Materials and Methods

Different types of chemicals used in this study are given in Appendices; however, the methods followed are discussed below:

A. ISOLATION AND IDENTIFICATION OF MICROORGANISMS

Urdbean, *Vigna mungo* variety Type-27 was procured from the market and sown in field in July 2010. This variety has been recommended for central and western tracts of Uttar Pradesh. The test pathogen and endophytic bacteria were isolated from root nodules and identified following the methods as given below:

1. Isolation of *Macrophomina phaseolina*

*V. mungo* growing areas were surveyed, plants showing disease symptoms were gently uprooted and put in sterile polyethylene bags. Roots were carefully observed for appearance of charcoal like symptoms. Similarly abnormal wrinkled seeds of urad were also used for isolation of the pathogen. *M. phaseolina* was isolated from above root and seed samples following the blotter technique and agar plate methods as described below:

1.1 Blotter Technique (de Tempe, 1963)

Circular discs of blotting paper equal to a diameter of Petri plate (90 cm) were cut and moistened with sterile distilled water then placed into the Petri plates. These were autoclaved at 15 PSI for 30 minutes. Diseased root pieces (1 cm long) were cut and sterilized with 0.5% NaOCl solution for 3-5 minutes and washed for about 8-10 times with sterilized distilled water to remove traces of NaOCl. These root pieces were dried with sterile blotting paper and five pieces were aseptically transferred on the surface of
moistened blotting paper and incubated at 28°C for 5-6 days for appearance of fungal colonies.

1.2. Water Agar Plate Method (Muskett and Malone, 1941)

Diseased root material and urad seeds were surface sterilized as described earlier. Samples were aseptically placed into the sterilized and cooled down 2% water agar plates. All plates were incubated at 28°C for 5-6 days, and appearance of fungal colonies around seeds was observed.

1.3. Identification of Fungal Pathogens

Characteristics of *M. phaseolina* were identified following mycological literature available in the laboratory and comparing with standard culture obtained from the Division of Plant Pathology, Indian Agriculture Research Institute (IARI), New Delhi (India).

1.4. Maintenance of Fungal Pathogen

A number of methods are used for maintaining fungal culture in a viable condition over a long period of time. However, the fungal culture was maintained on sterilized potato dextrose agar (PDA) medium in slants and stored in a refrigerator at 4°C for further use. These cultures were periodically transferred into fresh medium at an interval of three months.

2. Isolation and Identification of Endophytic Bacteria (Vincent, 1970)

*Vigna mungo* variety Type-27 was procured from the market and sown in a small experimental plot in July 2010. This variety has been recommended for central and western tracts of Uttar Pradesh. The plants are tall with dark green foliage, flowers are yellow and pods are green at early stage but black at maturity; seeds are medium-sized and black in colour.
Healthy *V. mungo* seedlings were collected from the experimental plot, put into sterile polyethylene bags and brought to the laboratory. Bacterial strains were screened as described by Vincent (1970). Root nodules were removed carefully and washed with sterile distilled water followed by surface sterilization with 5 ml of 95 % ethanol and again rinsed with sterile distilled water. Washed nodules were surface sterilized with 0.5 % NaOCl for 2-3 minutes and again washed with sterile distilled water about 8-10 times to remove traces of NaOCl. Nodules were crushed with a sterile glass rod in sterilized culture tube containing 1 ml of sterile distilled water. This suspension was serially diluted up to $10^{-5}$. 1 ml suspension from each dilution was transferred into the plates of different pre-prepared media (CrYEMA medium, Bacillus agar medium, King’s B medium and Nutrient Agar medium). All these plates were incubated at 28±1°C for 4-5 days. Colonies were obtained with different characters on different medium, which were observed and maintained on NAM plates or slants at 4°C for further use.

The cultures were identified following the Bergey’s Manual of Determinative Bacteriology (Holt *et al.*, 1994) and compared against the standard cultures of * Bradyrhizobium* sp. NAIMCC-B-00262 [obtained from National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Azamgarh, U.P.], *Bacillus subtilis* MTCC 441 and *Pseudomonas* sp. MTCC 129 [procured from Microbial Type Culture Collection Centre (MTCC), Institute of Microbiological Techniques (IMTECH), Chandigarh, India].

**B. CHARACTERIZATION OF ENDOPHYTIC BACTERIA**

Both physical and physico-chemical characterizations of bacterial isolates were done by based on the following parameters:

1. **Physical Characterization**

   Physical characterization of isolates included the culture characteristic, motility test and measurement of generation time.
1.1. Cultural Characteristic

Nodule isolates were streaked on nutrient agar medium (NAM), yeast extract mannitol agar (YEMA) medium, Bacillus agar medium (BAM) and King’s B (KB) medium. Colony morphology (like colony shape, colony appearance, colony forms, colony elevation, colony margins and colony colour, etc.) of the bacterial isolates was recorded.

1.2. Motility

Hanging drop method was used to determine motility test. One drop of exponentially grown culture of bacterial isolates was placed in the centre of a cover slip, this cover slip was inverted and put over the well of a cavity slide in such a way that the drop does not move or contact the side wall of the well. The cavity was sealed with paraffin wax and drop was observed for bacterial motility under 100X objective of compound light microscope (Dubey and Maheshwari, 2011).

1.3. Generation Time

Bacterial isolates were inoculated in 50 ml of their relevant broths in conical flasks and incubated in rotary shaker at 150 rpm at 28±1°C for 48 h. The growth (turbidity) was observed and O.D. was measured at 610 nm in a UV-VIS spectrophotometer (Shimadzu, Model UV-1601 after every 12 h intervals. Their generation time was calculated by using the following formula:

\[
\text{Generation Time} = \frac{(T2 - T1)}{3.3 \left( \log_{10} \text{OD}_2 - \log_{10} \text{OD}_1 \right)}
\]

Where,

\( T2 - T1 = \) time interval taken at any two points in the log phase of growth.

\( \log_{10} \text{OD}_2 - \log_{10} \text{OD}_1 = \) difference between the \( \log_{10} \) values of OD at time \( T2 \) hours to \( \log_{10} \) value of OD at time \( T1 \) hours.
2. Physiological and Biochemical Characterization

For physiological and biochemical characterization all the bacterial isolates were grown on desired solid as well as liquid media and incubated at 28±1°C. Log phase (exponential growth phase) cultures (10^8 cfu ml^{-1}) were used for inoculation. All the tests were done in triplicates.

2.1. Gram’s Staining

Smears of each bacterial isolate were separately prepared on a clean glass slide and heat-fixed after drying. One drop of crystal violet solution was put onto the smear and allowed to react for 45 sec. Excess stain was washed off with sterile water. Then one drop of Gram’s iodine solution was put and allowed to react for 45 sec. Then it was washed with water followed by dipping in absolute alcohol in a 100 ml beaker for 1 minute. Thereafter, one drop of safranine (counter stain) was applied over the smear, and allowed to react for 1 min. It was washed gently with sterile water, air dried, mounted in glycerine and examined under oil immersion.

2.2. Himedia Enterobacteriaceae Identification Kit

Himedia Enterobacteriaceae Identification Kit was used to perform the following tests:

ONPG (ortho-nitrophenyl-galactoside), nitrate reduction, H_2S production, indole and esculin hydrolysis, etc.

Broth media were prepared and inoculated with the bacterial isolates then incubated at 37°C for 4-6 h at 150 rpm until inoculum attained 0.1 OD at 620 nm by density adjustment with 2-3 ml sterile saline. Kits were opened aseptically, and 50 μl inocula from each bacterial isolate were put in each well of both strips (I and II). All strips were incubated at 35-37°C for 18-24 h. Development of any colour was recorded as the positive or negative result.
2.3. Catalase Activity (Graham and Parker, 1964)

A drop of 48 h old culture of all bacterial isolates was aseptically dropped on a clean glass slide. Then a drop of hydrogen peroxide (3%) was added to it. Liberation of effervescence of oxygen around the bacterial colonies was observed and recorded.

2.4. Oxidase Activity (Kovaks, 1956)

Few drops of p-aminodimethylaniline oxylate (1%) were dropped on the 48 h old culture of bacterial isolates put on a clean glass slide and examined for development of colour.

2.5. Gelatin Hydrolysis (Sadowsky et al., 1983)

Solid media such as YEMA, BAM and NAM containing 0.4% (w/v) gelatine were prepared, autoclaved and poured onto sterile Petri dishes. Then the plates were separately inoculated by exponentially grown culture of each bacterial isolate. All plates were incubated at 28±1°C for 7 days. The plates were observed for liquification of gelatin.

2.6. Urea Hydrolysis (Lindstrom and Lehtomaki, 1988)

Culture tubes containing 5 ml of sterilized different broth media (altered with 2% urea and 0.012% phenol red) were separately inoculated with log phase culture (12 h old) of bacterial strains and incubated for 7 days at 30°C. Appearance of red colour in tubes indicated urea hydrolysis.

2.7. Citrate Utilization (Koser, 1923)

To perform this test, modified Simmon’s citrate broth medium was prepared by adding sodium citrate (in place of carbon source) and bromothymol blue (25 mg l⁻¹). The plates were inoculated with log phase culture of bacterial isolates and incubated at 28±1°C for 48 h.
2.8. Growth in Presence of KNO$_3$ (El Idrissi et al., 1996)

The capability of bacterial isolates to grow in presence of 8% KNO$_3$ was detected by separately inoculating these isolates onto the desired nutrient plates containing 8% KNO$_3$. The plates were incubated for 7 days at 28±1°C for future growth of bacterial colonies.

2.9. Poly Hydroxy Butyrate (PHB) Accumulation (Navarini et al., 1992)

Respective broth media were prepared for different isolates, inoculated with 0.5 ml of log phase (10$^8$ cells ml$^{-1}$) culture of bacterial isolates separately and incubated in a rotary shaker at 150 rpm at 28°C for 96 h. Each culture was centrifuged at 10,000 rpm for 45 min and supernatant was collected. 2 volume of chilled acetone was added in supernatant to precipitate the exopolysaccharide (EPS). The extracted EPS was dried at 45°C till constant weight was achieved.

2.10. MR-VP Test

MR-VP broth was prepared and transferred into tubes which were inoculated with bacterial culture and incubated at 24°C for 2 days. 5 drops of methyl red indicator was dropped into each tube of first set. Tubes were observed for development of red colour. In another set of MR-VP broth, 2-3 drops of VP reagent was dropped and after shaking to 30 second tubes were exposed to the air and observed for development of crimson ruby pink colour (Dubey and Maheshwari, 2011).

2.11. Starch Hydrolysis

Petri plates containing starch medium were inoculated with bacterial isolates and incubated at 28±1°C for 72 h. Plates were observed for development of clear halo around the colonies. Iodine solution (1 ml) was poured in each plate and observed for clear zone against the blue-black background.
2.12. Effect of Temperature

YEMA, Bacillus agar medium and nutrient agar medium containing plates were prepared and inoculated with exponentially grown culture of all the bacterial isolates. All plates were incubated at different temperature regimes viz., 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C for 48 h in a rotary shaker at 150 rpm. Plates were examined for the presence of bacterial growth.

2.13. Effect of pH

Petri plates containing sterilised YEMA, Bacillus agar medium and nutrient agar medium were prepared by adjusting different values of pH viz., 4, 5, 6, 7, 8, 9, 10 and 11, with 1N HCl or 1N NaOH. All plates were separately inoculated with bacterial isolates and incubated at 28 ± 1°C for 48h. Thereafter, the plates were examined for bacterial growth.

2.14. Tolerance of 1-6% NaCl (El-Idrissi et al., 1996)

YEMA, Bacillus agar medium and nutrient agar medium were separately supplemented with different percentage of NaCl, autoclaved and poured into sterilised Petri dishes. The plates were inoculated with bacterial isolates and incubated at 28±1°C for 48 h for the appearance of bacterial growth; these colonies were recorded.

2.15. Utilization of Carbon Sources (El Idrissi et al., 1996)

Himedia Carbohydrate™ Kit (Himedia Laboratories Pvt. Ltd., Mumbai, India) was used to determine the 35 carbon source utilization consisting of monosaccharides pentoses, monosaccharides, hexoses, disaccharides, trisaccharides, polysaccharides, organic compounds and sugar alcohol. HiCarbohydrate™ Kit (Himedia) contained following carbon sources:

**Strip ‘A’**- Lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose.
Strain ‘B’- Inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α-methyl-D-glucoside, and ribose.

Strain ‘C’- Rhamnose, cellobiose, melizotose, α-methyl-D-mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, malonate, sorbose.

Desired broth media were prepared for all isolates. Pure culture from single colony of bacterial isolates was inoculated in all types of broth media separately and incubated at 37°C for 4-6 h until inoculum density attained 0.5 OD at 620 nm. Kits were opened aseptically and 50 µl inoculums of each strain of all bacteria were aliquoted in each well (as per manufacturer’s instructions).

The test is based on the principle of pH change after substrate utilization. When stripes were incubated for 24 hours, the isolates utilized different carbon sources via different metabolic processes and resulted in change in colour of each well.

3. Biolog Metabolic Finger-printing

For the first time, Garland and Mills (1991) applied Biolog MicroPlates to discriminate microbial communities from a variety of largely different habitats according to their utilization patterns of 95 sole carbon sources. Since that, this approach has been used in a number of studies on communities from soil and from rhizosphere or subsurface cores. The reason for the increasing popularity of this method is its applicability to screen rapidly community for changes.

Strains were tested for ability to metabolize carbon sources using Biolog GN microtiter plates (Microlog 2, Version 4.2, Biolog Inc, Hayward, CA). This plate consists of different 95 carbon sources as given in ‘Results’ (see Fig. 13).

Each 96 well microtiter plate has 95 wells that contain a single substrate per well with one water negative control well, for a total of 96 substrates. A single colony of each strain cultured on nutrient agar (NA) and incubated for 24h at 28°C. Cells were grown to mid-log phase for each respective isolate and then pelleted for 5 min at 7,000 rpm at 4°C. The media supernatant was discarded and the pellets were washed for three
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Characterization of endophytic rhizobacteria from Vigna mungo (L.) Hepper and their role in biocontrol of Macrophomina phaseolina (Tassi) Goid.

C. MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

Genomic DNA of the isolates VR1 and VR2 were isolated and characterized under the following steps:

1. Isolation of Genomic DNA

The genomic DNAs of the strains VR1 and VR2 were isolated separately following Sambrook and Russel (2001). A single colony was inoculated into the YEM broth and incubated over night at 28°C. The broth culture was centrifuged at 10,000 rpm for 5 minute at 28°C and the pellet was collected by discarding the supernatant. The
pellets were washed by TE buffer (10 mM Tris- HCl, 1mM EDTA at pH 8.0); this process was repeated twice. The cell pellets were re-suspended into 0.5 ml SET buffer (15 mM NaCl, 25 mM EDTA of pH 8.0, 20 mM Tris HCl of pH 8.0). 10 μl (10 mg/ml), lysozyme was added to the above suspension and incubated at 37°C for 30-60 minutes. After incubation 0.1 ml of 10% SDS and 10 μl of proteinase K (15 mg/ml) were added to above suspension and the contents were incubated at 55°C for 60 minutes. 10μl of RNase was added and incubated at 37°C for 30 minutes. Thereafter, 0.3 ml of 5M NaCl and equal volume of Tris water saturated phenol : chloroform : isoamyl alcohol (25: 24: 1) were added and the contents were incubated at room temperature for 30 minute followed by gentle overtaxing. Then the mixture was centrifuged at 10,000 rpm for 15 min at room temperature. Aqueous layer was removed in fresh tube. To the aqueous phase 0.1 ml of the sodium acetate (pH 4.8) and 1ml of chilled absolute ethanol were added followed by gentle extraction and incubated at room temperature for 30 minutes. The mixture was centrifuged at 10,000 rpm at 4°C for 5 minutes. The pellets were washed with 70% ethanol and again centrifuged at 10,000 rpm at 4°C for 5 minute. This step was repeated twice. The DNA was spooled out into eppendorf tubes and washed twice with 70% ethanol. After Washing, the alcohol was evaporated and DNA was re-dissolved in 30 μl TE buffer (or milli Q water) and stored at 4°C.

1.1. Quantification of Genomic DNA by Gel Electrophoresis

The quality and quantity of the genomic DNA was checked on agarose gel electrophoresis. The principle of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. When an electrical potential is applied on the DNA, it moves toward the positive pole. The 1X TAE buffer was prepared by diluting 50X TAE buffer. 100 ml 1X TAE buffer was added to 0.8% agarose by melting in a microwave oven. Then 2 μl ethidium bromide was added after cooling the agarose gel (ethidium bromide is intercalating dye and carcinogenic, hence this gel should be handled with care by wearing gloves). After cooling the solution was
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Characterization of endophytic rhizobacteria from Vigna mungo (L.) Hepper and their role in biocontrol of Macrophomina phaseolina (Tassi) Goid.

poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The gel was 3-5 mm thick. There were no air bubbles under or between the teeth of comb. While the gel was cooling, DNA sample were prepared by adding 1µl of tracking dye to 5µl of each sample.

The gel on the tray was placed horizontally into the electrophoresis chamber and flooded the top of the gel with fresh running buffer (TAE) to cover the gel to depth of about 1 mm. The solution (DNA sample with dye) was sucked into the pipette, the tip was placed in top of the well and the solution was gently expelled into the well. The lid and power leads were placed on the apparatus, 60 V current was applied, and current flowing was confirmed by observing the bubbles coming out from the electrodes.

1.2. Quantification of DNA by Spectrophotometric Method

DNA yield was determined by measuring the concentration of DNA in the sample by its absorbance at 260 nm.

The DNA was diluted using water (water is used as diluent while measuring the DNA concentration because the relationship between absorbance and concentration is based on extinction coefficients calculated for nucleic acids in water). The spectrophotometer was switched on and the blank was set using water. The diluted DNA sample was transferred in cuvette and placed in spectrophotometer. The ‘Sample’ button was clicked and the reading was recorded (includes the DNA yield, A260, A260/A280, etc.). This process was repeated thrice to get an accurate data. The ratio of the readings at 260 nm and 280 nm provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. For a pure DNA the A260/A280 ratio should be in between 1.8 and 2.0. Since the purity is considerably influenced by pH, therefore, use of 10 mM Tris-HCl as the diluents is better than water.

Calculation

Concentration of DNA (µg/ml) = DNA yield in µg/ml × A260 × dilution factor
Total amount (µg) = concentration × volume of sample in milliliters

Absorbance of the solution at wavelengths was measured at 260 nm and 280 nm. The ratios A230/A260 and A280/A260 were calculated. A good DNA concentration using the relationships for single stranded DNA, 1 OD at 260nm = 50 µg/ml. This estimate is influenced by contaminating substances like RNA and very low molecular weight DNA in the solution. Hence a working stock of samples of about 100 µl with concentrations of 10ng/µl was prepared.

1.3. PCR Amplification of 16S rRNA Gene

Amplification of 16S rDNA was carried out in total volume of 100 µL reaction mixture. The sequence of the primers used was PA: 5’-AGAGTTTGATCCTGGCTCAG-3’; PH: 5’-AAGGAGGTGATCCAGCCGCA-3’ (Banglore Genei). The reaction mixture consisted of pure genomic DNA (50 ng), Taq buffer (10X), dNTP mix (2.0 mM), primers (10 ng/µl), and 1 U Taq polymerase. DNA amplification was done in a Bio-Rad thermal cycler with the following temperature profile: an initial denaturation at 94°C for 5 min (39 cycles of denaturation for 30 sec at 94°C), annealing at 52°C for 40 sec and extension at 72°C for 1min, and a final extension at 72°C for 1 min. 40 cycles was performed. The PCR product was analyzed on 1.2% agarose in a TAE buffer. A 1 kb ladder DNA (Bangalore Genei) was used as size marker (for detail see Appendix).

1.4. Gel Electrophoresis

After the completion of amplification, 2 µl of loading dye (1X) was added to each PCR tube. Agarose gel (1.2 %), with ethidium bromide (1µl/ 100 ml) added to stain PCR products, was prepared; 3µl of amplified DNA molecules present from the PCR tubes were loaded into the gel. Electrophoresis of DNA molecules was carried out in 1X TAE buffer at 65 V for 2 hours.
1.5. 16S rDNA Gene Sequencing

The electrophoresed PCR product was sequenced by using ABI 3130 XL Sequencer. Similarity of 16S rRNA gene sequences by Sanger protocol was performed with the basic local alignment search tool BLASTn programme run against the BLAST database of GenBank (National Center for Biotechnology Information, NCBI) website (http://www.ncbi.nlm.nih.gov) to check the similarity pattern. After BLAST similarity search, the 16S rRNA gene sequence was submitted to NCBI database for accession number.

D. IN VITRO NODULATION TEST

1. Seed Germination and Nodulation

Nodulation test of bacterial isolates VR1, VR2, VR11 and VR13 was carried out in culture tubes in vitro. Healthy urad seeds were surface sterilized with 5% NaOCl and washed 6-8 times to remove traces of chemical. Five seeds were placed on surface of sterilized water agar medium (1%) in Petri plates and covered with sterile wet cotton pad. These plates were incubated in dark at 30±1ºC to allow germination.

A germinated single seed was transferred onto the surface of nitrogen-free nutrient agar medium in culture tubes of 50 ml capacity in three replicate. Then the tubes were covered with a black paper. Exponentially grown culture (10^8 cfu ml^-1) of isolates VR1, VR2, VR11 and VR13 was separately inoculated in each tube in three replicates. The un-inoculated seeds served as control. All the tubes were incubated at 30ºC for 15-20 days, and data were recorded thereafter.

E. PLANT GROWTH PROMOTING ATTRIBUTES

Different tests of plant growth promoting properties were performed as described below:
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1. IAA Production

Exponentially grown cultures of isolates were grown in broth medium at 28°C and 150 rpm for 24h. Cultures were separately centrifuged at 10,000 rpm for 10-15 minutes at 4°C. Supernatant was transferred into fresh tubes. 100 µl of 10 mM O-phosphoric acid was added to 2 ml of supernatant, and change in colour was recorded.

2. HCN Production (Bakker and Shippers, 1987)

For detection of HCN production, exponentially grown cultures of strains were streaked on agar plates supplemented with or without 4.4 g glycine l⁻¹. Filter paper soaked with 0.5% picric acid in 1% Na₂CO₃ were transferred in the upper lids of plates, which were sealed with parafilm. The plates were incubated at 28±1°C for 72 h along with a control, and changes in colour were recorded.

3. Phosphate Solubilisation (Pikovskaya, 1948)

Bacterial isolates were separately spotted on Pikovskya’s agar plates for detection of phosphate solubilization. Plates were incubated at 28±1°C for 72 h. Formation of clearing zones around the colonies due to solubilization of inorganic phosphate by bacterial isolates were recorded.

4. Siderophore Production

Siderophore production was detected on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). Bacterial isolates (24 h old culture) were spotted on CAS medium and all plates were incubated at 28±1°C for 48 h. Development of orange halos, if any, around the colonies due to siderophore production was recorded.

4.1. Detection of Type of Siderophore - CAS broth was prepared and transferred in culture tubes, which were then inoculated separately with 1 ml of each bacterial culture
and incubated at 28 ±1°C for 24h. Contents of each tube were centrifuged at 4°C for 15 minutes at 10,000 rpm and supernatant was collected. 1 ml of 1mM FeCl₃ solution was added to the supernatant. OD of supernatant was measured at 450 nm and 500 nm using UV-VIS spectrophotometer (Shimadzu, Japan).

Development of orange colour indicated hydroxamate type of siderophore which showed maximum absorption at 450 nm, whereas wine colour was indicative of catechol type of siderophore with maximum absorption at 500 nm (Neilands, 1981). Determination of catechol-phenolic type siderophores was done following the methods of Arnow (1937) and Rioux et al. (1986) using 2, 3-dihydroxybenzoic acid as the standard. Presence of hydroxamate type of siderophores was determined following the method of Gibson and Magrath (1969) by assessing the absorption spectrum of 48 h supernatant at 400 nm, spectrophotometrically.

4.2. Siderophore Assay

The universal chemical assay was done to examine siderophore as described by Schwyn and Neilands (1987). CAS assay solution was prepared, and 6 ml of 10 mM HDTMA solution was diluted with double glass distilled water to make the volume to 100 ml. A mixture of 1.5 ml iron (III) solution (1 mM FeCl₃.6H₂O in 100 mM HCl) and 7.5 ml of 2 mM aqueous CAS solution was slowly added under stirring. Anhydrous piperazine (4.307 g) was dissolved in water and 6.25 ml of 12 M HCl was carefully added to it. The volumetric flask was rinsed with this buffer solution (pH 5.6) which was then filled with water to afford 100 ml of CAS assay solution. The CAS shuttle solution was obtained by adding 5-sulfosalicylic acid to the above solution at a concentration of 4 mM.

For siderophore production by bacteria a special medium was prepared, which consisted of MM9 salts, Tris buffer, casamino acids (0.3%), L-glutamic acid (0.05%), (+) biotin (0.5 ppm) and sucrose (0.2%). Bacterial cultures were inoculated in this medium and incubated at 28°C for 48 h under rotating conditions; its cell density was monitored at 610 nm using a spectrophotometer. After incubation, culture supernatant
(0.5 ml) was mixed with CAS assay solution (0.5 ml). An un-inoculated medium served as control. The absorbance of the mixture was measured at 630 nm when colour reached the equilibrium. A calibration curve of 2,3-dihydroxybenzoic acid was prepared as a standard under the same conditions. This curve was used to determine the concentration of siderophore in cultures.

5.3. Time required for Siderophore Production

Following the above method for quantification of siderophore production, samples from the bacterial broth was withdrawn at every 24 h intervals for 168 h for the quantitative determination of siderophore. A plot was prepared comparing siderophore production with respect to time.

5. ACC Deaminase Activity

Respective broth media (according to bacterial isolates) were prepared and poured in 50 ml Erlenmeyer flasks, autoclaved and inoculated separately with log phase ($10^8$ cells ml$^{-1}$) cultures of each isolate. The flasks were incubated at 28°C for 96 h in a rotary shaker at 150 rpm. Each culture was centrifuged at 8,000 rpm for 10 min at 4°C and pellets were collected, washed with sterile distilled water and re-suspended in sterile distilled water. Plates containing minimal medium (amended with 3 mM ACC) were separately spotted with the suspension of each culture. The inoculated plates were incubated at 28±1°C for 3-4 days (Honma and Shimomura, 1978) along with positive (minimal medium with $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source) and negative control (minimal medium without ACC). All the plates were examined and compared with other plates for ACC deaminase production.

F. ANTAGONISTIC PROPERTIES IN BACTERIAL ISOLATES

Antagonistic properties of bacteria were tested against $M.\ phaseolina$ in vitro (by assay of chitinase activity, growth inhibition of interacting colonies by dual culture
method (and development of deformities in fungal hyphae), and using cell-free culture filtrates of bacterial antagonists (for measuring the zones of inhibition, mycelia dry mass, sclerotia germination and hyphal development).

1. Chitinase Activity

Chitinase activity was measured as below:

1.1. Preparation of Colloidal Chitin (Berger and Reynoldes, 1988)

Colloidal chitin was prepared by slowly dissolving 10 g of crab shell chitin (Sigma) in one liter of concentrated HCl under stirring conditions at 4°C. The viscous mixture was obtained incubated at 37°C on a water-bath until the decrease of viscosity of the mixture. Sterile distilled water (4 liters) was added to this mixture and left overnight at 4°C. The supernatant was slowly decanted and the precipitate was collected on a filter paper. Then this precipitate was washed several times with sterile distilled water to attain a neutral pH (7.0). The saturated colloidal chitin was air-dried and dissolved in 250 ml sterile distilled water before use.

1.2. Assay of Chitinase Activity

Defined medium was used to detect chitinase activity having colloidal chitin as sole carbon source following the method of Renwick et al. (1991). Bacterial isolates were spotted on the plates containing defined medium supplemented with colloidal chitin, and incubated at 28±1°C for 5-6 days. Thereafter, plates were observed for development of clear zones around bacterial colonies.

2. In vitro Antagonistic Properties by Dual Culture Method

Antagonistic property of all bacterial isolates was tested against M. phaseolina following dual culture method and cell-free extract method. Abnormalities and deformity caused in fungal mycelia were observed under light and electron microscope, and photomicrographs were taken.
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Antagonistic property of *Bradyrhizobium*, *Bacillus* and *Pseudomonas* isolates against *M. phaseolina* was tested by using dual culture technique (Skidmore and Dickinson, 1976). 5 day-old mycelial disc (5 mm diam.) cut from actively growing margin of pathogen was placed in the centre of sterilized modified medium containing YEMA and Czapek’s Dox agar (CDA) in a ratio 1:1. A loop full exponentially grown culture of all bacterial isolates was spotted 2 cm juxtaposed from the fungal disc and incubated at 28±1°C for 5 days. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies as compared to control (without bacterium). The zone of inhibition was recorded and growth inhibition (%) was calculated by using the formula:

\[
\text{Inhibition} \% = \frac{\text{Radial growth in control (C)} - \text{Radial growth in dual culture (T)}}{\text{Radial growth in control (C)}} \times 100
\]

Where, C = radial growth in control

T = radial growth in dual culture

2.1. Microscopic Examination of Fungal Mycelia

Some distortion, abnormality and defect aroused due to antagonistic activity of bacterial isolates in fungal mycelia were observed under compound microscope. Fungal mycelia were picked up with the help of a sterile needle or forceps from the edge of zone of inhibition and placed onto a clean glass slide upon which a drop of lacto-phenol was put. Slide was observed under a compound microscope (Olympus BX 51 TRF). If any abnormality, deformity or defect occurred, the images were captured by using Image Analyzer (Biovis, India).
2.2. Scanning Electron Microscopy (SEM) of Fungal Mycelia from Zone of Interaction

For preparation of scanning electron microscope (SEM) samples, mycelia were collected, with the help of a sterile needle, from the zone of interaction between two microorganisms. Mycelia were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) at 48°C, and washed thrice in phosphate buffer (10 min each). Then, samples were dehydrated by serially passing through 70, 80, 90 and 100% ethanol (5 min at each stage) and finally in 100% ethanol at room temperature. Thereafter, ethanol was replaced with liquid CO₂ and the samples were air dried as described by Lopez-Llorca and Valiente (1993). The samples were mounted on stubs followed by coating with gold; the coated specimens were observed at 15 kV in a LEO 485 VP SEM and photomicrographs showing deformities/abnormalities in fungal mycelia were taken.

3. Antagonistic Activity of Cell-free Culture Filtrates of Bacterial Isolates

Inhibitory effect of cell-free culture filtrates of the bacterial isolates were evaluated based on colony growth inhibition, mycelia biomass yield and sclerotia germination and hyphal development from the germination sclerotia of M. phaseolina.

3.1. Preparation of Cell-free Culture Filtrates

YEM broth, Bacillus broth and nutrient broth were prepared and separately inoculated with respective exponentially grown culture (10⁻⁸ cells /ml) of bacterial isolates then incubated in rotating shaker at 150 rpm at 28±1°C up to 5 days. Broth cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatant was collected separately in 50 ml beakers and few drops of chloroform were added to each beaker to kill the bacterial cells if any. Culture filtrate of each isolate was left open under the hood of laminar flow for 1 hour to evaporate the chloroform vapours. The filtrate was used directly for colony growth inhibition of M. phaseolina, measuring
inhibition in mycelia dry weights, and in preparation of agar plates for sclerotial germination.

3.2. Effect of Cell-free Culture Filtrates on Colony Growth of *M. phaseolina*

Two wells each of 5 mm diam. were made onto the modified media, 2 cm away from the centre. 5 days old mycelial disc (5 mm diameter) was placed in the centre of each sterile plate. 0.5 ml of cell free culture of all bacterial isolates was aliquotted into each well. Plates were incubated at 28±1°C for 5 days, and then observed for development of zone of inhibition. Colony growth inhibition (%) of the pathogen was recorded as described earlier.

3.3. Effect of Cell-free Culture Filtrates on Mycelia Dry Weight Yield

Czapek Dox broth (CDB) was prepared and sterilized at 121±1°C for 20 min. For estimation of mycelial yield cell-free culture filtrate of all strains of *Bradyrhizobium* and *Bacillus* along with standard culture of *Bradyrhizobium* sp. NAIMCC-B-00262 and *Bacillus subtilis* MTCC 441 was added separately in 50 ml pre-sterilized CDB in three replicates so as to get 15, 30 and 45 % concentration (v/v). Sterilized distilled water was added in the same concentration in control sets in triplicate. The flasks were inoculated with 5 agar blocks (each of 5 mm diam.) cut from actively growing margin of *M. phaseolina*. Each culture was filtered through pre-weighed Whatman filter paper No. 1 after incubation for seven days at 30±1°C. The mycelial mat of each treatment was dried at 85°C for 24 h to determine the mycelial dry weight yield. Loss (%) in mycelial dry weight was calculated by using the formula : 100 × (C-T)/C, where C = mycelial dry weight in control, T= mycelial dry weight in treatment.

4. Effect of Cell-free Culture Filtrates (CFCF) of Bacterial Isolates on Sclerotia Germination of *M. Phaseolina*
M. phaseolina sclerotia are the surviving propagules and source of infection; therefore, break of its survival is necessary.

4.1. Production (harvesting) of Sclerotia

Cellophane disc method of Ayanru and Green (1974) was used to harvest the sclerotia of M. phaseolina. Cellophane paper discs (90 cm diam.) were cut and boiled in 100 ml distilled water in a beaker for 30 min to remove plasticizers. Then cellophane discs were aseptically removed and spread onto the surface of sterilized and solidified potato dextrose agar (PDA) medium in Petri dishes. An agar block (5 mm diam.) cut from the margin of actively growing culture of M. phaseolina was put in the centre of these Petri dishes and incubated at 30±1°C for 5 days in dark. Thereafter, each cellophane paper was aseptically removed from each Petri dish and scrubbed with a blade to harvest the mycelia mat containing sclerotia. The mixture of mycelia and sclerotia were dried overnight at 35-40°C onto sterile filter paper, mashed using a sterile pestle and mortar, and passed through a sieve of 50 µm pour size. The sclerotial powder was transferred into a vial and stored at 4°C for further use.

4.2. Study of Sclerotia Germination

Water agar medium (2%) was prepared and sterilized at 121°C for 30 min. Cell-free culture filtrate of each bacterial isolate prepared as above was poured into 20 ml culture tubes each containing 2% water agar medium in three replicates so as to get 15, 30 and 45% concentration (v/v) of filtrate of each isolate. The control plates were devoid of any filtrate.

Sclerotia germination was studied following the standard ‘tube dilution’ method of Baily and Scott (1974) modified by Dwivedi and Dubey (1986). A small amount of sclerotia prepared as above were put in 100 ml beaker, washed with sterile phosphate buffer (0.1 M, pH 6.5), centrifuged at 1000 rpm for 3 min and decanted to remove mycelia fragments and exogenous inhibitors. Then 50 ml sterile distilled water was poured into the beaker to prepare sclerotial suspension (300 sclerotia/ml). One ml
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Characterization of endophytic rhizobacteria from Vigna mungo (L.) Hepper and their role in biocontrol of Macrophomina phaseolina (Tassi) Goid.

4.3. Effect of Culture Filtrate of Bacterial Isolates on Hyphal Development

Effect of cell-free culture filtrate of each *Bradyrhizobium* isolate on development of hypha/mycelia was studied after 48 h of incubation as described by Dubey (1992). In both the case 400 sclerotia per treatment were counted in three replicates. Germination was defined as the production of hypha(e) by a sclerotium to about half of its diameter. The germinating sclerotia were examined under light compound microscope (Olympus BX 51 TRF), and images of mycelial/sclerotial deformities, if any, were captured by using an Image Anlyser (Biovis)

G. BIOINOCULANTS AND THEIR EFFECT ON PLANT GROWTH AND YIELD

Inocula of bacterial antagonists and *M. phaseolina* were prepared for pot trials as described below:

1. Interaction(s) among the Bacterial Isolates (*in vitro*) (Pierson and Weller, 1994)

Bacterial lawn was prepared by seeding of nutrient agar plates separately with exponentially grown culture of VR1, VR2, VR11 and VR13. After 20 minutes, plate having lawn of VR1 strain was spotted by the freshly grown broth cultures of VR2, VR11 and VR13, separately. Similarly, plate containing lawn of VR2 strain was spotted by freshly grown broth cultures of VR1, VR11 and VR13. Similar step was also repeated with VR11 and VR13 strain. All the inoculated plates were incubated at 28±1°C for 72 h along with a control plate (devoid any inoculum) and examined for
zone of inhibition/non-inhibition around the spotted culture to record antagonistic/synergistic interaction between two bacterial strains.

1.1. Effect of Cell-free Culture Filtrate (CFCF) of \textit{Bradyrhizobium} sp. strain VR2 on growth of \textit{Bacillus} sp. VR11 (Samavat et al., 2011)

Based on the results obtained from the above experiment, it seemed to find out the effect of cell-free culture filtrates of \textit{Bradyrhizobium} sp. strain VR2 on growth of \textit{Bacillus} sp. strain VR11 \textit{in vitro}.

\textit{Bradyrhizobium} sp. strain VR2 was grown at 28±1°C in YEM broth for 72 hours at 150 rpm on a rotary shaker following the method of Samavat et al. (2011). Similarly, \textit{Bacillus} sp. strain VR11 was also grown in 250 ml Erlenmeyer flasks containing 50 ml Bacillus broth medium on a rotary shaker for 72 h. Culture of \textit{Bradyrhizobium} sp. strain VR2 was centrifuged at 7,000 rpm to get cell-free culture filtrates. Thereafter, 2 ml cell suspension of \textit{Bacillus} sp. strain VR11 and 4 ml cell-free culture filtrate of \textit{Bradyrhizobium} sp. strain VR2 were poured in a sterilized culture tubes containing 4 ml of Bacillus broth medium, in three replicates. The control set consisted on 4 ml Bacillus broth medium, 4 ml YEM broth and 2 ml of \textit{Bacillus} sp. strain VR11 cell suspension. All the tubes were incubated at 25°C on a rotary shaker. The growth of \textit{Bacillus} sp. strain VR11 was measured after 24, 48, 72 and 96 h intervals in terms of turbidity (optical density) at 600 nm by using a spectrophotometer (Shimadzu, Japan).

2. Antibiotic Sensitivity Test and Development of Intrinsic Antibiotic-resistant Marker Strains

2.1. Antibiotic Sensitivity Test

YEMA, BAM and King’B media were prepared and poured into the sterile Petri plates. Bacterial lawn was prepared by pipetting 0.5 ml of the late exponential phase culture of each isolate onto the agar plates, and then inoculum of each bacterium was spread evenly onto the surface of agar media with the help of sterile spreader. After 15
minutes readymade antibiotic Octadiscs (Hi media, Mumbai) was placed on the surface of bacterial lawn of each bacterial isolates. All the Petri plates were incubated at 28±1°C for 72 h and observed for sensitivity or resistance against individual antibiotic on the basis of zone of inhibition around each disc.

Himedia Octadiscs (OD006R and OD042R) consisted of the following antibiotics:

Chloramphenicol (30 mcg), nalidixic acid (30 mcg), furazolidine (50 mcg), norfloxacin (10 mcg), oxytetracycline (30 mcg), cephalexin (30 mcg), cephotaxime (30 mcg), co-trimoxazole (25 mcg), ciprofloxacin (10 mcg), nitrofurantoin (300 mcg), norfloxacin (10 mcg), netilin (30 mcg) and ofloxacin (5 mcg).

2.2. Development of Intrinsic Antibiotic-resistant Marker Strains

For the development of a resistant marker, individual antibiotic-resistant strain of B. japonicum strain VR1, Bradyrhizobium sp. strain VR2 and Bacillus sp. strain VR11 was selected based on antibiotic sensitivity test. Then resistant marker strains were developed by subjecting the individual bacterial culture successively from low concentration to high concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg ml⁻¹) of antibiotics, for example furazolidone for selection of B. japonicum strain VR1fur⁺, nalidixic acid for Bradyrhizobium sp. strain VR2nal⁺, and norfloxacin (for selection of Bacillus sp. strain VR11nor⁺. The seedlings of urad, raised from seeds bacterized with B. japonicum strain VR1fur⁺, Bradyrhizobium sp. strain VR2nal⁺, and Bacillus sp. strain VR11nor⁺, were sampled for root colonization after 30, 45, and 60 days after sowing, and the bacterial population on the root was measured.

3. Preparation of Bacterial Consortia (co-inoculation of Bacterial Isolates)

Broth media were prepared according to bacterial isolates and separately inoculated by bacterial isolates (VR1, VR2 and VR11). All the flasks were incubated at 28±1°C for
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Characterization of endophytic rhizobacteria from *Vigna mungo* (L.) Hepper and their role in biocontrol of *Macrophomina phaseolina* (Tassi) Goid.

24 h at 150 rpm in a rotary shaker to attain 10^8 cfu ml^{-1} of cell density. Co-inoculation of bacterial isolates was done in nutrient broth in the following combinations:

**Consortium A** - *B. japonicum* strain VR1^{fur+} + *Bradyrhizobium* sp. strain VR2^{nal+} + *Bacillus* sp. strain VR11^{nor+}

**Consortium B** - *B. japonicum* strain VR1^{fur+} + *Bradyrhizobium* sp. strain VR2^{nal+}

**Consortium C** - *B. japonicum* strain VR1^{fur+} + *Bacillus* sp. strain VR11^{nor+}

**Consortium D** - *Bradyrhizobium* sp. strain VR2 + *Bacillus* sp. strain VR11^{nor+}

All combinations were incubated in shaking incubator at 28±1°C for 48-72 h at 150 rpm. After 6 h of intervals, samples of the culture were aseptically taken to determine the cell density. 0.1 ml of aliquots of above culture was spread on nutrient agar plates containing separately 100 µg ml^{-1} of furazolidone (for selection of *B. japonicum* strain VR1^{fur+}), nalidixic acid (for *Bradyrhizobium* sp. strain VR2^{nal+}), and norfloxacin (for selection of *Bacillus* sp. strain VR11^{nor+}). The property of antibiotic resistance of bacterial isolates was selective so this made easy to enumerate the bacterial isolates when it was in combination (Consortium A, B, C and D). All the plates were incubated at 28±1°C for 48-72 h.

4. Seed Bacterization

The method of Weller and Cook (1983) was followed for seed bacterization. YEMA and NAM broths were prepared and separately inoculated with bacterial isolates (VR1, VR2 and VR11) and their consortia (A, B, C and D) were incubated on a rotary shaker at 28±1°C for 48 h for inoculum preparation. The cultures were centrifuged at 7,400 rpm for 15-20 min at 4°C to get cell pellets. Supernatant was discarded and cell pellet were washed and re-suspended in sterile distilled water.

The cell suspension of each isolate obtained so was used for direct seed bacterization. There are two methods for seed coating, direct coating method or slurry method. Slurry method was followed for seed inoculation. CMC
(carboxymethylcellulose) (1%) solution and cell suspension of bacterial isolates were
mixed in the ratio of 1 : 0.5 to prepare slurry. Seeds (50 g) of Vigna mungo were surface
sterilized and mixed with 50 ml CMC slurry to coat the containing bacterial strains on
seed surface (Gupta et al., 2002). Seeds coated only with CMC slurry served as control.
Seeds coated with slurry were properly missed and dried in shade to adher the cells on
seed surface; then seeds were ready for sowing in pots.

5. Preparation of Inoculum of M. phaseolina

Mycelia of M. phaseolina were inoculated in pre-sterilized moist oat (Avena sativa)
grains in flasks of one litre capacity, and incubated at 30°C for 5 days to produce mass
inoculum for soil infestation. After 5 days, the grains were removed from the flask and
dried aseptically. The grains containing mycelia plus sclerotia were powdered that acted
as inoculum. The grain-based inoculum of M. phaseolina was mixed in formalin-
sterilized garden soil to make the inoculum level to about $10^5$ cfu g$^{-1}$ dry soil.

6. Effect of Microbial Isolates / Consortia on Growth and Yield of V. mungo in Pots

Experiment was conducted to study the effect of best three selected bacterial isolates
viz., VR1, VR2 and VR11 on seed germination, seedling growth and biomass yield of
V. mungo. Garden soil was procured and sterilized with formalin keeping in an air tight
container and left for 5 days. Thereafter, soil was taken out and put in on sterile
polythene sheet to evaporate the residues of formalin. Plastic pots (24 × 12 × 12 cm)
were filled with 1 kg garden soil (75.3% sand, 13.2% silt, 11.5% clay, 0.241% total
organic matter, 0.097% total organic C, pH 6.4, and 40% water holding capacity)
infested with with inocula of M. phaseolina as described above. Bacterised seeds (10
seeds per pot) of V. mungo were sown in soil containing inocula of M. Phaseolina (and
without M. phaseolina) along with control (seeds without inoculum). The experiment
was designed as below:
Materials and Methods

Characterization of endophytic rhizobacteria from *Vigna mungo* (L.) Hepper and their role in biocontrol of *Macrophomina phaseolina* (Tassi) Goid.

Pots were arranged in triplicates according to the above given designs. Pots were observed for seedling germination (on 15\textsuperscript{th} day after sowing). The plants were watered with tap water whenever required to maintain soil moisture at 60 % of its water holding capacity (WHC). The disease incidence was noted as percentage of the plants showing charcoal rot after 30 and 60 days of sowing. Development of charcoal rot symptoms was observed by using a hand lens (10X). Plant length, shoot length, root length, dry weight of root and shoot were observed and recorded after 30 days of sowing, then data were analysed statistically.

The following parameters were recorded on 30 and 60 DAS:

- Seed germination percentage (%)
- Shoot length (cm)
- Root length (cm)
- Dry shoot weight (g)
- Dry root weight (g)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Soil (without <em>M. phaseolina</em>) + non-bacterized seeds</td>
</tr>
<tr>
<td>T1</td>
<td>Non-bacterized seeds + <em>M. phaseolina</em></td>
</tr>
<tr>
<td>T2</td>
<td><em>M. phaseolina</em> + <em>Bradyrhizobium</em> sp. (Vigna) VR2\textsuperscript{nal+}</td>
</tr>
<tr>
<td>T3</td>
<td><em>M. phaseolina</em> + <em>B. japonicum</em> VR1</td>
</tr>
<tr>
<td>T4</td>
<td><em>M. phaseolina</em> + <em>Bacillus</em> VR1\textsuperscript{nor+}</td>
</tr>
<tr>
<td>T5</td>
<td><em>M. phaseolina</em> + <strong>Consortium A</strong> (B. japonicum VR1, <em>Bradyrhizobium</em> sp. VR2\textsuperscript{nal+} + <em>Bacillus</em> VR1\textsuperscript{nor+})</td>
</tr>
<tr>
<td>T6</td>
<td><em>M. Phaseolina</em> + <strong>Consortium B</strong> (B. japonicum VR1+ <em>Bradyrhizobium</em> sp. VR2\textsuperscript{nal+})</td>
</tr>
<tr>
<td>T7</td>
<td><em>M. Phaseolina</em> + <strong>Consortium C</strong> (B. japonicum VR1+ <em>Bacillus</em> VR1\textsuperscript{nor+})</td>
</tr>
<tr>
<td>T8</td>
<td><em>M. Phaseolina</em> + <strong>Consortium D</strong> (Bradyrhizobium sp. VR2\textsuperscript{nal+} + <em>Bacillus</em> VR1\textsuperscript{nor+})</td>
</tr>
</tbody>
</table>
Materials and Methods

- Nodulation
- Vigour index
- Percent disease reduction (% DIR)

Seed germination (%) was recorded on 15 DAS, and percent seed germination was calculated by using the following formula:

\[
\text{Seed germination} (\%) = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds sown}} \times 100
\]

Shoot and root length of ten plants (from each pot) were measured by using a scale (in cm) from tip of plant to the end of stem and from the collar region to the end of root on 30 DAS and 60 DAS. Average of shoot and root length of ten plants was recorded.

Dry weight (g) of shoot and root of ten plants was measured by weighing them on an electronic balance after drying in hot air oven at 70°C for 24 h. Average of shoot- and root- weight was noted.

The roots of uprooted plants were washed. Number of nodules (per plant) was counted. Average number of nodules per ten plants was recorded at 30 and 60 DAS.

Vigour index was calculated by multiplying seed germination and total plant length as per Abdul-Baki and Anderson (1973).

\[
\text{Vigour index} = \text{Germination} (\%) \times \text{plant length (shoot length + root length)}
\]

Per cent reduction in disease (% DIR) was calculated at 30 and 60 days of growth by using the following formula:

\[
\% \text{ DIR} = \frac{\text{No. of plants infected in control} - \text{No. of plants infected in treatment}}{\text{No. of plants infected in control}} \times 100
\]
7. Root Colonization Study

Antibiotic resistant marker strains viz., VR1\textsuperscript{fur+}, VR2\textsuperscript{nal+}, and VR11\textsuperscript{nor+} were recovered on the growth medium containing 100 µg ml\textsuperscript{-1} of furazolidone, nalidixic acid, norfloxacin, respectively.

Plants were carefully removed with a shovel, and soil particles adhering to the roots were gently taken out. Care was taken not to remove soil particles tightly adhered to the roots. The roots were then cut into 1cm-long pieces; 1 g root segments were dipped into 5 ml sterilized distilled water and vortexed 4-5 times to release the rhizosphere bacteria into the water. Dilution of above suspension was made and poured into the pre-sterilized plates containing YEMA medium and Bacillus agar medium supplemented with antibiotic (100 µg ml\textsuperscript{-1} of furazolidone, nalidixic acid, norfloxacin separately) to measure the bacterial population. The inoculated plates were incubated at 28±1°C for 24 h; then log cfu g\textsuperscript{-1} root segment was counted.

H. STATISTICAL ANALYSIS

The data were analysed statistically by using ANOVA and LSD, standard deviation and standard error of the mean of replicates.

Dendograms were obtained by computing Jaccard’s similarity coefficients and UPGMA (Un-weighted Pair Group Method with Arithmetic means) cluster analysis using MEGA (molecular evolutionary genetics analysis) Software version 4.0 (Tamura \textit{et al.}, 2007), and computer package NTSYS-pc (Numerical Taxonomy and multivariate analysis SYStem) Version 2.02e (Rohlf, 1997), respectively. Dendograms of biochemical characteristics were obtained by using MVSP (Multi Variate Statistical Package) Version 3.21 Software.