

Isolation and Screening of Indigenous Rhizobia from Black Gram Cultivated in Fallow Rice Soils for Plant Growth Promoting Traits

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ABSTRACT

Bio fertilisers are relatively safer, environmentally friendly and a cost-effective approach to chemical fertiliser usage. The selection of bacterial strains with multiple beneficial characteristics is important to maximise their effectiveness on the host plant. In the present study, four native and indigenous rhizobial strains (VM-2, VM-8, VM-9 and VM-15) were isolated from root nodules of black gram (*Vignamungo*) cultivated in fallow rice soils of Andhra Pradesh, India. All the four strains were screened *in vitro* for their plant growth-promoting (PGP) characteristics viz. production of indole acetic acid (IAA), exopolysaccharide (EPS), hydrogen cyanide (HCN) and phosphate solubilisation. The results indicated that the rhizobial strains varied in their plant growth promoting activities. All the four strains produced IAA, EPS and also solubilised the insoluble phosphate. The amount of IAA produced varied from strain to strain and relatively high amounts were recorded in VM-8 (43.4 µg/ml) followed by VM-15 with 43.1 µg/ml. Maximum EPS production was recorded in VM-9 (527 mg/ml) followed by VM-8 (483 mg/ml). The phosphate solubilisation efficiency of *Rhizobium* strains on solid media ranged between 16% and 17%. In liquid medium, strain VM-2 recorded maximum solubilisation (799µg/ml) followed by VM-8 (372µg/ml). All the strains except strain VM-8 were HCN producers. Among these three strains, VM-2 and VM-15 showed strong HCN production. These isolates were identified as *Rhizobium* sp. strain VM-2 (KJ 704783), *Brady rhizobium* sp. strain VM-8 (KJ 704784), *Brady rhizobium* sp. strain VM-9 (KJ 704785) and *Achromobacter* sp. strain VM-15 (KJ501696) after 16S rRNA sequencing. The pot culture experiment showed that VM-8, VM-9 and VM-15 inoculated plants had good results both

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in inoculated sterilised and inoculated unsterilised soils than the plants grown in sterilised uninoculated soils and control soils. The VM-2 strain showed moderate results under plant inoculation test. This study suggests that these four native rhizobial strains of PGP can be used as bio fertilisers as well as a bio control agent for enhancing the yield of blackgram in rice fallows.

Keyword: Rice fallows, black gram, plant growth promoting characteristics.

INTRODUCTION

Blackgram (*Vignamungo*) is a short duration crop belonging to the Leguminaceae family. It is also called urad bean. Our (1993) reported that millions of people in many countries are consuming it as a part of their diet and is a cheap source of protein (17-34% seed protein). Reddy *et al.* (2011) reported that this legume increases soil fertility by fixing 38 kg N/ha/year in soil from atmosphere. It is mainly cultivated in the rice fallows after rice cultivation to conserve soil nutrients and utilise the left-over soil moisture present in the rice fallows. Cultivation of legumes in rice fallows can prevent the loss of soil nitrate and additionally capture atmospheric nitrogen through biological nitrogen fixation process (George *et al.* 1992).

Most of the rhizospheric microorganisms promote plant growth and development either directly (nitrogen fixation, phosphate solubilisation and plant growth regulators) or indirectly (by controlling the pathogenic microorganisms) and are referred to as plant growth promoting rhizobacteria (PGPR). Besides symbiotic nitrogen fixation, *Rhizobium* can also produce phytohormones like Indole acetic acid (Halda-Alija 2003), siderophores and HCN, thereby decreasing the damage due to plant pathogens and ultimately improving plant growth and yield (Deshwal *et al.* 2003; Weller and Cook 1983; Raajmakers *et al.* 1999; Kranthi Kumar and Raghu Ram 2016; Manasa *et al.* 2017).

Phosphorous is one of the most important macro nutrients that plays an important role in plant metabolism (Sashidhar and Podile 2010). Several microorganisms in the rhizosphere (rhizobacteria) solubilise inorganic phosphate by the production of organic acids (Rodriguez and Fraga 1999). Rhizobia is also a good phosphate solubiliser and tends to increase phosphorous availability to plants by solubilising the insoluble phosphates (Halder *et al.* 1990; Johri *et al.* 2003).

Another important characteristic feature of PGPR is EPS production which helps in nitrogen fixation by protecting the dinitrogenase enzyme from high oxygen concentrations (Tank and Saraf 2003). The EPS produced by the *Rhizobium* species play a prominent role in the *Rhizobium*-legume symbiosis (De and Basu 1996) particularly in root hair infection and nodule formation (Phillip-Hollings worth *et al.* 1989; Kranthi Kumar and Raghu Ram 2016).

Less information is available on these important plant growths promoting traits of native or indigenous *Rhizobium* strains isolated from blackgram particularly cultivated in rice fallows. The present investigation was undertaken

to isolate and screen the indigenous Rhizobium bacteria from root nodules of *Vignamungo* plants for their PGPR activities like IAA, EPS, HCN production and phosphate solubilisation followed by plant inoculation test.

MATERIALS AND METHODS

Isolation of Rhizobium Strains from Blackgram Root Nodules

In the present investigation, the nodulated roots of mature black gram plants cultivated in rice fallows of Krishna and Guntur districts of Andhra Pradesh, India were collected. Rhizobium strains were isolated from freshly collected healthy root nodules on yeast extract mannitol agar (YEMA) medium with 0.1% Congo red. The pure cultures of all isolates were maintained on YEMA slants and preserved at 4°C (Vincent 1970). The identity of the strains was confirmed by tests such as Gram staining, growth on culture media such as YEMA with Congo red (Vincent 1970; 1982), Hofer's alkaline broth and Glucose Peptone Agar (Vincent 1970), Ketolactose test (Bernaertz and Deley 1963) and nodulating ability on homologous hosts (Somasegaran and Hoben 1985).

Screening of Rhizobial Strains for Their Plant Growth Promoting Activities

Indole Acetic Acid (IAA) Production

IAA production was determined by the (Gorden and Weber, 1951) method. For IAA production, all the four strains were grown separately in 100 ml conical flasks containing 30 ml of YEM broth (Skerman 1959) supplemented with L-tryptophan (1.5 mg/ml) at pH 7.0 in triplicate on a rotatory shaker for 54 h at 30±2°C. Bacterial growth was determined by taking optical density (OD) at 540 nm using a Spectrophotometer (Elico-CI 157). The broth cultures were centrifuged at 5000 rpm for 20 min and the cell free supernatant was analysed for IAA extraction according to Sinha and Basu (1981). To the 10 ml of supernatant, 2 ml of Salkowsky's reagent (0.5 M FeCl₃ in 35% perchloric acid) was added and the mixture was left in the dark for 30 min. The development of pink colour indicated IAA production and the optical density was measured at 540 nm using a spectrophotometer. The yield of IAA was calculated by using the standard graph of authentic IAA (Merck). Data on three replications was maintained.

Exopolysaccharide (EPS) Production

The *Rhizobium* strains were inoculated into Erlenmeyer flasks containing 100 ml of YEM broth supplemented with 1% Mannitol. The inoculated flasks were incubated at 30±2°C on a rotator shaker at 300 rpm for 72 h. After incubation, the culture broth was centrifuged at 3000 rpm and the supernatant was mixed with two volumes of chilled acetone. The crude polysaccharide developed was collected by centrifugation at 3000 rpm for 30 min. The EPS was washed with distilled water and acetone alternatively, then transferred on to a filter paper and weighed after overnight drying at 105°C (Damery and Alexander 1969). Data on three replications was maintained.

Phosphate Solubilisation

The phosphate solubilising ability of the strains was tested on Pikovskaya's solid agar medium (Pikovskaya 1948) with Tricalcium phosphate (TCP) as insoluble phosphate source. The solubilisation efficiency (SE) on solid agar medium was expressed in terms of SE (%) (Sri Ram Kumar and Kannapiran 2011; Srivastava *et al.* 2004). The strains which showed a solubilisation zone on solid agar medium were further tested in flasks containing 100ml of Pikovskaya's broth having an initial pH 7. One ml of the inoculum was inoculated into the broth and the flasks were incubated on a rotary shaker (200rpm) at $28\pm 2^\circ\text{C}$ for 72h. The supernatant was separated from the bacterial cells by centrifugation of flasks at 3000rpm. Later the final pH of the supernatant was measured and the liberated P_2O_5 was estimated by adding 2.5 ml of Barton's reagent to 10ml aliquot of the clear culture supernatant and the volume was made up to 50 ml. After 10 min, the resultant yellow colour was read in a calorimeter at 430 nm (Jackson 1973) and the liberated P_2O_5 was estimated by comparing the values with a standard curve prepared with K_2HPO_4 . Data on three replications was maintained.

Hydrogen cyanide (HCN) Production

All the isolates were screened for their ability to produce HCN. Production of HCN was assayed by the method given by Miller and Higgins (1970) with slight modifications. Actively growing bacterial cultures were streaked on YEMA plates supplemented with 4.4 g glycine/L. Filter paper soaked in 0.5% picric acid and 1% Na_2CO_3 was attached to the upper Petri dish lids and the plates sealed with parafilm. Plates without inoculum served as control. HCN production was estimated after seven days of incubation at room temperature, by observing a colour change in the filter paper from yellow to light brown (low), brown (moderate) or reddish brown (strong). Data on three replications was maintained.

PCR Amplification and Partial Sequencing of 16S rRNA Gene

The amplification of PCR and sequencing of 16S rRNA gene of the four isolates, VM-2, VM-8, VM-9 and VM-15, was done by using the commercial services of Macrogen Inc. Korea.

Phylogenetic Analysis of Bacterial Strains

The gene sequences of VM-2, VM-8, VM-9 and VM-15 were submitted to BLAST for comparison with Gen Bank sequences employing the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/GenBank/>). For the phylogenetic analysis, Gene Sequences greater than 600 bp in length were used.

Plant Inoculation Test

Pot culture experiment and experimental design

Symbiotic efficiency and persistence of inoculated Rhizobium in soil are necessary for the success of an inoculation program. Screening for these traits is therefore an important component of inoculation studies. A pot culture experiment was carried

out using the most cultivated rice fallow black gram variety, LBG-752, in the Botanical garden of Acharya Nagarjuna University, Guntur, Andhra Pradesh, India to evaluate the effect of indigenous *Rhizobium* strains (VM-2, VM-8, VM-9 and VM-15) isolated from rice fallows on the growth, nodulation, nitrogen fixation and yield of black gram. All the pots used in this experiment were of uniform size (27×25 cm) and 5 kg of soil was used in all pots. The experiment was conducted in RBD with three replications and three treatments. The treatments were as follows: Treatment-1: Seed inoculation with isolated native strains in sterilised soil; Treatment-2: Seed inoculation with isolated native strains in unsterilized soil; Treatment-3: Growth of seedlings in sterilised soil without inoculation; and Control: Growth of seedlings in unsterilized soil without inoculation.

Materials used

The materials used include rice fallow soils collected from different rice fields, black gram seeds obtained from Regional Agriculture Research Station (RARS), Lam, Rhizobium cultures, autoclave, earthen pots, plastic tag for labeling, broth culture for multiplying the Rhizobium strain and electric orbital shaker.

Soil sterilisation

Rice fallow soils collected from different rice fields were heat sterilised using (electric soil steriliser at 65°C for 90 min) and then autoclaved for 30 min at 130 kpa and 121°C. Then the soil was left to cool and stored in air tight bags.

Sowing and inoculation

Seeds of black gram LBG-752 which were uniform in size, shape and weight were surface sterilised with 1% mercuric chloride (HgCl₂) for 3-4 min and were repeatedly washed with sterilised water. No fertilizer was applied at the time of sowing. For seed inoculation, seeds were coated with a paste of *Rhizobium* inoculum containing approximately 10⁸ cells per seed (Somasegaran and Hoben 1994) and eight such seeds were sown per pot containing 5 kg soil for Treatments 1&2. The non-coated sterilised seeds were sown in pots of Treatment-3 and Control. The experiment was conducted under natural conditions by following all agronomic practices which were uniform and normal for all the treatments.

Data Collection

Data was collected from all the treatments at 35 DAS and at 50% flowering stage on morphological and yield characters such as number of nodules, nodule fresh weight (mg), nodule dry weight (mg), leg haemoglobin content (µg/ml), root length (cm), shoot length (cm), root fresh weight (gm), root dry weight (gm), shoot fresh weight (gm), shoot dry weight (gm), number of leaves per plant, number of branches per plant, number of clusters per plant, number of pods per plant, seeds per pod, pod length (cm), nodule nitrogen (%), root nitrogen (%), shoot nitrogen (%), leaf nitrogen (%), seed nitrogen (%), seed protein and seed yield per plant.

Statistical Analysis

Statistical analysis of the PGPR data was performed by using SPSS software (version 2.0). Correlation coefficient and ANOVA were calculated for the PGPR data wherever necessary. The data on pot inoculation was statistically analysed using AGRISTAT software. Correlation coefficients between traits regarding pot experiment were calculated by MINITAB 16 software.

RESULTS AND DISCUSSION

Isolation of Rhizobial Strains

Four isolates were obtained from the nodules of blackgram plants grown in the rice fallows of Krishna and Guntur districts of Andhra Pradesh, India. The *Rhizobium* colonies on Congo red medium appeared as white, round, transparent, and elevated with entire margin. They were Gram-negative rods and did not grow on Hofer's medium and glucose peptone agar. All the strains were negative for the production of 3-ketolactose from lactose and were finally confirmed as rhizobia by the nodulation test (Satyanandam *et al.* 2014).

Screening of Rhizobial Strains for Various Plant Growth Promoting Activities

In this study all the four strains were screened in vitro for their plant growth promoting properties like Indole Acetic Acid production, EPS production, Phosphate solubilisation and HCN production. The results revealed that all the four strains were IAA, EPS producers and phosphate solubilisers. Except for strain VM-8, all the other strains showed HCN production (Table 1).

TABLE 1
Plant growth promoting activities of different rhizobial isolates

Strains	IAA	EPS	Phosphate solubilisation	HCN
VM-2	+	+	+	+
VM-8	+	+	+	-
VM-9	+	+	+	+
VM-15	+	+	+	+

'+' indicates positive

'-' indicates negative

IAA Production

All the four strains showed IAA production. The amount of IAA produced varied from strain to strain and relatively high amounts were recorded in VM-8 (43.4 µg/ml) followed by VM-15 with 43.1 µg/ml incubated for 54 h when YEM medium was supplemented with 1.5 mg/ml L-tryptophan. A low amount of IAA was produced by VM-9 (35.0 µg/ml) and VM-2 (19.0 µg/ml) respectively (Table 2). In earlier

reports, the *Rhizobium* sp. isolated from root nodules of *Dalbergial anceolaria* produced a high amount of IAA at 2.5 mg/ml L-tryptophan concentration (Ghosh and Basu 2002) while the *Rhizobium* sp. from root nodules of *Roystonearegia* produced a maximum amount of IAA at 3 mg/ml L-tryptophan concentration (Basu and Ghosh 2001). Kranthi Kumar and Raghu Ram (2016) reported that *Ensifer* sp. isolated from *Vigna trilobata* produced a maximum of 42.5 µg/ml of IAA in the presence of L-tryptophan 2mg/ml concentration. Manasa *et al.* (2017) mentioned that out of 15 Rhizobial strains isolated from different legume crops such as groundnut, black gram, green gram, soy bean and redgram, 11 were able to produce IAA. Further, out of 11 isolates, the *Rhizobium* strain from ground nut showed maximum IAA (24.12 µg/ml).

TABLE 2
Production of IAA by *Rhizobium* strains from *Vigna mungo*

S. No	Name of strain	IAA production (µg/ml)
1	VM-2	19.0
2	VM-8	43.4
3	VM-9	35.0
4	VM-15	43.1

Notes: Each value in the table is a mean of three replicates
F-calculated (4.256); F-tabulated (1.925); significant at 5% level

EPS production

Maximum EPS production was recorded in VM-9 (527 mg/ml) followed by VM-8 (483mg/ml). The lowest EPS production was recorded by VM-2 (341mg/ml) followed by VM-15 (287 mg/ml) (Table 3). The above results clearly indicate that these isolates are considered as copious EPS producers. The *Rhizobium* strain isolated from the root nodules of *Crotalaria saltiana* produced 16 µg/ml (Mukhurjee *et al.* 2011) while that of *Rhizobium* DL 10 from *Dalbergia lanceolaria* produced maximum EPS 765 µg/ml (Ghosh *et al.* 2005) and *Rhizobium* strain from blackgram produced maximum EPS 346 mg/l (Mandal *et al.* 2007).

Phosphate Solubilisation

All the four strains of VM-2, VM-8, VM-9 and VM-15 produced a clear zone around the colonies after 24 h of incubation on Pikovskaya's agar medium, which gradually increased up to 72h. The solubilisation efficiency (SE) of *Rhizobium* strains on solid media ranged between 16% and 170%. The *Rhizobium* strain VM-2 showed maximum solubilisation efficiency (Figure 1) followed by VM-8, VM-15 and VM-9. In liquid medium, *Rhizobium* strain VM-2 recorded maximum solubilisation (799µg/ml) followed by VM-8 (372µg/ml), VM-15(353µg/ml)

TABLE 3
EPS production by *Rhizobium* strains from *Vigna mungo*

S.No	Name of strain	EPS production (mg/100 ml)
1	VM-2	341
2	VM-8	483
3	VM-9	527
4	VM-15	287

Notes: Each value in the table is an average of three replicates
F-calculated (5.637); F-tabulated (2.295); significant at 5% level



Figure 1. Phosphate solubilised zone of *Rhizobium* strain VM-2

and VM-9 (261 µg/ml) (Table 4). A drop in a pH was accompanied by phosphate solubilisation. Phosphate solubilising microorganisms dissolve insoluble phosphates by the production of inorganic or organic acids and/or by a drop in pH value (Sperber 1958; Rodriguez and Fraga 1999; Sridevi *et al.* 2007; Kranthi Kumar and Raghu Ram 2016).

In earlier reports, solubilisation efficiency (SE) of *Rhizobium* isolates from *Cassia absus*, *Vigna trilobata* and three strains from *Sesbania sesban* on solid media ranged between 33% and 150%. In liquid medium, maximum solubilisation was recorded with *Rhizobium* isolate from *Cassia absus* (620 µg/ml) (Sri devi and Mallaiah 2009) while the *Rhizobium* sp isolated from root nodules of *Crotalaria retusa* recorded maximum solubilisation (840 µg/ml) in liquid medium (Sri devi *et al.* 2007). In the study of Muhammad Adnan (2016) it was observed that 21% of the tested rhizobia were phosphate solubilising bacteria. Among 15 *Rhizobial* isolates, 7 isolates were able to solubilise phosphate on Pikovskaya's

TABLE 4
Solubilisation of tricalcium phosphate by *Rhizobium* strains from *Vigna mungo*

S. No	P ₂ O ₅ liberated strain	(µg/ml)
1	VM-2	799
2	VM-8	372
3	VM-9	261
4	VM-15	353

Notes: Each value in the table is an average of three replicates
Significant at 1% ($p = 0.000$)

media containing Tricalcium phosphate as phosphate source. The solubilisation efficiency of *Rhizobium* strains on solid media ranged between 38% and 270% (Manasa *et al.* 2017).

Hydrogen Cyanide Production

Among the four strains screened, except for VM-8 strain, the other three strains (VM-2, VM-9 and VM-15) produced HCN. Among these three strains VM-2 and VM-15 showed strong HCN production by a change in colour of filter paper from yellow to reddish brown (Figure 2) and Strain VM-9 showed low HCN production by change in colour of filter paper from yellow to light brown (Table 5). Control plate did not show any colour (Figure 3).



Figure 2. HCN production by VM-2

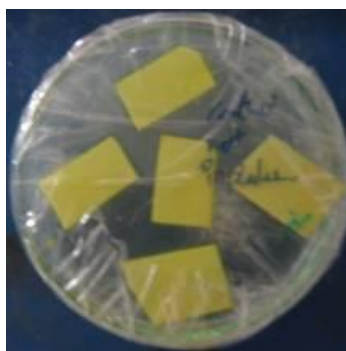


Figure 3. HCN control plate

TABLE 5
Production of hydrogen cyanide (HCN) by *Rhizobium* strains

S. No.	Strain	Colour
1.	VM-2	Reddish brown
2.	VM-8	–
3.	VM-9	Light brown
4.	VM-15	Reddish brown

The isolates of *Rhizobium meliloti* from ground nut were able to produce HCN (Arora *et al.* 2001). Thirty-three isolates (7.26%) from 454 rhizobial isolates had the ability to produce HCN as reported by Pellock *et al.* (2002). Yogendra *et al.* (2013) reported that out of the 25 *Rhizobium* strains tested, only one strain produced hydrogen cyanide (HCN). Muhammad *et al.* (2016) reported that their studies on PGPR features of the *Rhizobium* strains obtained from different summer legumes, only 9% of the tested rhizobial strains produced HCN. Kranthi Kumar and Raghu Ram (2016) reported that four *Rhizobium* strains out of six strains isolated from *Vignatrilobata* showed HCN production. Monika *et al.* (2017) reported that four rhizobial strains from 14 rhizobial isolates had the ability to produce HCN. Out of 15 *Rhizobium* isolates, eight produced HCN. Further, out of eight, the *Rhizobium* strain obtained from red gram exhibited strong HCN production and the *Rhizobium* strains obtained from ground nut scored as moderate for HCN production (Manasa *et al.* 2017).

Phylogenetic Analysis of Four Representative Isolates

The phylogenetic analysis of the four gene sequences of 16S r RNA of VM-2, VM-8, VM-9 and VM-15 was blasted against the nucleotide database of the NCBI and the sequences were aligned with a set of published sequences on the basis of the conserved primary sequence and also by nucleotide BLAST similarity search analysis. Based on the 16S rRNA gene sequences, the strain VM-2 showed a close relation with *Rhizobium* sp. strain, VM-8 and VM-9 with *Bradyrhizobium* sp. and VM-15 with *Achromobacter* sp. The 16S rRNA sequences were deposited in NCBI with the accession numbers KJ 704783 (VM-2), KJ 704784 (VM-8), KJ 704785 (VM-9) and KJ 501696 (VM-15).

The above results clearly indicate that the strains belong to Rhizobiaceae (VM-2), Bradyrhizobiaceae (VM-8, VM-9) and Alcaligenaceae (VM-15) families which are phylogenetically distinct.

Plant Inoculation Test

Pot culture experiment

Among the different morphological and yield parameters studied under pot culture experiment (Figure 4), indigenous strains such as VM-8, VM-9, and VM-15 inoculated plants showed good results both in inoculated sterilised and Inoculated unsterilised soils than the plants grown in sterilised uninoculated soils and control soils both at 35 DAS and at 50% flowering stage. The strain VM-2 showed moderate results among the different parameters studied under pot culture experiment.

Similar reports of variation among the native or indigenous rhizobial strains inoculation in different crops on different parameters have been reported by so many authors. Arroyo *et al.* (1998) inoculated common bean with native Bradyrhizobia, Pant and Prasad (2004) treated soybean with native Bradyrhizobia while Hungria *et al.* (2015) and Samudin and Kuswantoro (2018) inoculated

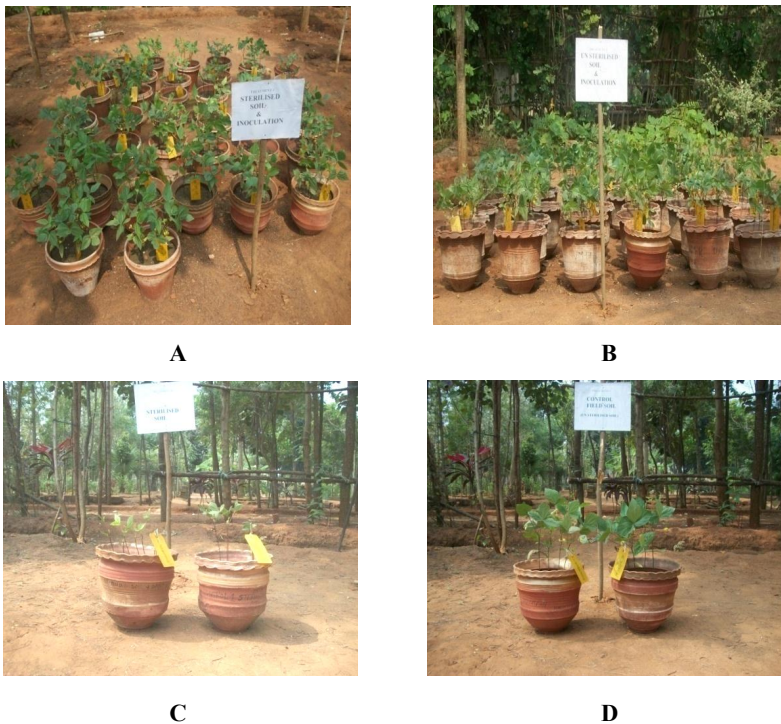


Figure 4. Experimental view of pot experiment

- A.** *Treatment 1: Seed inoculation with isolated native strains in sterilised soil.*
- B.** *Treatment 2: Seed inoculation with isolated native strains in unsterilised soil.*
- C.** *Treatment 3: Growth of seedlings in sterilised soil without inoculation*
- D.** *Control : Growth of seedlings in unsterilised soil without inoculation*

soybean with native *Rhizobium*. Other researchers carried out studies on the Bengal gram (Bhattarai and Maskey 1992; Tippannavar and Desai 1992) the soybean (Palaniappan *et al.* 1997), black gram (Neemar *et al.* 2007), chick pea (Yadav *et al.* 2011), green gram (Bhat *et al.* 2010) and in dry bean (Karaca and Uyanoz 2012).

CONCLUSION

Based on our study, it is concluded that all the four isolates (VM-2, VM-8, VM-9 and VM-15) exhibited plant growth promoting traits like production of IAA, EPS, HCN and phosphate solubilisation. Inoculation of most cultivated rice fallow black gram variety LBG-752 with these four indigenous rhizobial strains promoted plant growth which could be directly attributed to the beneficial effects from biological N₂ fixation and phytohormones, EPS production and indirectly to phosphate solubilisation. These strains belong to *Rhizobium* sp., *Bradyrhizobium* sp. and *Achromobacter* sp. respectively. In this investigation, *Achromobacter* sp. (VM-15) is reported for the first time to nodulate the Indian blackgram. This study, therefore suggests that these four native rhizobial strains of PGP potential can be used as biofertilisers as well as bio control agent for enhancing the yield of blackgram in rice fallows.

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