Summary

*Vigna mungo* (L.) Hepper (uradbean) is one of the most highly prized pulses of India and widely used as staple crop. It is a summer pulse crop with short duration (90 to 120 days), high nutritive value and digestibility (El Karamany, 2006). According to the reports pulse consumption is more than its production. For the last fifty years, pulse production could not keep pace with the rapidly increasing population and per capita availability has progressively declined from 60 g in 1950-51 to 32 g at present. As a result of which, contribution of pulses in the national food basket reduced from 17% to 7% (Bakhru, 1997).

Many fungi and bacteria are associated with plants which may be harmful or beneficial. There are different kinds of soil-borne fungal phytopathogens which infect *Vigna mungo*, among them *Macrophomina phaseolina* (Tassi) Goid. has been found the most destructive pathogen that causes a number of diseases among which charcoal rot disease is very common. Moreover, it causes seedling blight, stem rot and pod rot, and attacks more than 500 plant hosts (Sinclair, 1982). Use of chemical fertilizers or fungicides is neither profitable nor recommendable because they create imbalances in soil, microbial community, and environment via food chain to the human. The use of plant growth promoting rhizobacteria (PGPR) having antagonistic property are very good alternative to the fungicides and they biologically control symbiotically or non-symbiotically to the fungal pathogen(s) associated with plant and are safe to the soil, environment and humankind. Seed inoculation with endophytic PGPR before sowing, allows decrease in diseases and susceptibility to the environmental stress.

In the light of above background the objectives of the proposed study are:

1. To isolate the endophytic bacteria from root nodules of *V. mungo*.
2. To identify and characterization them morphologically, biochemically, and by using modern molecular techniques.
3. To determine both direct and indirect plant growth promoting traits present in selected bacterial strains.

4. To carry out sequence comparison of root nodule bacteria by using modern molecular tools.

5. To assess the antagonistic potential of root nodule bacteria against the charcoal-rot pathogen, *Macrophomina phaseolina*.

6. To evaluate the effect of consortia of endophytic bacteria on growth and yield of *V. mungo* in pot trials.

In the present investigation the charcoal rot fungus was isolated by using blotter and water agar methods, identified by comparing culture characteristics with the standard isolates. Pathogenicity test carried out in pot trials confirmed as *M. phaseolina*. Similarly, 20 endophytic bacterial isolates were screened from root nodules of *V. mungo*; among those 16 isolates were selected and characterized on the basis of morphological, physiological and biochemical characteristics.

Bacterial isolates VR1 - VR10 growing on NAM, YEMA and CrYEMA were Gram-negative rods, non-endospore formers, motile, and positive for PHB accumulation, catalase activity, indole production and esculin hydrolysis. Among VR1-VR10 none of the isolates hydrolysed urea (except VR10). Some of the isolates showed oxidase production, indole production and esculin hydrolysis. Only VR5 and VR10 tolerated 8% KNO₃ solution, but none of them resulted in positive response for MR-VP test. Isolates VR4, VR6 and VR10 did not utilize citrate.

The isolates grown on Bacillus agar medium were Gram-positive, rod shaped, motile, and produced endospores. All the isolates showed negative result for methyl red and positive for H₂S production. All the isolates VR11-VR14 were capsulated, accumulated PHB, and were positive for urease- and oxidase production, and nitrate reduction. Isolates VR11 and VR 13 did not utilize starch. Except VR14 none of the
isolates were positive for gelatine-hydrolysis. All the isolates were negative for 8% KNO$_3$ tolerance, methyl red test, Voges Proskauer (except VR15) and citrate utilization.

Bacterial isolates screened on King’s B medium were Gram−negative rods, non-capsulated, non-endospore forming and motile bacteria. They were positive for catalase production, PHB accumulation, esculin hydrolysis, oxidase (except VR20), indole production (VR15) and citrate utilization (except VR15). None of the isolates were able to produce H$_2$S. All isolates produced fluorescent pigment, except VR16 which was positive for nitrate reduction, urease production and starch hydrolysis. All the isolates were negative for MR-VP test (except VR 15 and VR20 which were VP positive), and gelatin hydrolysis.

NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) Version 2.02e software was used to obtain a dendrogram from 19 bacterial isolates VR1−VR20 and standard cultures of Bradyrhizobium sp. NAIMCC-B-00262, Bacillus MTCC-441 and Pseudomonas sp. MTCC-129 with UPGMA (unweighted pair-group method with arithmetic mean) clustering method based on Jaccards coefficient. This software analyses the cluster and establishes phylogenetic relatedness among all the bacterial isolates VR1-VR20 and the standard cultures on the basis of physico-chemical characteristics performing UPGMA analysis.

Jaccard’s coefficient dendrogram analysis classified the isolates into the three major groups, YEMA/CrYEMA isolates in one group, KB medium isolates in second group and Bacillus agar isolates in third group. The dendrogram showed 92 % similarity between VR1 and VR2, and 87.9% similarity between the standard strain Bradyrhizobium sp. NAIMCC-B-00262 and the node of VR1-VR2. In the second group the isolates VR15 and VR20 showed 85% similarity, but Pseudomonas sp. MTCC-129 showed 79.1 % similarity with node of VR15-VR20. In the third group the isolates VR11 and VR13 were 85% similar, and Bacillus MTCC-441 showed 76.4% similarity with node 2 of VR11 and VR13.
All the isolates (VR1 to VR20) showed copious growth at 28°C temperature, 7-8 pH and tolerated 1-3% salt concentration. On the basis of similarity established by UPGMA analysis the isolates VR1-VR10 may be designated as Bradyrhizobium sp. VR1–VR10, the isolates VR15-VR20 as Pseudomonas sp. VR15–VR20, and the isolates VR11-VR14 as Bacillus sp. VR11–VR14.

Carbon utilization test of all the isolates was done using a CarboKit (Himedia, Mumbai) to measure utilization of different 35 carbon sources on the basis of colour change of the given carbon source due to pH change. All the isolates utilized xylose (except VR6, VR9 and VR10), rhamnose, mannose (except isolates VR15-VR16), maltose (except VR3, VR6 and VR10), sucrose (except VR1, VR2 and VR10) and glycerol (except VR5 and VR20). None of the isolates was able to utilize L- and D-arabinose, sorbose, melezitose, inulin (except isolates VR11-VR14), sodium gluconate, salicin, glucosamine, α-methyl-D-glucoside, α-methyl-D-mannoside and dulcitol.

Based on UPGMA, cluster analyses all the isolates were done where they formed eighteen clusters showing their phylogenetic similarity. Isolate VR2 and standard strain Bradyrhizobium sp. NAIMCC-B-00262 were identical that showed 83.3% similarity with B. japonicum VR1. Strains VR3 and VR4 were 74.6% similar. Isolate VR11 was 95% similar with that of VR13, and these two were 92.4% similar with the standard strain Bacillus subtilis MTCC-441. The isolate VR16 was 88.2% similar with standard strain Pseudomonas sp. MTCC-129, and this node showed 83% similarity with VR19.

Based on similarities in carbon utilization the bacterial metabolic fingerprints established the phylogenetic relationships by using BioLog GN2. Isolates Bradyrhizobium japonicum strain VR1 and Bradyrhizobium sp. (Vigna) strain VR2 showed 100% similarity in utilizing the carbon sources, hence both of the strains are similar. Similarly VR4 and VR5 showed 82% similarity in utilization of carbon sources. Besides, isolate VR6 and node of VR4 and VR6 shows 77% similarity in utilizing the various sources of carbon. Lastly, VR3 and node of VR6 and VR4–VR5 were 68.5% similar for utilizing carbon sources.
Phylogenetic analysis of isolates VR1 and VR2 was carried out based on 16S rRNA gene sequence. The 16S rRNA gene sequence of VR1 and VR2 comprised of 1374 nucleotides and 1257 nucleotides, respectively (NCBI Gene Bank Accession Numbers are: VR1 = JX001401, and VR2 = JX001402). The isolate VR1 showed 100% sequence similarity with Bradyrhizobium japonicum EU333382 and Bradyrhizobium sp. NR042177, and VR2 showed 100% sequence similarity with Bradyrhizobium sp. AB681396 and Bradyrhizobium elkanii AB672634.

Therefore, the isolate VR1 can be designated as Bradyrhizobium japonicum strain VR1, and the isolate VR2 can be designated as Bradyrhizobium sp. (Vigna) strain VR2.

All the isolates were tested for nodulation test on nitrogen free nutrient agar medium among those only Bradyrhizobium japonicum VR1 and Bradyrhizobium sp. (Vigna) strain VR2 showed positive result by forming small pinkish nodules developed on young roots after 15 days of incubation.

All the isolates of Bradyrhizobium, Bacillus and Pseudomonas were used to studied their both direct and indirect plant growth promoting (PGP) activities. All the isolates were found to be positive for IAA production and phosphate solubilization (except VR10). None of the isolate produced HCN. Chitinase activity was shown by only two isolates of Bradyrhizobium VR1 and VR2, all the isolates of Bacillus and only Pseudomonas VR15. Positive result for siderophore production was shown by eight isolates; among those two were Bradyrhizobium strains VR1 and VR2, Bacillus strains VR11-VR14 and Pseudomonas strains VR15 and VR19. Quantitative assay of siderophore production was done; the maximum quantity of siderophore (hydroxamate type) was produced by VR2 (37 µg/ml) followed by isolate VR11 (35 µg/ml), VR13 (34 µg/ml) and VR1 (33 µg/ml). ACC deaminase production was observed in only four isolates viz., Bradyrhizobium isolates VR1 and VR2, and Bacillus isolates VR13 and VR14.

Growth of M. phaseolina was inhibited by Bradyrhizobium isolates VR1, VR2, VR3, VR4 and VR5, Bacillus isolates VR11-VR14, and Pseudomonas isolate VR15.
when compared to control. In dual cultures, VR1, VR2, VR11 and VR13 inhibited the radial growth of *M. phaseolina* by 50.5%, 71.5%, 78.6% and 60.2%, respectively. Cell-free culture filtrates of these isolates have also inhibited the radial colony growth of *M. phaseolina*. But the fungal growth inhibition in dual culture was more pronounced than that of cell-free culture filtrate method.

Light microscopy and scanning electron microscopic studies displayed the destructive post-interaction events in the hyphae, mycelia and sclerotia of *M. phaseolina* caused by *Bradyrhizobium* sp. VR2 and *Bacillus* sp. VR11. Several deformities were observed, such as hyphal fragmentation, cytoplasm vacuolation, hyphal shrinkage, mycelial lysis, fragmentation and lysis with totally collapsed hyphae. Many sclerotia in zone of interaction lost cell pigments due to secondary metabolite production by the antagonistic isolates VR2 and VR11 resulting in structural integrity and loss of vigour. The brown to black coloured sclerotia became abnormal after losing its pigment.

The cell-free culture filtrates (CFCF) of VR1, VR2, VR11 and VR13 inhibited growth, mycelia yield, sclerotia germination and hyphal development of *M. phaseolina*. The increase in concentration of CFCF resulted in a decline in mycelia dry weight. *Bradyrhizobium* isolates VR1, VR2 and *Bacillus* isolates VR11 and VR13 showed inhibitory effect on mycelia production of *M. phaseolina* at all the concentration of (15%, 30% and 45%). Complete inhibition of mycelia yield of *M. phaseolina* was recorded at 45% concentration of CFCF of *B. japonicum* VR1, *Bradyrhizobium* sp. VR2, *Bacillus* sp. VR11 and VR13 when compared to control.

Sclerotia germination of *M. phaseolina* gradually increased with incubation time (48h, 72h and 96h) at all the concentration (15%, 30% and 45%). Inhibition of sclerotia germination was more pronounced at 45% concentration CFCF of *Bradyrhizobium* isolates VR1, VR2, *Bacillus* sp. VR11 and VR13 as compared to control. CFCF of the standard strains, *Bradyrhizobium*-B-00262 and *Bacillus subtilis* MTCC-441 also inhibited sclerotal germination.
Germination of sclerotia of *M. phaseolina* led to the development of hyphae on water agar medium. In turn these germinating sclerotia have also produced small-sized secondary sclerotia singly or in chain. The number of sclerotia producing >7 hyphae was decreased with increasing the concentration of CFCF of isolates. CFCF of isolates VR1, VR2, VR11 and VR13 completely inhibited the sclerotia germination and hyphal development at 45% concentration due to loss in vigour of sclerotia. Both the standard strains *Bradyrhizobium*-B-00262 and *Bacillus subtilis* MTCC-441 also showed complete reduction of hyphal development at 45% of concentration.

Primarily, all the strains were allowed to interact with each other *in vitro* to evaluate their synergistic or antagonistic effect before consortia formulation. *B. japonicum* VR1, *Bradyrhizobium* sp. VR2, *Bacillus* sp. VR11 and VR13 showed synergistic interaction among each other. Besides, the *Pseudomonas* strains VR19 and VR20 also did not inhibit each other, thus they showed synergism. Moreover, the presence CFCF of *Bradyrhizobium* sp. strain VR2 positively enhanced the growth of *Bacillus* sp. strain VR11 with incubation time when compared to control.

*B. japonicum* VR1, *Bradyrhizobium* sp. VR2 and *Bacillus* sp. VR11 effectively showed PGP properties; hence they were selected for antibiotic sensitivity test for several antibiotics and found to be resistant against number of antibiotics. *Bradyrhizobium japonicum* VR1, *Bradyrhizobium* sp. (*Vigna*) VR2 and *Bacillus* sp. VR11 was further used to develop the highest level of tolerance to furazolidone, nalidixic acid and norfloxacin, *i.e.* 100 µg ml⁻¹ separately. Thus acronyms were: *Bradyrhizobium japonicum* VR1fur⁺, *Bradyrhizobium* sp. (*Vigna*) VR2nal⁺ and *Bacillus* sp. VR11nor⁺. These marker strains were further used for seed bacterization and root colonization studies.

Based on the bacterial interaction study, three isolates VR1fur⁺, VR2nal⁺ and VR11nor⁺ were selected for seed bacterization alone or in form of consortium. The bacterized seeds were sown in plastic pots containing sterile soil with or without *M. phaseolina*. Maximum seed germination (88%), vegetative parameters [root length (8.9
cm) and shoot length (26.7 cm) their respective dry weights (0.661 g and 3.523 g), nodule numbers (12/plant) and vigour index were recorded in T5 (M. phaseolina+VR1fur+VR2nal+VR11nor+) followed by T2 (VR2nal+) (86%), T4 (VR11nor+) (82%), T3 (VR1fur+) (80%), T8 (M. phaseolina+VR2nal+VR11nor+) (78%), T6 (M. phaseolina+VR1fur+VR2nal+) (77%), T7 (M. phaseolina+VR1fur+VR11nor+) (76%) and T1 (M. phaseolina) (62%). Presence of Bradyrhizobium sp. VR2 nal+ showed the maximum seed germination and vegetative parameters in all the treatments 30 DAS followed by Bacillus sp. VR11nor+ and B. japonicum VR1fur+.

After 60 days, all vegetative plant parameters (root and shoot length and their respective dry weights, nodule numbers per plant) were gradually increased in all the treatments when compared to control. It was found that treatment T5 (M. phaseolina+VR1fur+VR2nal+VR11nor+) gave the best result of all growth parameters followed by T2 (VR2 nal+). Bradyrhizobium sp. The isolate VR2 resulted in the highest enhancement of growth parameters either alone or in combination with the other strain (VR1 and VR11).

The maximum disease reduction was recorded in T5 (66.7% and 68.7%) followed by T8 (46.7% and 51.2%), T6 and T7, 30 and 60 DAS when compared with control. Bradyrhizobium sp. VR2nal+ was found to be a good root colonizer when compared to B. japonicum VR1fur+ and Bacillus sp. VR11nor+ either alone or in form of consortium. Based on the population recovery as log cfu g⁻¹ root segments it was found that consortium in T5 (M. phaseolina+VR1+VR2+VR11) was the best followed by that in T8 (M. phaseolina VR2+VR11), T6 (M. phaseolina+VR1+VR2) and T7 (M. phaseolina+VR1+VR11) after 30 and 60 days of sowing.

On the basis of the results obtained in the present investigation it may be concluded that Bradyrhizobium sp. strain VR1, Bradyrhizobium sp. (Vigna) strain VR2, and Bacillus sp. strain VR11 are not only the aggressive root colonizers of V. mungo, but also a potential plant growth promoting rhizobacteria and biocontrol agent of M. phaseolina.