

The activity of the enzyme gelatinase for hydrolyzing gelatin was tested by gelatin liquefaction. The test cultures were stab inoculated into nutrient gelatin deep tubes, incubated at refrigerated condition for 48 h and observed for gelatin liquefaction.

3.8.2. Starch hydrolysis (Aneja, 2006)

Starch hydrolysis test was done to study the activity of amylase. The endophytic isolates were streaked on nutrient agar plates containing 2 per cent insoluble starch and incubated at room temperature. Hydrolysis of starch was tested by flooding with iodine solution and the plates were observed for the presence of clear zones surrounding the colonies and considered for positive reaction.
3.8.3. Cellulose degradation (Samanta, 1989)

The activity of the enzyme cellulase for hydrolysis of cellulose was tested. The media was used according to Samanta (1989). The endophytic isolates were streaked on cellulose agar minimal medium containing cellulose and incubated at 30°C in inverted position for 2-5 days. After incubation plates were flooded with 0.2 per cent aqueous Congo red and destained with 1M NaCl for 15 minutes. The plates were observed for the presence of clear zones surrounding the colonies after 30 minutes. Clear zone surrounding the colony indicated cellulase activity.

3.8.4. Pectin degradation (Aneja, 2006)

The production of the enzyme pectinase by the endophytic bacterial isolates was tested by using Hankin’s medium. The autoclaved medium was poured into sterile petri plates and allowed to solidify. The endophytic isolates were aseptically inoculated on one each of pH 7 and pH 5 by streaking and the plates were incubated at 30°C for 48-72 hours. All the plates were flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide. The formation of a clear zone around the growth on both the media indicated positive result for pectinase.

3.9. Functional properties of the endophytic bacterial isolates

3.9.1. Polysaccharide production

The endophytic isolates were tested for their ability to produce polysaccharide in the plate assay. Modified nutrient media with high sucrose content (5%) was used. The isolates were streaked on the solidified agar and the plates were incubated at 30°C for 4-7 days. The thick viscous mass formed over the streak was visually scored and the
isolates showing viscous formation were considered as positive for polysaccharide production.

3.9.2. Growth of endophytic isolates on N free media

The ability of the endophytic isolates to grow in N free media was tested by streak plate method. Jensen’s medium (Jensen, 1954), N free malate medium (Okon et al., 1977) and Waksman No. 77 medium (Waksman, 1937) were used for the study as the carbon source of these media are different. The endophytic isolates were streaked on the above mentioned agar media and the plates were incubated at 30°C for 4-7 days. Care was taken to avoid the presence of any extraneous nitrogen in the media and glassware. The glasswares used were washed thoroughly with chromic acid and with double distilled water. The agar used was Himedia Laboratories Agar Powder (Bacteriological) with < 0.005% nitrate. The water used for media preparation was double distilled water and other media components were also of high purity to avoid the presence of nitrogen. The isolates which were able to form glistening colonies in the nitrogen free media were designated as positive for N fixation.

3.9.3. Phosphate solubilisation ability of the isolates

3.9.3.1. Phosphate solubilisation by endophytic bacteria in agar media

Microorganisms capable of producing a halo/clear zone due to solubilization of inorganic phosphate in the surrounding medium by organic acids were selected as potential phosphate solubilizers. They were screened in the laboratory by a plate assay. The endophytic isolates were streaked on Sperber’s agar (Sperber, 1958) and the plates were incubated at 30°C for 4-7 days. The isolates showing the clearing zone
were considered as P solubilizers. The clearing zone was measured in mm and recorded.

3.9.3.2. Estimation of inorganic phosphate solubilised by endophytic isolates

As the plate assay is not considered a reliable method in determining a strain as phosphate solubilizer, the pure cultures were further screened in liquid medium containing Ca$_3$(PO$_4$)$_2$, at a concentration of 5 g L$^{-1}$ as insoluble P source. Each of the endophytic isolates were inoculated to 100 ml of Pikovskaya broth (Pikovskaya, 1948) and incubated for 2 weeks at 28$^\circ$C. The broth cultures were centrifuged at 9000 rpm for 20 min. The supernatant was used for estimation of inorganic phosphorus by the molybdenum blue method (Murphy and Riley, 1962). The amount of phosphate released in the flasks after incubation was estimated in comparison with a set of uninoculated controls.

One millilitre of the supernatant of each isolate was taken in a 50 ml volumetric flask to which 10ml of chloromolybdic acid was added and mixed thoroughly. The volume was made up to three-fourth with distilled water and 0.25 ml of chlorostannous acid was added. Immediately the volume was made up to 50 ml with distilled water. After 15 min the blue colour developed was read on a spectrophotometer at 610 nm using reagent blank. Standard curve was prepared using different concentrations of standard potassium dihydrogen phosphate solution. The amount of inorganic phosphorus released was calculated from the standard graph.
3.10. **Intrinsic antibiotic resistance of endophytic isolates** (Clower and Hay, 1968)

The intrinsic antibiotic resistance of the endophytic isolates was determined against 50, 100, 150 and 200 ppm of Streptomycin and Kanamycin. The endophytic isolates were grown in nutrient broth for 48 h at 30°C under shaker conditions and 1.5 ml culture was taken in microfuge tube and centrifuged at 10,000 x g for 5 min. The pellet cell was washed thrice with 1% saline solution and suspended in 1 ml of sterile saline solution. To 50 ml of sterilized and cooled media, appropriate concentration of antibiotics were added and plated in sterile petriplates. After solidification, plates were divided into 4 quadrants on the back of petriplate and by using sterile tooth picks each part was inoculated with different endophytic strains under sterile condition. Control plates were maintained separately by excluding the antibiotics in the medium. After 2-3 days of incubation at 30°C, the growth was recorded.

3.10.1. **Development of UV resistant antibiotic marker strain of endophyte**

An antibiotic resistant marker strain was developed by Uv irradiation for further identification and confirmation of the endophytic nature of the isolated endophytic bacteria. The uv resistant antibiotic marker strain was developed by following the method of Saz et al. (1952).

3.10.1.1. **Preparation of cells for mutagenic treatment**

The endophytic isolates VUE13 and GME16 were selected as they were naturally resistant to 200 ppm Kanamycin. The wild VUE13 and GME16 as well as the colonies picked up from 200 ppm Kanamycin plates designated as 200KV and 200KG were used. 2 ml of 24 hour old bacterial culture (10^8 cfu/ml) prepared in nutrient broth medium was centrifuged aseptically for 10 min. The bacterial cells were suspended in
50 ml sterile distilled water. The suspension was then diluted up to 10^{-3} times.

### 3.10.1.2. Ultraviolet irradiation of the isolates

The diluted suspension was used for irradiation. Ten millilitre of the above diluted suspension was transferred to sterile 9 cm petri dishes. The petri plates were placed under the UV lamp at a distance of 30 cm (emitting the energy of 1.6x10^2 J/m^2/s) for 15 min. At the conclusion of the irradiation period, the cultures were transferred to sterile tubes and iced. The range of resistance in the irradiated material was determined directly by plating one millilitre of the bacterial suspension into 100 ml nutrient agar, containing varying amounts of antibiotic viz. 150 ppm, 200 ppm, 300 ppm, 400 and 500 ppm. Unexposed isolates were also maintained in the Kanamycin plates as control. Both exposed as well as unexposed cells were plated in control plates without antibiotic. The plates were incubated for 24 hours at 30^0 C. After 24 hours the plates were examined for the bacterial growth and the results recorded. The colonies that came up in the 500 ppm Kanamycin plate were considered as antibiotic resistant mutants and designated as marker strains for Kanamycin resistance and labeled 500KGX.

### 3.10.2. Establishment of the endophytic marker strain 500KGX on the seeds and shoots of soybean (Glycine max)

To determine the ability of isolated bacterial endophytes to recolonize soybean seeds, healthy seeds were washed thoroughly in running tap water for 5 min. They were surface disinfected by 70 % (w/v) ethanol wash for 90 s, sodium hypochlorite for 3 min and 70 % (w/v) ethanol wash for 60 s. This treatment was followed by four thorough rinses with sterile distilled water. The surface sterilized seeds were soaked in a 24 hour old culture of the endophytic isolate 500KGX for 6 hours. The initial population of 500KGX as well as the population in
seeds after soaking for 6 hours was recorded by serial dilution upto $10^{-7}$ and plating in nutrient agar with 500ppm Kanamycin. The soaked seeds were placed in sterile agar flasks under aseptic conditions and the tubes were incubated in dark for 3 days for germination and then placed in light under green house conditions. Control flasks were maintained with uninoculated surface sterilised seeds. After 10 days the shoot was excised from the seedling aseptically and surface sterilized with sodium hypochlorite for 30 s and washed 5 times in sterile distilled water. The cut segments were placed in nutrient agar containing 500ppm Kanamycin and the plates were incubated at 30$^0$C for 48 hours. The bacterial growth around the cut segments was recorded. Also one gram of shoot sample was macerated under aseptic conditions with a sterile mortar and pestle in 9 ml of sterile water. It was serially diluted up to $10^{-7}$ and each dilution was pour plated (1 ml) on nutrient agar media with 500 ppm Kanamycin. The plates were incubated at 30$^0$C for 48 hours. The colonies appearing in the 500 ppm plates were recorded.

3.11. **Plant growth promotion effect of endophytes**

3.11.1. **Effect of marker strain 500KGX on seedling growth of tomato**

Treatments : Four

Design : CRD

Replications: Five

**Treatment details:**

T1 – Non-surface sterilized seeds on plain agar

T2 - Surface sterilized seeds on plain agar

T3 - SS seeds on antibiotic agar media

T4 - 500KGX inoculated SS seeds on plain agar
3.11.1.1. Surface sterilization of tomato seeds

Surface sterilisation of tomato seeds (cv. Vaibhav) was carried out to eliminate all the epiphytic microorganisms present in the seeds. They were surface sterilized with 70 % ethanol for 1 min, 3 % Sodium hypochlorite for 3 min followed by 70 % ethanol wash for 1 min. The seeds were rinsed in sterile distilled water three times and blot dried.

3.11.1.2. Seed bacterisation

Five surface sterilised seeds were placed in agar tubes containing Kanamycin 500ppm to kill all the endophytic microorganisms in the seeds. Surface sterilised seeds were also inoculated with bacterial suspension of 500KGX to confirm the plant growth promoting activity of endophytes. These seeds were placed in plain agar tubes. Similarly five surface sterilised seeds were placed in autoclaved plain agar tubes as negative control and non surface sterilised seeds were maintained in plain agar as positive control. The tubes were incubated in dark for 3 days for germination and then placed in light under green house conditions. The growth of the inoculated seeds was compared to uninoculated control tubes.

3.11.2. Evaluation of the effect of endophytic bacterial isolates on seedling growth of paddy

3.11.2. 1. Preparation of bacterial inoculum (Thompson, 1996).

The endophytic bacteria were grown on NA broth with constant shaking at 150 rpm for 48 h at room temperature (30°C) to approximately $10^6$ cfu/ml. The bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min, and bacterial cells were resuspended in sterile distilled water. This was used as bacterial inoculum.
3.11.2. 2. **Seed bacterization**

Paddy seeds were surface sterilized with 70 % ethanol for 1 min, 3% Sodium hypochlorite for 5 min followed by 70 % ethanol wash for 1 min, rinsed in sterile distilled water thrice and dried overnight under sterile air stream. Required quantity of seeds were soaked in ten millilitre of bacterial suspension containing $10^6$ cfu/ml for 12 h and dried under shade. The seeds soaked in sterile distilled water were maintained as control.

3.11.2. 3. **Seed germination test**

Plant growth – promoting activity of bacterial endophytic strains were assessed based on the seedling vigour index by growing the seeds in cups with sand as substrate. Rice seed bacterization was done as described earlier. One seed was placed on small cups filled with autoclaved river sand. The cups were watered with sterile water and incubated in growth chamber for 30 days. Each treatment had ten replications. The root and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated. The root and shoot length of individual seedlings were measured and germination percentage of seeds was also recorded. The vigour index was calculated by using the formula as described by Abdul-Baki and Anderson (1973).

Vigour index = per cent germination x seedling length (shoot length + root length)
3.11.3. Production of plant growth regulators by endophytic bacterial isolates

3.11.3.1. Gibberellic acid (GA) production

3.11.3.1.1. Bioassay for GA production by endophytes: Starch agar halo test.

This bioassay is based on the principle that GA induces denovo synthesis of amylase in germinating seeds. Stock solutions of GA were prepared $10^{-3}$ to $10^{-7}$M. 24 hour old endophytic bacterial culture ($10^8$cfu/ml) were inoculated in 25 ml nutrient broth and incubated at $30^\circ$ C for 10 days. The cells were centrifuged at 5000rpm to remove the cell mass and the culture filtrate was used for the bioassay. Pre germinated paddy seeds were cut into two and the embryo less half seed was incubated in 5ml of culture filtrate for 6 to 8 hours. Then they were transferred to petriplates with starch agar medium. The half seed was placed with the cut surface touching the medium and the plates were incubated for 24-48 hours. Then the seeds were removed and the plates were flooded with iodine solution. Seeds soaked in sterile water served as control and those soaked in different concentrations of GA solutions served as standard. The clear halo formed was measured and compared with the control plate. The standard graph was plotted with concentration of GA and diameter of zone. From the standard graph GA production of the endophytic isolates was calculated.

3.11.3.1.2. Estimation of GA by Spectrophotometry

3.11.3.1.2.1. Extraction of Gibberellins from culture filtrate (Tien et al., 1979)

Nutrient broth was prepared and 100 ml quantities were dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized at 15 psi for 15 min. One ml of the standard inoculum ($10^9$ cells ml$^{-1}$) of endophytic
isolates was added to each flask and incubated at 30°C in a shaker. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 14 days of incubation, 25 ml of the sample was withdrawn and the cells were spun at 5000 x g for 15 min in a centrifuge (Remi, India). The supernatant was acidified to pH 2.5 using 5N hydrochloric acid and partitioned with equal volumes of ethyl acetate for five times in a separating funnel. The ethyl acetate phase was dried at 32°C and the residue was redissolved in 2 ml of distilled water.

3.11.3.1.2.2. Spectrophotometric estimation of Gibberellins (GA)

Amount of GA present in the supernatant of the culture was determined by the method of Paleg (1965). Fifteen ml of ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added. After 2 min, 2 ml of potassium ferrocyanide solution was added and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 per cent hydrochloric acid and the mixture was incubated at 20°C for 75 min. The blank was prepared with 5 per cent hydrochloric acid. The absorbance was measured at 254 nm in a spectrophotometer.

3.11.3.2. Indole Acetic Acid (IAA) production

3.11.3.2.1. Bioassay for IAA production by endophytic isolates: Cucumber root elongation bioassay (Loper and Schroth, 1986)

Healthy seeds of cucumber were selected for the study. They were soaked in water for six hours and allowed to germinate in filter paper. Stock solutions of IAA were prepared upto 10⁻³ strength. 24 hour old endophytic bacterial culture (10⁹cfu/ml) were inoculated in 25 ml nutrient broth and incubated at 30°C for 10 days. The cells were
centrifuged at 5000rpm to remove the cell mass and the culture filtrate was used for the bioassay. 6ml of the culture filtrate was added to petriplates and the selected seedlings were transferred into them (10 seeds/plate) and incubated for 48 hrs. Similarly different concentrations of IAA solution was used as standard. Sterile water served as control. After 48 hours measured the root length of the seedlings and tabulated the results. The standard graph was plotted with the concentration of IAA and the decrease in root length. The IAA production by the endophytic isolates was calculated from the standard graph.

3.11.3.2.2. Estimation of indole acetic acid (IAA) by spectrophotometry

3.11.3.2.2.1. Extraction of indole acetic acid (IAA) from culture filtrate (Tien et al., 1979)

Nutrient broth supplemented with 100 µg/ml L-tryptophan was prepared and 100 ml quantities were dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized at 15 psi for 15 min. The standard inoculum (10^6 cells ml^-1) of endophytic isolates was added to each flask(1 ml) and incubated at 30º C in a shaker. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 14 days of incubation, 25 ml of the sample was withdrawn and the cells were spun at 5000 x g for 15 min in a centrifuge (Remi, India). The supernatant was acidified to pH 2.5 using 5N hydrochloric acid and partitioned with equal volumes of ethyl acetate for five times in a separating funnel. The ethyl acetate phase was dried at 32º C and the residue was redissolved in 2 ml of distilled water.
3.11.3.2.2. Spectrophotometric estimation of IAA (Gordon and Weber, 1951)

A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by 4 ml of Salper’s reagent and incubated in darkness for 1 h at 28 °C. The intensity of the pink colour developed was read in spectrophotometer at 535 nm. By referring to a standard graph prepared with chemical grade of indole-3-acetic acid, the quantity of IAA in the sample was determined.

3.11.3.3. Cytokinin production by endophytic bacteria

3.11.3.3.1. Extraction of cytokinins

The endophytic bacterial culture was grown in nutrient broth medium for 14 days in a shaker at 250 rpm at 30°C. The culture was centrifuged at 12000 rpm for 30 min and the cell free culture filtrate was extracted twice with equal amount of n-butanol. The supernatants were pooled. The n-butanol fractions were kept for evaporation. After evaporation the cytokinin fraction was dissolved in 2 ml of HPLC grade methanol and filter sterilized using 2µm bacterial filter.

3.11.3.3.2. Bioassay for cytokinins by endophytic isolates: Cucumber cotyledon greening bioassay (Fletcher et al., 1982)

Cucumber seeds were planted in vermiculite and germinated in dark at 28 °C. The cotyledons from 5-day-old plants were excised in dim green light, weighed and uniformly floated in 7-cm Petri dishes containing 5 mL of culture filtrate. Benzyl adenine (BA) was used as standard. Petri dishes containing 5 mL of test solution containing 10⁻⁴ to 10⁻⁸ M of BA and 40 mM KCl served as control. BA was dissolved in 0.1 ml of ethanol and diluted with distilled water to given concentration. Cotyledons placed in a solution of 5 mL of 40 mM KCl was used as control. Each plate of sample and control were placed 10 pieces of cotyledons with the adaxial face
down. Their weight was on average 0.1700±0.0050 mg. All the dishes were returned to the dark at 28 °C for an incubation of 24 hours and then exposed to fluorescent light with an intensity of 11 mmol·m-2·s-1 for 3 hours at 28 °C. The cotyledons were extracted directly with 10 mL 95 % acetone - ethanol 2:1 (v/v) solution in dark for 24 hours. The absorbance of the extraction solutions were measured using UV755B spectrophotometer at 663 and 645 nm.

3.12. Compatibility of endophytic bacterial isolates with beneficial soil microorganisms

The endophytic isolates were tested for their compatibility with beneficial microorganisms by following the method described by Fukui et al. (1994). The compatibility was determined for Pseudomonas sp., Azotobacter sp., B. subtilis and B. megaterium by using nutrient agar medium. The beneficial soil bacteria and endophytic isolates grown in nutrient broth for a period of 24 hours (10^8 cfu/ml) was used as inoculum. One loopful of the test organism and the endophytic isolate was streaked on opposite side of the medium in the petriplates. The plates were incubated at 30 °C for 48 - 72 h. Compatibility was tested by overgrowth or by inhibition of the test organisms and observations were recorded.

3.13. Biocontrol efficiency of endophytic isolates against fungal pathogens of vegetables

3.13.1. Screening of endophytic bacterial isolates by dual culture plate assay (Dennis and Webster, 1971)

Endophytic bacterial isolates were screened for antagonist activity against four major pathogens of vegetables. The phytopathogenic fungi were the root pathogens Fusarium oxysporum, Pythium aphanidermatum
and *Rhizoctonia solani* and the leaf pathogen *Colletotrichum sp.* They were collected from the culture collection of NBAIM, Bangalore.

Antifungal activity was screened using dual culture method in which both endophytic bacteria and test fungi were inoculated in single Potato Dextrose Agar media plate. The test fungi (5 mm diameter disc) were inoculated at the centre of potato dextrose agar plate and 24 hour old culture of endophytic bacteria ($10^5$ cfu/ml) was spot inoculated at corner of the plate and incubated for four to eight days at 27°C. Antifungal activity was indicative as mycelia growth of test fungus inhibited in the direction of active endophyte, the level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony from the endophyte growth radius. The width of inhibition zone between the pathogen and endophyte was evaluated as inhibition zone and ranked as +: <2mm; ++: >2mm and −: no inhibition

3.13.2. Evaluation of endophytic bacterial isolates on growth inhibition of fungal plant pathogens (Opгенorth and Endo, 1983)

Measurement of fungal growth inhibition by mycelial mat weight determination was the method followed for this study. The endophytic isolates showing high inhibition of the pathogens in plate assay were used in liquid media. The direct measurement of a decrease in fungal weight can be determined by inoculating the culture flasks (with a known volume of broth) with the pathogenic fungus and the antagonistic endophyte together, followed by filtering off the mycelium, drying at 60°C for 48 hours to a constant weight. Potato dextrose broth which supports the growth of fungi and bacteria was used in this study. Each flask containing 100 ml broth was inoculated with 8 mm disc of the pathogenic fungi along with 1 ml of 24 hour old endophytic bacterial
culture (10^5 cfu/ml). Control flasks with only the fungus and only bacteria were maintained. The flasks were incubated at 30°C under static conditions for 10 days. After the incubation period the contents were filtered through a pre-weighed Whatman filter paper and the fresh weight was recorded. The filter papers were dried in an oven at 105°C for 48 hours and reweighed along with the mycelium to get the dry weight. The weight of the mycelium was calculated by subtracting the weight of the filter paper from the weight of the filter paper+mycelium. The reduction in weight of co inoculated flasks was determined by comparing with the control flasks.

3.13.3. Plant infection study (Gravel et al., 2005)

The best isolates which showed high inhibition of the pathogens was further used for plant infection studies. *Fusarium oxysporum*, *Rhizoctonia solani*, and *Pythium aphanidermatum* were used for testing the incidence of tomato damping off. The soil was infected with the root pathogen prior to sowing. Seeds of tomato (cv. Vaibhav) were surface sterilised and bacterisation of seeds was done with the endophytic isolates by the procedure given earlier. The inoculated seeds were sown in the sick soil. Uninoculated seeds soaked in sterile distilled water served as control. The seedling trays were maintained under greenhouse conditions. The number of healthy seedlings was recorded after 14 days. The experiments were done in triplicate with 10 seeds/ cup. The per cent of damping off was calculated as (TS-GS/TS) x100 where TS is the total number of seeds sown and GS is the number of seeds germinated.


Amplification of nitrogenase (*nifH*) gene of the best isolate, LEE19 was studied using *nifH* primer. *Azotobacter chroococcum* was used as positive control for *nifH* gene.
3.14.1. Isolation of total genomic DNA

The total genomic DNA from the GME16 mentioned above was isolated by the method given by Poly et al. (2001). 48 hr old bacterial culture was centrifuged in an eppendorf tube to get cell pellet. It was repeated 2-4 times to get enough cell mass in an eppendorf tube. 500 μl lysing buffer was added and the suspension was agitated vigorously in a vortexer for 3min. The tubes were incubated in water bath for 1 hour at 65°C. Then the suspension was centrifuged at 18000 rpm for 15 min at 4°C. The supernatant was transferred to fresh 2ml centrifuge tube. The phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to the supernatant (500 μl) and mixed well gently but thoroughly till the mixture turns white. The mixture was centrifuged at 18000 rpm for 15 min at 4°C. The clear supernatant just above the middle layer was transferred carefully to a fresh 2ml centrifuge tube. 1/10 vol of 3M Sodium acetate and 2 vol of absolute ethanol was added to the supernatant and mixed well. The nucleic acids were allowed to precipitate overnight for 24 hours at -20°C or 2hours. The nucleic acid precipitate was aggregated by centrifuging at 18000 rpm for 15 min at 4°C. The supernatant was decanted and any residual ethanol was drained off by inverting on absorbant tissue. 100 μl of 70 % ethanol was added and mixed well gently. The mixture was centrifuged at 13400 rpm for 15 min at 4°C. The supernatant was decanted and the excess ethanol drained out by inverting on absorbant tissue. The precipitate was dried at 50°C for 10 min. The precipitate was resuspended in 50-100 μl of TE buffer and stored in deep freezer at -20°C.

3.14.2. Amplification of DNA

For PCR, usually a primer is used with the aim to amplify specific segments of the largest genome in a random fashion. This method depends on the fact that specific sequences are common in a large
genome so that just by chance, at several unpredictable locations, two primers will anneal sufficiently close to one another on opposite strands of the template to amplify the intervening region. The DNA was amplified using Nif \( H \)-g1 primers (Helmut et al., 2004).

3.14.2.1. **Nif \( H \) primers sequence**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward</td>
<td>5'GGTTGTGACCCGAAAGCTGA-3',</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Reverse</td>
<td>5'GCGTACATGGCCATCATCTC-3'.</td>
<td></td>
</tr>
</tbody>
</table>

3.14.2.2. **The composition of the reaction mixture**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Particulars</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taq DNA polymerase buffer (10X)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>2</td>
<td>dNTP mixture</td>
<td>10mM</td>
</tr>
<tr>
<td>3</td>
<td>Forward primer</td>
<td>10 μm</td>
</tr>
<tr>
<td>4</td>
<td>Reverse primer</td>
<td>10 μm</td>
</tr>
<tr>
<td>5</td>
<td>Taq DNA polymerase</td>
<td>3 U/μl</td>
</tr>
<tr>
<td>6</td>
<td>Total DNA (50 ng)</td>
<td>100ng</td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume</td>
<td></td>
<td>25 μl</td>
</tr>
</tbody>
</table>

The reaction mixture was given a momentary spin in a Biofuge for thorough mixing of the cocktail components. The PCR tubes were then loaded in a thermal cycler (PTC-100™ Programmable Thermocycler MJ Research) for amplification.
3.14.2.3. The thermal cycler programme is as follows

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denaturation (initial)</td>
<td>94</td>
<td>1min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94</td>
<td>30s</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>50</td>
<td>1min</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72</td>
<td>30s</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72</td>
<td>10min</td>
</tr>
</tbody>
</table>

Total number of cycle (2-4) 40 cycles

3.14.3. Agarose gel electrophoresis

Agarose gel electrophoresis was performed based on the method given by Sambrook et al. (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. 1X TBE tank buffer (500 ml) was prepared to fill the electrophoresis tank and to prepare the gel. The open ends of the Pyrex gel casting plates were sealed and placed on a horizontal leveled platform. Agarose (0.8 per cent for genomic DNA and 1.5 per cent for PCR) was added to 1X TBE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature. Ethidium bromide was added at the rate of 5 μl per 50 ml to agarose solution and was allowed to completely mix. It was then poured into the gel mould and the comb was placed. It was allowed to solidify for half an hour at room temperature. After solidification the comb was removed carefully. The caste gel was placed in the electrophoresis tank containing 1X TBE buffer with the well near the cathode and submerged to a depth of one cm. Fifteen μl of the PCR product was mixed with 3 μl of 10X tracking dye and mixed well by pipetting in and out for 3 times. The mixture was loaded into the wells with the help of the micropipette. Two μl of lambda DNA marker (EcoR I and Hind III double digest) was loaded in one of the wells as a standard marker. The cathode and anode were connected to power pack using power cord and the
gel was run at a constant voltage of 50 volts. The negatively charged DNA molecules move towards the anode and get separated according to their molecular weight. The power was turned off when the marker reached the anode end and the gel was viewed in an UV trans illuminator and the banding pattern was analyzed. Gel photo documentation

After the separation of the PCR products with 1.5 per cent agarose gel, it was viewed and photographed using Alpha imager TM1200 documentation and analysis system.

3.15. Applications of endophytes

3.15.1. Application of endophytes in Nursery technology

3.15.1.1. Pre-plant bacterisation with endophytic isolates on the stem cuttings of the ornamental plant, *Hibiscus rosasinensis*.

3.15.1.1.1. General

The pot culture experiments as detailed below were conducted in the glass house of Department of Agricultural Microbiology, University of Agril. Sciences, GKVK Bangalore.

Garden land soil having the chemical properties of pH 7.4, EC 0.4 dS m⁻¹, available nitrogen 230 kg ha⁻¹, available P₂O₅ 10 kg ha⁻¹ and available K₂O 250 kg ha⁻¹ was passed through a 4 mm sieve and mixed along with farmyard manure in 2:1 proportion and filled in pots @ 10 kg pot⁻¹.

3.15.1.1.2. Source of cuttings and their preparation

Cuttings of Hibiscus 15 cm long and 2.5-3 cm thick were obtained from the Dept. of Horticulture, UAS, GKVK, Bangalore. A slant cut was
given at the distal end of the roots and that portion was kept in the media.

3.15.1.1.3. Source of endophytic isolates

Four endophytic isolates ECE6, HAE7, LEE18 and LEE19 which were already screened for their plant growth promotion ability by plant hormone production were used for this study.

3.15.1.1.4. Experimental details

Location : University of Agricultural Sciences, GKVK, Bangalore.

Design : CRD

Treatments : Eight

Replications: Three

No. of cuttings per replication : 5

3.15.1.1.4.1. Treatment details

T1 – Uninoculated control

T2 – Commercial formulation (Quicroot dip for 30-45 sec)

T3 – Chemical treatment (IBA 2500ppm for 30sec)

T4 – Ragi isolate ECE6

T5 – Sunflower isolate HAE7

T6 – Tomato isolate LEE18

T7 – Tomato isolate LEE19

T8 – Consortium of ECE6, HAE7, LEE18 and LEE19

3.15.1.1.5. Treatment of cuttings with endophytic isolates

Endophytic isolates viz. ECE6, HAE7, LEE18, LEE19 and the consortium of the above four was used for this study. A loopful of the
endophytic isolates were inoculated into the nutrient broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28 °C). After 48 h of incubation, the broth containing a population of $10^6$ cfu/ml was used for inoculation. The bacterial strains were grown separately and the four strains that are going to make up the combination were added equally (v/v) and finally mixed at the time of inoculation. The cuttings were dipped in 200 ml of the bacterial suspensions and incubated for 12 hours. Control was maintained by dipping the cuttings in water.

3.15.1.1.6. Treatment of cuttings with chemicals for rooting

Effect of endophytic isolates on rooting was compared with the commercial formulation Quicroot as well as with the standard chemical IBA.

3.15.1.1.6.1. Treatment of cuttings with Quicroot for rooting

Quic Root is a commercial formulation for rooting of cuttings from Ashwin Chemicals, Bangalore, which might be a combination of auxin (IBA) and other root promoting substances. The cut end of roots was dipped in this solution for 45 seconds.

3.15.1.1.6.2. Preparation of IBA stock solution

Chemical grade IBA was dissolved in little quantity of ethyl alcohol and the stock solution of IBA with a concentration of 25,000 ppm (25,000 mg IBA in one litre of distilled water) was prepared with distilled water. The working sample was prepared by making up 10 ml of this stock solution to 100ml with distilled water to make.

3.15.1.1.6.3. Treatment of cuttings with IBA

After required concentration of the growth regulator solution was prepared, the cuttings were dipped to 5 cm deep in the solution for 30
sec and were planted in the trays filled with rooting media, and placed inside the glass house.

3.15.1.1.7. After care

Watering was done regularly.

3.15.1.1.8. Observations recorded

After 90 days of the planting into the trays, the rooted cuttings were uprooted carefully and were dipped in water for 30 min to loosen the soil. These rooted cuttings were used for recording observations.

3.15.1.1.8.1. Root parameters

3.15.1.1.8.1.1. Rooting per cent

This parameter was calculated by taking the ratio of the number of rooted cuttings to the total number of cuttings planted and was multiplied by 100.

\[
\text{Rooting per cent} = \frac{\text{Number of rooted cuttings}}{\text{Total number of cuttings planted}} \times 100
\]

3.15.1.1.8.1.2. Length of the longest root

The length of the longest root was measured in centimeter and their mean was calculated to express the length of the longest root.

3.15.1.1.8.1.3. Fresh weight of roots

The fresh weight of roots was recorded using an electronic balance and the mean was calculated to express the fresh weight of roots in milligrams.
3.15.1.1.8.1.4. **Dry weight of roots**

The dry weight of roots was taken in milligrams by drying the roots in hot air oven at 50° C for 2 hours until constant dry weight was obtained and their mean expressed as the dry weight of roots.

3.15.1.1.8.2. **Shoot parameters**

3.15.1.1.8.2.1. **Sprouting per cent**

This parameter was calculated by taking the ratio of the number of sprouted cuttings to total number of cuttings planted and was multiplied by 100.

\[
\text{Sprouting per cent} = \frac{\text{Number of sprouted cuttings}}{\text{Total number of cuttings planted}} \times 100
\]

3.15.1.1.8.2.2. **Number of sprouts per cutting**

The number of sprouts was counted and their mean was used to express the number of sprouts per cutting.

3.15.1.1.8.2.3. **Length of shoot**

The length of shoot was measured in centimeter and their mean was used to express the length of shoot. The length was measured from point of initiation of the shoot to the growing tip.

3.15.1.1.8.2.4. **Number of leaves per cutting**

The number of fully opened leaves was counted and their mean was used to express the number of leaves.
3.15.1.2. Effect of endophytes on growth and establishment of tissue culture banana var. Nanjangudu rasabale

3.15.1.2.1. Experimental details

Location : University of Agril. Sciences, GKVK, Bengaluru
Crop : Tissue culture banana var. Nanjangudu Rasabale (primary hardening and secondary hardening stages)
Design : CRD
Treatments : 5
Replications : 5
No of plantlets / replication : 5

3.15.1.2.1.1. Treatment details

T1 : Control
T2 : Soybean isolate GME16
T3 : Tomato isolate LEE18
T4 : Tomato isolate LEE19
T5 : Consortium of the three

3.15.1.2.2. Preparation of individual and combination of endophytic bio-formulations

Endophytic isolates, viz. GME16, LEE18, LEE19 and combination of the above three were used for this study. A loopful of the endophytic isolates were inoculated into the nutrient broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28 °C). After 48 h of incubation, 200 ml of the broth containing 10^6 cfu/ml was used for inoculation. The bacterial strains were grown separately and the three
strains that are going to make up the consortium were added equally (v/v) and finally mixed at the time of inoculation.

3.15.1.2.3. Biotization of tissue culture banana plantlets

The micropropagated plantlets of banana var. Nanjangudu Rasabale were used for this study. Forty eight hour old cultures were used to inoculate micropropagated plantlets at the primary hardening stage. The bacterial suspensions were prepared (10^6 cfu/ml) as above. The tissue cultured plantlets from the bottles were immersed in 200ml suspensions for 12 h. Sterile distilled water served as control.

The roots of micropropagated plantlets while transferring from bottles to cups for primary hardening were dipped in the prepared bacterial suspension for 6 hours. The inoculated plantlets were planted in cups with coirpith substrate and incubated in the mist chamber. After 45 days, the plantlets were transplanted to the polybags filled with potting mixture for secondary hardening. At this time, endophytic bacterial suspensions were drenched in the tissue culture banana plantlets @1ml/plantlet. Tissue culture plantlets drenched in water served as controls. The plantlets were kept in the hardening chamber for 45 days and the growth parameters were recorded.

3.15.1.2.4. Observations

The following observations were recorded on morphological characters such as root length (cm), pseudostem height (cm), pseudostem girth (cm), number of leaves, and total leaf area.

3.15.1.2.4.1. Pseudostem height

The plantlet height was measured in centimeters (cm) from the ground level to tip of the plantlet after 45 days of planting.
3.15.1.2.4.2. Pseudostem girth

The girth of the pseudostem was measured in centimeters (cm) at the ground level after 45 days of planting.

3.15.1.2.4.3. Number of leaves per plant

The total numbers of leaves were recorded per plantlet after 45 days of planting.

3.15.1.2.4.4. Length of the leaf

The length of the third leaf from top was measured in centimetres from the end of petiole to the tip of the leaf.

3.15.1.2.4.5. Breadth of the leaf

The breadth of the leaf was measured at its broadest point and expressed in centimetres.

3.15.1.2.4.6. Total leaf area (Murray, 1960)

Total leaf area (TLA = L x B x K1 x N) was estimated non-destructively by multiplying the product of length (L) and breadth (B) of the third leaf by the factor 0.8 (K1), number of leaves (N) and expressed in cm².

3.15.1.2.4.7. Biomass production

The roots and shoots were separated after uprooting the plantlets. The shoot fresh weight and root fresh weight were recorded after uprooting the plants and expressed in grams.
3.15.2. Applications of endophytes in the field

3.15.2.1. Comparison of the effect of endophytic bacterial isolates on seedling growth of different varieties of aerobic rice

3.15.2.1.1. Experimental details

Variety: var. MAS26, var. MAS99, var. MAS946, var. MAS145
Design: CRD
Treatments: 5
Replications: 5

**Treatment details:**

T1: Soybean isolate GME16
T2: Tomato isolate LEE18
T3: Tomato isolate LEE19
T4: Consortium of GME16, LEE18 and LEE19
T5: Uninoculated control

3.15.2.1.2. Source of seeds

The seeds of aerobic rice var. MAS 26, var. MAS 99, var. MAS 946 and var. MAS 145 were obtained from the Dept. of Plant Breeding and Genetics, UAS, GKVK, Bangalore.

3.15.2.1.3. Source of endophytic isolates

Three endophytic isolates GME16, LEE18 and LEE19 which were already screened for their plant growth promotion ability and showing good GA, IAA and cytokinin activity were used for this study. A combination of the above three endophytic isolates were also used as one treatment.
3.15.2.1.4. Treatment of aerobic rice seeds with endophytic isolates

Endophytic isolates viz. GME16, LEE18, LEE19 and consortium of the above three were used for this study. A loopful of the endophytic isolates were inoculated into the nutrient broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28 °C). After 48 h of incubation, ten milliliter of the broth containing a population of $10^6$ cfu/ml was used for inoculation. The bacterial strains were grown separately and the three strains that are going to make up the combination were added equally (v/v) and finally mixed at the time of inoculation. The surface sterilization and seed bacterisation was carried out by following the procedure mentioned earlier.

3.15.2.1.5. Seedling growth study

The seedling growth was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Twenty treated seeds were kept over the presoaked germination paper. The seeds were held in position by placing another pre-soaked germination paper strip and gently pressed. The polythene sheet along with seeds was then rolled and incubated in growth chamber for 14 days. The following observations were recorded.

3.15.2.1.6. Observations recorded

3.15.2.1.6.1. Germination percentage

The percentage of germination of the seeds was calculated by recording the total no of seeds germinated to the total number of seeds sown after 14 days.

3.15.2.1.6.2. Shoot length

Ten normal seedlings were selected at random in each treatment for measuring the shoot length. The shoot length was measured from the
collar region to tip of the shoot and mean shoot length was expressed in centimeter.

**3.15.2.1.6.3. Root length**

Ten normal seedlings that were used for recording shoot length were also used to measure root length. Root length was measured from the collar region to the tip of the longest root in centimeter.

**3.15.2.1.6.4. Seedling length**

The seedling length of the plants was calculated as the total of the shoot length and root length and their mean was used to express the seedling length.

**3.15.2.1.6.5. Vigour index** (Abdul-Baki and Anderson, 1973).

The vigour index was calculated by using the formula

\[
\text{Vigour index} = \text{per cent germination} \times \text{seedling length (shoot length + root length)}
\]

**3.15.2.2. Effect of endophytic bacterial isolates on growth and yield of crop plants**

**3.15.2.2.1. General**

The pot culture experiments as detailed below were conducted in the glass house of Department of Agricultural Microbiology, University of Agril. Sciences, GKVK, Bangalore during 2011 – 2012 to study the effect of endophytic isolates on growth of three crops. Tomato, Paddy and cowpea were selected for the study.

**3.15.2.2.2. Experimental details**

Location : Dept. of Agril. Microbiology, UAS, GKVK, Bengaluru

Crops : Tomato var. Vaibhav
Aerobic rice var. MAS99
Cow pea var. KBC-2

Design : CRD
Treatments : 15
Replications : 3

**Treatment details**

T1- Control
T2 – NPK
T3 – 3 organisms+ spray
T4 – NPK + 3 organisms + spray
T5 - NPK + GME16 + spray
T6 - NPK + LEE18 + spray
T7 - NPK + LEE19 + spray
T8 – 50% NPK + 3 organisms + spray
T9 – 50% NPK + GME16 + spray
T10 – 50% NPK + LEE18 + spray
T11 – 50% NPK + LEE19 + spray
T12 – 75% NPK + 3 organisms + spray
T13- 75% NPK + GME16 + spray
T14 – 75% NPK + LEE18 + spray
T15 - 75% NPK + LEE19 + spray
3.15.2.2.3. Preparation of pot mixture

Garden land soil having the chemical properties of pH 7.4, EC 0.4 dS m\(^{-1}\), available nitrogen 230 kg ha\(^{-1}\), available P\(_2\)O\(_5\) 10 kg ha\(^{-1}\) and available K\(_2\)O 250 kg ha\(^{-1}\) was passed through a 4mm sieve and mixed along with farmyard manure in 2:1 proportion and filled in plastic pots @ 10 kg pot\(^{-1}\). Soil samples were mixed well to obtain homogenous mixture and subjected to two cycles of wetting and drying.

3.15.2.2.4. Source of seeds

The seeds of the tomato hybrid var. Vaibhav were obtained from the Dept. of Horticulture, UAS, GKVK, Bangalore. The seeds of paddy var. MAS99 were obtained from the Dept. of Plant Breeding and Genetics, UAS, GKVK, Bangalore. The seeds of cowpea var. KBC2 were obtained from the Dept of Agronomy, UAS, GKVK, Bangalore.

3.15.2.2.5. Source of endophytic isolates

Three endophytic isolates GME16, LEE18 and LEE19 which were already screened for their plant growth promotion ability based on the germination studies and by plant hormone production were used for pot culture studies.

3.15.2.2.6. Treatment of tomato seeds with endophytic isolates

Endophytic isolates viz. GME16, LEE18, LEE19 and combinations of the above three were used for this study. The endophytic isolates were grown in broth culture to a population of \(10^6\) cfu/ml by the method given earlier. The bacterial strains were grown separately and the three strains that are going to make up the combination were added equally (v/v) and finally mixed at the time of inoculation. The surface sterilization and seed bacterisation was carried out by following the procedure mentioned earlier.
3.15.2.2.7. Phyllosphere spray (PS)

The phyllosphere spray was given at the flowering stage of the crop. The standard inoculum of the test endophytic isolate combination was diluted at 1:1 ratio with sterile water and sprayed on the leaf at early morning or evening to have uniform wetting.

3.15.2.2.8. Imposition of treatments and fertilizer application

Recommended Dose of fertilizers (UAS, Bangalore)

<table>
<thead>
<tr>
<th>Crop</th>
<th>NPK (kg/ha.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>100: 76:45</td>
</tr>
<tr>
<td>Aerobic rice</td>
<td>50:50:50</td>
</tr>
<tr>
<td>Cowpea</td>
<td>20:30:10</td>
</tr>
</tbody>
</table>

The fertilizers were added as per treatment details. The nitrogen was applied as urea in two splits *i.e.* 50 per cent as basal and remaining 50 per cent as top dressing at 25th day after sowing and calculated quantities of Diammonium phosphate and Muriate of potash was applied. Phllosphere spray was taken up at 90 days of crop growth.

3.15.2.2.9. Watering

The watering of the plants was done at regular intervals based on crop requirement.

3.15.2.2.10. Harvesting

120 days after sowing the crops were harvested.

3.15.2.2.11. Observations recorded

The following observations *viz.*, germination percentage, seedling growth parameters, plant growth parameters, plant dry matter production, and finally yield parameters were recorded.
3.15.2.2.11.1. Germination per cent

The percentage germination of the seeds was calculated by recording the total no of seeds germinated to the total number of seeds sown after 7 days of sowing.

3.15.2.2.11.2. Shoot length

Ten normal seedlings were selected at random in each treatment for measuring shoot length. The shoot length was measured from the collar region to tip of the shoot and mean shoot length was expressed in centimeter.

3.15.2.2.11.3. Root length

Ten normal seedlings that were used for recording shoot length were also used to measure root length. Root length was measured from the collar region to the tip of the longest root in centimeter.

3.15.2.2.11.4. Seedling length

The seedling length of the plants was calculated as the total of the shoot length and root length and their mean was used to express the seedling length.

3.15.2.2.11.5. Vigour index (Abdul-Baki and Anderson, 1973).

The vigour index was calculated by using the formula

\[
\text{Vigour index} = \text{per cent germination} \times \text{seedling length (shoot length + root length)}
\]

3.15.2.2.12. Observations recorded for tomato

3.15.2.2.12.1. Plant height

The height of the plant from the cotyledonary node to the tip of the main stem was measured in a plant in each replication and the means
were worked out and expressed in cm during 60 days, 90 days and 120 days.

3.15.2.2.12.2. Number of branches

The branches that arise from the main stem were counted at intervals; the mean was worked out and expressed as number.

3.15.2.2.12.3. Number of leaves per plant

The total number of leaves in the plant was counted, the mean was calculated and expressed as number.

3.15.2.2.12.4. Plant dry matter production

The shoots, roots and leaves were first washed and then dried in shade for 24 to 36 hours. Then they were dried in hot air oven at 50°C until constant dry weight was obtained and the average dry weight of the plant was expressed in grams.

3.15.2.2.12.5. Yield parameters

3.15.2.2.12.5.1. Mean number of fruiting clusters per plant

The number of fruiting clusters per plant was counted from randomly selected plants in each replication and the average number was calculated.

3.15.2.2.12.5.2. Mean number of fruits per plant

The red ripe fruits harvested from randomly selected plants were counted and expressed as mean number of fruits per plant.

3.15.2.2.12.5.3. Mean individual fruit weight

Mean fruit weight was obtained by dividing the mean yield per plant by mean number of fruits per plant and expressed in g.
3.15.2.2.12.5.4. Mean yield per plant

All the red ripe fruits harvested from randomly selected plants were weighed, the mean was calculated and mean for each plant was expressed as mean yield in g/plant.

3.15.2.2.13. Observations recorded for paddy

3.15.2.2.13.1. Plant height

The plant height of five plants were measured from the ground level to tip of the topmost leaf at early stages (15, 30, 60, 90 DAT), up to the tip of main panicle at maturity and the average height was expressed in centimetres.

3.15.2.2.13.2. Number of tillers per plant

Total number of tillers were counted in each plant and averaged for five plants.

3.15.2.2.13.3. Number of leaves per plant

Total number of leaves were recorded per plant separately and averaged for five plants.

3.15.2.2.13.4. Plant dry matter production

The shoots, roots and leaves were first washed and then dried in shade for 24 to 36 hours. Then they were dried in hot air oven at 50°C until constant dry weight was obtained and the average dry weight of the plant was expressed in grams.

3.15.2.2.13.5. Yield parameters

3.15.2.2.13.5.1. Number of panicles per plant

Total number of panicles were recorded per plant separately and averaged for five plants.
3.15.2.2.13.5.2. Panicle length

Panicle length from five tillers selected from randomly labelled plants was recorded from base to the tip of the panicle. The mean value was calculated and expressed in centimetres.

3.15.2.2.13.5.3. Panicle weight

Panicle weight from five tillers selected from randomly labeled plants was recorded; the mean value was calculated and expressed in grams.

3.15.2.2.13.5.4. Grain yield per plant

The weight of the grains in the panicles per plant from five tillers selected from randomly labeled plants was recorded and the mean was expressed in grams.

3.15.2.2.14. Observations recorded for cowpea

3.15.2.2.14.1. Plant height

The plant height was measured in centimeters (cm) from the ground level to tip of the main stem of the plant at monthly intervals.

3.15.2.2.14.2. Number of primary branches per plant

The number of branches arising directly from the main stem was counted at intervals.

3.15.2.2.14.3. Number of leaves per plant

Total number of leaves was recorded per plant at monthly intervals.
3.15.2.2.14.4. Plant dry matter production

The shoots, roots and leaves were first washed and then dried in shade for 24 to 36 hours. Then they were dried in hot air oven at 50°C until constant dry weight was obtained and the average dry weight of the plant was expressed in grams.

3.15.2.2.14.5. Yield parameters

3.15.2.2.14.5.1. Number of flowering clusters per plant

The total numbers of flowering clusters per plant were counted from randomly selected plants in each replication and the average number was calculated.

3.15.2.2.14.5.2. Number of pods per plant

The total number of pods per plant was counted at the time of harvest

3.15.2.2.14.5.3. Pod Length

Pod length in centimeters (cm) was measured from the randomly selected five pods at the time of harvest and their mean was calculated.

3.15.2.2.14.5.4. Number of seeds per pod

Number of seeds per pod was taken as a mean number of seeds of five randomly selected pods at the time of harvest.

3.15.2.2.14.5. Mean yield per plant

All the pods harvested from randomly selected plants were weighed, the mean was calculated and mean for each plant was expressed as mean yield in g/plant.
3.16. Statistical analysis

The results of the experiments were subjected to statistical scrutiny as per the methods detailed by Panse and Sukhatme (1985). Data were subjected to the analysis of variance (ANOVA) at two significant levels (< 0.05 and < 0.01) and means were compared by Duncan’s multiple range test (DMRT).