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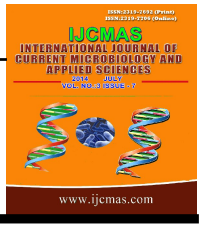

Original Research Article

Evaluation of selective Rhizobacteria as a Bioinoculant on Green gram (*Vigna radiate* L.) R. Wilczek

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A B S T R A C T

Plant growth promoting rhizbacteria (PGPR) colonize the plant roots and enhance plant growth by a wide variety of mechanisms. The objective of this study was to characterize the rhizobacterium from different crop fields and assessed their effects in green gram. Of the 64 total isolates, 13 isolates exhibit multiple plant growth promoting trait such as IAA production, phosphate solubilisation, siderophore and hydrogen cyanide production and own strong antagonism against fungal pathogens. Based on the trait four isolates PPG3, MNM3, SPSC3 and VYSC1 were selected for determining growth promoting activity in green gram. The bioassay was conducted under *in vitro* conditions in pot culture and significant results were observed in seed germination, root and shoot elongation followed by high biomass content. Molecular characterization studies (16s rRNA) fits the isolates with the *Pseudomonas* sp., *P. aeruginosa*, *P.stutzeri*, *Acinetobacter*, *A.soli*, Alcaligenes, *Enterobacter*, *Bacillus* sp., *B.cereus* and *B.subtilis* respectively. The results of this work indicate that the selectively isolated strains prove their potentiality in green gram growth and future studies should carried out to different crops and in field condition to develop as an effectual bioinoculant agent.

Keywords

Green gram,
Rhizobacterial isolate,
pot culture,
Antifungal

Introduction

Continuous application of pesticides and fertilizers in agriculture has increased pathogen resistance and environmental pollution that necessitate the need to develop an ecologically significant approach for plant protection and production. In ecological view, microbes interact with plant with various degrees of dependence either beneficial (PGPR, endophytes) or harmful (pathogens). The study of plant beneficial microbial interaction is now being applied to develop crop protection methods based on use of beneficial microorganisms or stimulation of plant defense response. (Montesinos, et al., 2002).

Plant growth-promoting rhizobacteria (PGPR) are a group of microorganisms able to colonize the rhizosphere or roots of many plant species, conferring beneficial effects

on their host (Kloepper, 1993). The interaction of PGPR and plant is not completely understood. However, many PGPR isolates seems to have positive outcome on their plants such as uptake of nutrient phosphorus via phosphate solubilization, synthesizing stimulatory phytohormones indole-3-acetic acid (IAA), or aiding in the control of the deleterious effects of pathogens by producing inhibitory substances, excluding them from the roots by competition or by inducing systemic resistance (Kloepper, et al., 1980; Lugtenberg, et al., 2004). Several bacterial genera such as *Azotobacter*, *Azospirillium*, *Pseudomonas*, *Acetobacter*, *Burkholderia* and *Bacillus* are confessing as PGPR. (Glick, 1995)

The use of PGPR as microbial inoculants for plant growth is consistently improving and successfully employed in commercial crops such as wheat, rice and maize (Ashrafuzzaman, et al., 2009; Sandhya, et al., 2010; Khalid, et al., 2004). Eventhough the immediate response to soil inoculation with PGPR varies considerably with soil types, plants, climate and other environmental factors. Sometimes, the inoculated PGPR cannot survive better in the soil because it must adapt to the indigenous microbial flora (Bashan and Holguin, 1997). Isolating of native strains adapted to that environment and their studies contribute to the formulation of an inoculant to be used in region crops.

Therefore, the objective of this study is to isolate rhizobacterial strains from different crop fields and characterize their plant growth promoting traits in green gram.

Materials and Methods

Sample collection and Bacterial isolation

The soil samples were collected from agriculture land having crops such as *Zea mays L.* (Maize), *Oryza sativa L. (*Rice), *Arachis hypogaea L.* (Groundnut), *Helianthus annuus* (Sunflower), *Musa paradisiaca L.* (Banana) and *Saccharum officinarum L.* (Sugar cane), Tiruchirappalli district, Tamil Nadu, India. (altitude: 78m; latitude: 10.81 °N; longitude: 78.69 °E). The soil samples were serially diluted and spread on nutrient agar medium at 30 °C for 2 days and examined for the presence or absence of microorganismal growth colony. The colonies were selected according to their time of growth and morphology (color, size, shape) and maintained in 20 % (v/v) glycerol solution and stored at 4 ºC.

Qualitative and Quantitative studies of IAA production

In this study, all the isolates were selected for the qualitative analysis of IAA production (Ivanova, et al., 2001). Quantitative analysis of the test isolates was analyzed by the method of Lopper and Scroth (1986) at different concentrations of L - tryptophan (0, 50, 150, 300, 400 and 500 µg/ml). Development of pink colour indicates IAA production. Optical density was taken with the help of spectrophotometer at 530nm. Based on the qualitative and quantitative analysis of IAA production, the isolates shown positive results were screened for other PGP activity.

Screening of selected test isolates for PGP traits

The phosphate soubilization activity of each isolate was determined by using Pikovskaya's agar medium (Pikovskaya, 1948) with 0.5mg as tri-calcium phosphate as an inorganic source. The plates were incubated at 28°c at 72 hr and the formation of clear halo around the colonies indicated phosphate solubilisation assay.HCN

production was done by the method of Lorck (1948). Development of orange to red color indicates positive for HCN production. Siderophore productions were assayed for the test isolates in th**e** Chrome azurol S agar medium, described by Schwyn and Neilands (1987). Development of deep blue to yellow - orange halo around the growth was positive for siderophores production. Further, the test isolates were screened for antifungal activity against *R. solani, F. oxysporum* by agar well diffusion method. Diluted spore suspension of the fungi was spread on Muller Hinton [MH] agar plates and wells of 8 mm diameter were punched into the agar medium and filled with 200 µl of bacterial cultures. Nutrient broth was taken as negative control and 100 µg/ml antifungal antibiotic, nystatin, was used as positive control. The antifungal activity was evaluated by measuring the growth of inhibition zone.

Identification and Phylogenetic analysis of Rhizobacteria

Isolates were characterized and sequenced at molecular level. The 16S rRNA of the bacterial genomic DNA was amplified by the fluorescent dye terminator method (ABI Prism TM Bigdye TM Terminator cycle sequencing ready reaction kit v.3.1; Applied Biosystems, USA) using the Primers 1F (5'-AGCGGCAGACGGGTGAGTAATG-3'), FGPS1509R (5'- AAGGAGGGGAT CCAGCCGCA - 3'). The PCR products were purified by Qiaquick PCR purification kit and run in an Qiaquick column and sequenced using Big dye terminator (Applied Bio System). The completed 16s rRNA sequence were aligned and their closest relative sequence were ascertained using BLAST search and multiple sequence alignment was done using CLUSTAL X. Phylogenetic dendogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1,000 data sets using MEGA 3.1 (Molecular Evolutionary Genetic Analysis). The 16s rRNA sequences of each isolated were deposited in the NCBI GENBANK database under the corresponding accession numbers.

Evaluation of test isolates on growth of green gram

In this study, based on efficient performance in PGP trait assay, four isolates were selected and evaluated for plant growth study. Pots were surface sterilized with 20% sodium hypochlorite solution and filled with sterile loam soil [pH 7.8, EC 0.21 dS $m^{-1,}$ available nitrogen 230 kg ha^{-1}, available P_2O_5 10 kg ha^{-1} and available K_2O 250 kg ha^{-1}] and sieved in 4 mm pore size sieve and mixed along with farmyard manure in 2:1 proportion and filled in the respective pots. The seeds of green gram were surface sterilized with 1% mercuric chloride for 3 min followed by successive washing with sterile distilled water. The seeds were added to test cultures grown in their respective medium for 48 h containing at least 10^6 cells per ml and kept at 30 min in the culture and shade dried before sowing as described by Holland and Polacco [1994]. Aseptic conditions were maintained throughout the process. After imbibitions of seeds of respective bacterial treatment in pot culture, experiments were conducted under *in vitro* conditions and control was maintained with 3 replicates. The pots were maintained at 20ºC - 22 ºC with 13-11 h natural light, 50% relative humidity. After 20^{th} day all the seedlings were pulled out with entire roots intact and washed with tap water to remove the sand particles. The seedlings were then analyzed for shoot length, root length and plant fresh biomass was noted. After recording the observations the seedlings were kept in an incubator at 80 ± 2 ºC for 48

h until it reached a constant weight and plant dry matter production was recorded.

Statistical analysis

To evaluate the result of different amendments on plant growth of PGPR, data were subjected to one way analysis of variance [ANOVA] and significant differences means were compared using Tukey's test [$p < 0.05$]. Before analysis, the bacterial populations were transformed to log 10 [x+1]. The statistical package SPSS 11 was used for all analyses.

Results and Discussion

Rhizobacteria isolation and characterization

In this study, rhizobacteria were isolated and identified from the agriculture lands in Tiruchirappalli district, Tamil Nadu, India . Totally 64 (42.66 %) rhizobacterial isolates were isolated from 150 soil samples. Among them, 14 isolates from *Z. mays,* 14 from *O. sativa L,* 5 from *A. hypogaea L.,* 10 from *M. paradisiaca L,* 8 from *S. officinarum L.* and 13 from *H. annuus* were obtained.In collected soil samples pH varies from 6.7 to 8.4 and the organic matter content was ranging from 0.80 to 5.74 % and the electric conductivity was measured from 0.34 to 4.33 %. The morphological variation and other disparity in biochemical characteristics were given in table: 1

PGP traits of the isolates

The PGP traits of the bacterial isolates are showed in the table: 2 &3. As a preliminary screening all the 64 isolates were checked by qualitative estimation of IAA. From the study, 13 isolates were shown positive for IAA production. These isolates were further selected and quantified using tryptophan at different concentration range 0-500µg/ml. At 0µg/ml concentration, IAA production was not observed; the addition of tryptophan at different concentration an increase in IAA production was observed in the isolates. Isolate MNM3 (75.7 ± 0.340), PPG3 (90.3 ± 0.26), SPSC3 (70.2 ± 0.36) and VYSC1 (78.7 ± 0.36) recorded maximum IAA production at 500 µg/ml concentration. Among 13 isolates, few isolates were able to soubilize phosphate, produce siderophore as well as hydrogen cyanide and inhibiting growth of *R.solani, F.oxysporum*. Table: 2 & 3.

Identification and phylogenetic analyses of rhizobacteria

DNA Sequencing of 16s rRNA was done for all 13 isolates. Based on the nucleotide sequences each of the isolates was analyzed in BLAST to search for their closest identity in GenBank database. The sequence of the isolates was then aligned using Clustal X, with the sequence of their closest identity retrieved from database. (*Pseudomonas aeruginosa*, *Pseudomonas* sp. *Pseudomonas stutzeri Bacillus subtilis*, *Bacillus pumilus Bacillus cereus Acinetobacter soli Acinetobacter* sp., *Alcaligenes faecalis, Alcaligenes* sp., and *Enterobacter* sp). The phylogenetic position of the recovered isolates within different 5 genera of PGPR is presented in Fig: 1. The figure indicates all the sequenced isolates were clustering with their respective genus. All the sequenced isolates were then submitted in GenBank database and their respective corresponding number were represented in table:4

Evaluation of plant growth promoting ability in green gram

As from the above study, four isolates (PPG3, MNM3, SPSC3 and VYSC1) have multiple PGP traits were selected for

inoculation with green gram seeds under *invitro* conditions. The result of test isolates on seed inoculation was evaluated by % of seed germination, shoot length, root length, total biomass and dry weight of plant. The percentage of seed germination was significant in all the inoculated seeds with that of control. All the isolate inoculated seeds show an increase in growth of plant; compare with control. Strain MNM3 is the efficient isolate in our study recorded significantly higher vigour index 2995, increases root and shoot length, total dry weight and biomass of the plant when compare with the other isolates. (Table: 5)

Isolation and identification of rhizobacteria was studied from the agriculture lands in Tiruchirappalli district, Tamil Nadu, India. A PGPR isolated from a region cannot do in the same way in other soil and climatic conditions (Duffy, et al., 1997). Therefore it is important to search for region-specific microbial strains that can be used as a potential bioinnoculant.

Studies of multiple PGP activities among PGPR have been reported by some other workers while; such findings on indigenous isolates of India are very few in reports (Gupta, et al., 1998). In this study, we have isolated 64 isolates from rhizosphere soils, 13 isolates were selected based on the qualitative assay of IAA production. Similar observations of qualitative and quantitative estimation of IAA have been reported by other researchers. (Djuric, et al., 2011, Farah, et al., 2006). In our study, PPG3 isolate exhibits a high IAA production followed by MNM3, PPG3, SPSC3 and VYSC1. The important aspect to be noted in our study is isolate PPG3 posses high IAA production and our study is the first to report the *Azotobacter* isolates from Tamil Nadu. Only a few reports have been reported the diversity and IAA production of *Azotobacter* from tropical soils of India. The production of IAA was found dependant upon bacterial isolates and concentration of tryptophan. Such findings have direct practical application, although intrinsic ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant (Arshad and Frankenberger, 1993). In this study the isolates showed multiple PGP traits phosphate solubilisation, siderophore, HCN production and antagonism against fungal pathogens. It is a well known verified that phosphorus solubilisation influences overall plant growth and development (Jones and Darrah 1994). Plants use bacterial iron-siderophore complexes for obtaining nutrient (iron) from soil (Glick and Bashan 1997). For example, *Pseudomonas* siderophore producers promoted rootlet elongation on cucumber grew under gnotobiotic conditions (Bellis and Ercolani 2001). In this study, few isolates were shown HCN detection; believe to be a prominent source in biocontrol aspect. The antifungal activity of the isolates is due to the production of siderophores and HCN or synergistic interaction with other metabolites. Several reports have shown that production of siderophores, other secondary metabolites and lytic enzymes by *Pseudomonas* strains was effective in controlling the plant root pathogens including *F. oxysporum* and *R. solani* (Nagrajkumar, et al., 200, et al., 2004; O'Sullivan and O'Gara, 1992)

The potent PGP attributing rhizobacterial isolates were sequenced and characterized with BLAST search. *Pseudomonas, Enterobacter, Acetobacter, Alcaligenes and Bacillus* genera were dominated in our rhizosphere soil sample that grouped into their family Bacillaceae, Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae and Alcaligenaceae grouped into two major divisions Proteobacteria and Firmicutes.

Table.1 Grouping of Rhizobacterial isolates according to Morphological and Biochemical studies

Characteristics	Fluorescent *Pseudomonas* spp.	*Bacillus* spp.	*Acinetobacter* spp.	*Alcaligenes* spp.	*Enterobacter*
Pigmentation	Florescent green	-	-	-	-
Colony morphology	Button shaped	Serrated margins	Irregular edge	Thin, irregular edge	Round
Gram reaction	-ve	+ve	-ve	-ve	-ve
Cell Shape	rods	rods	circle	rods	rods
Motility	+	+	+	+	+
Indole test	+	+	+	+	+
MR test	-	-	-	-	-
VP test	-	-	-	-	+
Catalase	+	+	+	+	+
Citrate	+	+	-	+	+
Oxidase	+	-	+	+	-
Carbohydrate utilization-glucose	73.80	77.77	86.66	72.72	77.77
lactose	19.04	22.22	59.99	54.54	44.44
Sucrose	35.10	77.77	46.62	36.36	-
Mannitol	-	72.02	26.40	-	-

Table.2 Plant growth promotion traits of recovered rhizobacterial isolates

Strain name	Phosphate solubilization	Siderophore production	HCN production	Antifungal activity	
				R.solani	*F.oxysporum*
VPG2	-	+	+	20.35 ± 1.64	20.14 ± 1.61
MNM3	+	+ + +	+	22.32 ± 1.42	22.52 ± 1.23
MRR1	+	+	-	12.14 ± 0.21	15.18 ± 0.81
MSB1	-	-	-	11.62 ± 0.34	13.17 ± 0.25
ALB2	-	-	-	13.41 ± 0.42	11.10 ± 0.43
VYSC1	+	+ +	+	18.26 ± 1.35	21.27 ± 1.74
SPSC3	+	+ +	+	19.42 ± 1.32	21.25 ± 1.52
AKB2	-	+	-	10.22 ± 0.41	13.31 ± 0.58
PVS2	+	-	+	14.36 ± 0.71	14.16 ± 0.67
NKR5	+	-	+	15.36 ± 1.31	16.47 ±1.25
NKM7	-	-	+	18.13 ± 1.22	16.35 ± 0.55
MPM3	-	-	+	17.36 ± 1.46	13.31 ± 0.42
PPG3	+	+ + +	+	21.42 ± 1.62	24.44 ± 1.70

(+) positive (-) negative + 0.3-0.5cm ++ 0.6-0.9 cm +++ >1cm

Table.3 Production of IAA by selected bacterial isolates at different concentration of tryptophan

Isolate codes	IAA production (µg/ml ± SD) at different tryptophan concentration (µg/ml)					
	0	**50**	**150**	**300**	**400**	**500**
VPG2	ND	05.4 ± 0.14 [b]	13.5 ± 0.13 [c]	25.4 ± 0.24 [c]	31.6 ± 0.35 [c]	37.2 ± 0.27 [c]
MNM3	ND	12.4 ± 0.23 [a]	30.1 ± 0.23 [a]	54.2 ± 0.36 [a]	66.1 ± 0.23 [a]	75.7 ± 0.34 [a]
MRR1	ND	07.3 ± 0.12 [b]	22.6 ± 0.26 [b]	41.3 ± 0.24 [b]	49.2 ± 0.34 [b]	57.1 ± 0.24 [c]
MSB1	ND	09.6 ± 0.14 [b]	24.7 ± 0.23 [b]	47.6 ± 0.26 [b]	55.3 ± 0.37 [b]	62.6 ± 0.25 [b]
SPSC3	ND	10.1 ± 0.15 [a]	27.3 ± 0.24 [a]	52.2 ± 0.25 [a]	61.9 ± 0.24 [a]	70.2 ± 0.36 [a]
MPM3	ND	06.2 ± 0.11 [b]	16.2 ± 0.21 [c]	30.1 ± 0.32 [c]	37.4 ± 0.28 [c]	40.6 ± 0.37 [c]
ALB2	ND	06.7 ± 0.13 [b]	18.5 ± 0.27 [b]	34.1 ± 0.35 [c]	41.0 ± 0.38 [c]	47.4 ± 0.38 [c]
AKB2	ND	06.4 ± 0.12 [b]	17.4 ± 0.24 [c]	32.7 ± 0.38 [c]	37.3 ± 0.24 [c]	46.1 ± 0.31 [c]
PVS2	ND	08.1 ± 0.14 [b]	22.1 ± 0.28 [b]	40.8 ± 0.31 [a]	47.3 ± 0.26 [b]	54.7 ± 0.27 [b]
NKR5	ND	05.8 ± 0.11 [b]	16.3 ± 0.24 [c]	30.7 ± 0.32 [c]	36.2 ± 0.32 [c]	44.9 ± 0.33 [c]
NKM7	ND	08.3 ± 0.13 [b]	21.7 ± 0.29 [b]	40.2 ± 0.36 [b]	47.2 ± 0.42 [b]	55.6 ± 0.29 [b]
VYSC1	ND	12.1 ± 0.24 [a]	32.6 ± 0.31 [a]	61.9 ± 0.32 [a]	70.7 ± 0.38 [a]	78.7 ± 0.36 [a]
PPG3	ND	15.3 ± 0.26 [a]	38.5 ± 0.35 [a]	74.3 ± 0.27 [a]	82.4 ± 0.31 [a]	90.3 ± 0.26 [a]

ND- Not detected

Table.4 Relative analyses of recovered isolates with their closely related species using 16s rRNA sequence

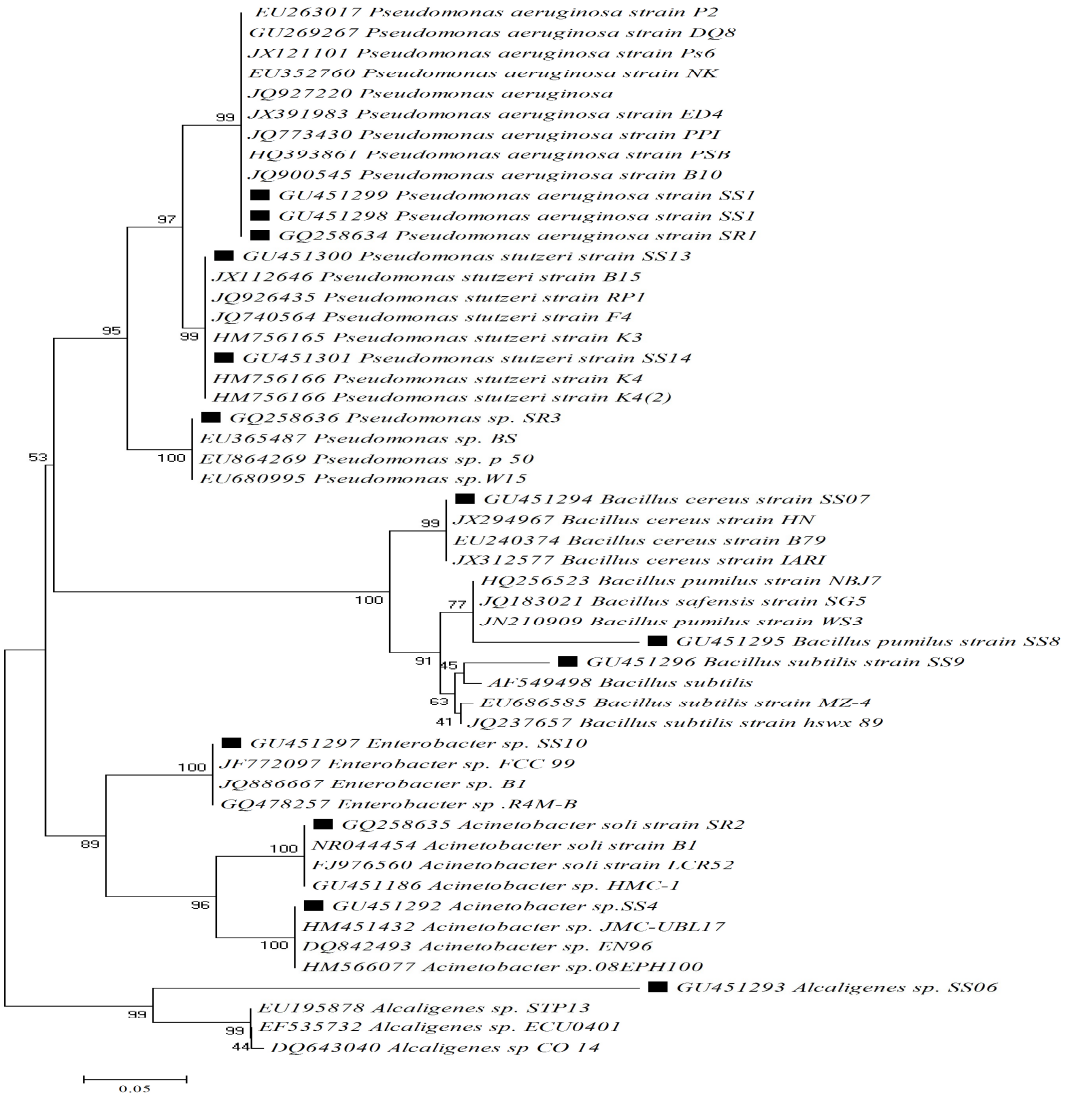
Isolate code	Length of 16s rRNA sequenced	GenBank Accession No	Close relative in database	% seq identity
VPG2	566	GU451295	JN391161	96%
MNM3	1493	GQ258636	EU747696	98%
MRR1	554	GU451296	FJ430065	96%
MSB1	1453	GQ258635	HQ407279	99%
ALB2	760	GU451297	JQ682634	99%
VYSC1	1050	GU451293	AB681169	93%
SPSC3	714	GU451294	JQ818413	99%
AKB2	659	GU451299	HQ393861	99%
PVS2	699	GU451298	JQ839146	99%
NKR5	1318	GQ258634	HQ288928	97%
NKM7	592	GU451300	JQ838017	99%
MPM3	610	GU451301	HM756166	99%
PPG3	579	GU451292	AY902243	99%

Table.5 Effect of rhizobacterial isolates on growth of green gram seedlings

Treatments	Green gram				
	% of seed germination	Shoot length (cm)	Root length (cm)	Total biomass (mg plant^{-1})	Dry wt (mg plant^{-1})
Control	80 ± 6.3^{b}	12.4 ± 2.1^{c}	06.2 ± 1.4^{c}	240 ± 15.4^{c}	36 ± 2.3^{c}
MNM3	100 ± 0.0^{a}	20.9 ± 3.5^{a}	10.5 ± 1.0^{a}	427 ± 21.9^{a}	50 ± 4.2^{a}
PPG3	100 ± 0.0^{a}	16.2 ± 3.2^{b}	09.4 ± 1.5^{ab}	285 ± 11.2^{c}	42 ± 4.1^{b}
SPSC3	100 ± 0.0^{a}	15.4 ± 2.8^{b}	07.5 ±1.8^{b}	272 ± 16.3^{c}	43 ± 2.8^{b}
VYSC1	100 ± 0.0^{a}	14.2 ± 2.5^{c}	08.4 ± 1.3^{b}	372 ± 14.7^{b}	47 ± 3.1^{a}

Results obtained were of mean of triplicates. Data were analysed using one-way analysis of variance and treatment means were compared (P B 0.05%)

Figure.1 Neighbour joining Phylogenetic analysis of 16s rRNA gene sequences and their closest phylogenetic neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position



These results were agree with the Krishna kumar et al. (2011) screened a total of 80 rhizobacterial isolates from coastal agricultural ecosystem of cultivated vegetable rhizosphere soils from Andaman and Nicobar islands, India and Deepa et al. (2010) reported the PGPR from non rhizospheric soils of western Ghats, Kerala from south India.

The present investigation on the plant growth promotion by seed bacterization has been demonstrated by the isolates PPG3, MNM3, SPSC3 and VYSC1. Among the isolates MNM3 inoculated seed exhibits a high elongation activity in root and shoot growth followed by VYSC1 respectively. This is mainly due to the ability of the isolate to produce IAA that positively influences root growth and development, thereby enhancing nutrient uptake and improved phosphorous nutrition influences overall plant growth and development (Jeffries, et al., 2003). Indole Acetic Acid (IAA) produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik 1986).

L-tryptophan is a precursor for synthesis of IAA in plants; likewise it is also a precursor for rhizosphere bacterium, when adding to its bacterial cultures promotes and increases IAA synthesis (Park, et al 2005; Tsavkelova, et al., 2007). In our study, the selected isolates were confer with multiple PGPR traits as IAA production, phosphate solubilisation, HCN and siderophore production that over all influences the plant growth and development in green gram. The results obtained indicate that the recovered isolates are a prominent resource as a PGPR. Future studies determine their efficiency in various crops and in greenhouse experiments.

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