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Original Research Article

Isolation, characterization of plant growth promoting bacteria from the plant *Chlorophytum borivilianum* and *in-vitro* screening for activity of nitrogen fixation, phospthate solubilization and IAA production

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A B S T R A C T

Several microbes promote plant growth and many microbial products that stimulate plant growth have been marketed. Plant growth promoting rhizobacteria (PGPR) are the soil bacteria that colonizes on plant root enhance plant growth and live symbiotically with plant. Microbes can promote plant growth by the regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients (like phosphate) and including resistance against plant pathogen. Present study deals with the isolation of rhizobactor and selection of plant growth promoting bacteria via their biochemical screening like N_2 fixation, phosphate solubilization and indole acetic acid production. In total 30 bacterial isolates were selected randomly from rhizosphere of plant *Chlorophytum borivilianum* (Safed Musli) and screened *in vitro* for plant growth promoting properties. Out of 30 isolated 10 samples are scored for positive growth promoting activity, in which all 10 isolates showed positive result for N_2 fixation on solid media but only 8 isolates showed N_2 fixation activity on liquid media, one isolate gave positive result for phosphate solubilization activity and only one isolate showed production of indole acetic acid (IAA) that give the OD 1.493 i.e. 100ng/ µl with respect to standard curve of IAA.

Keywords

PGPR,
IAA,
N_2 fixation,
Antagonistic activity,
Phosphate solubilization

Introduction

During the late 19th and early 20th centuries inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers. Due to the increase in human population, fertilizers were used to increase crop production to meet rising demands for food. The hazardous nature of chemical fertilizers for the environment has led to a resurgence of interest in the use of bio-fertilizers for enhanced crop yield and environmental sustainability. In the past 10–15 years close to 4,000 publications have appeared in the field of plant growth promoting bacteria (Bashan and Holguin, 1998). Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate

plant growth by a plethora of mechanisms. Rhizosphere is the centre to microbial and nutrient dynamics and describes the zone of soil surrounding roots of plant species which release organic substances. Bacteria that are present in the rhizosphere and enhance plant growth by any mechanism are referred to as plant growth promoting rhizobacteria (PGPR) (Vessey, 2003; Arnou, 1953).

Rhizosphere bacteria promote plant growth and yield either directly or indirectly (Kloeppr *et al.*, 1989; Glick, 1995). First the direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of the uptake of nutrients from the environment (Glick *et al.*, 1999). The direct growth promoting activities are as follows i) nitrogen fixation ii) solubulization of phosphorus iii) sequestering of iron by production of siderophores iv) production of phytohormones such as auxins, cytokinins, gibberellins and v) lowering of ethylene concentration (Kloepper *et al.*, 1989; Glick, 1995; Glick *et al.*, 1999). The capacity to produce the phytohormone indole-3-acetic acid (IAA) is widespread among bacteria that inhabit diverse environments such as soils, fresh and marine waters, plant and animal hosts. Three major pathways for bacterial IAA synthesis have been characterized that remove the amino and carboxyl groups from the α-carbon of tryptophan via the intermediates indolepyruvate, indoleacetamide, or indoleacetonitrile; the oxidized end product IAA is typically secreted (Patten *et al.*, 2013). *Pseudomonas putida* 1290 is a model organism for the study of bacterial degradation of the plant hormone indole-3-acetic acid (Scott *et al.*, 2013). Use of coinoculants of *Pseudomonas*, *Azotobactor* and *Bacillus* with Mussoorie Rock Phosphate(MRP) could make phosphorus availability to equivalent 50 kg of P_2O_5/ha applied in the form of Single Super Phosphate (SSP). The efficient phosphate solubilizers are *Pseudomonas* species viz., *Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas fluorescence and Pseudomonas putida* (Kumar and Dangar, 2013). Strains of *Bacillus altitudinis, Pseudomonas monteilii,* and *Pseudomonas mandelii* formed associations with rice plants and fixed nitrogen (Habibi *et al.*, 2014).

Second indirect mechanism of plant growth promotion by PGPR include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal compound iv) production of fungal cell wall lysing enzymes v) competition for sites on roots and induced systemic resistance (Weller and Cook, 1986; Dunne *et al.*, 1993; Liu *et al.*, 1995). Food crop productions highly effected by pathogenic microorganisms affect plant health are major threat to ecosystem stability worldwide. Agriculture producers became more and more dependent on agrochemicals as a reliable method of crop protection helping with economic stability of their agriculture practices. However increases in chemical inputs causes several negative effects, i.e., development of resistant pathogen varieties, their non-target environmental impacts, growing cost and accumulation of chemicals in edible plant parts. (Compant *et al.*, 2005). Soil borne fungal diseases pose serious constraints on agro-productivity.

Biological control is non-hazardous strategy to control plant pathogens and improve crop productivity. PGPR (plant growth promoting rhizobacteria) have long been used as plant disease control agents. PGPR produced a wide range of secondary compounds that may act as signals—that is, allelochemicals that include metabolites, siderophores, antibiotics, volatile metabolites, enzymes and others. Their mode of action and molecular mechanisms provide a great

awareness for their application for crop disease management (Saraf *et al.*, 2014).

The importance of plant growth promoting rhizobacteria in growth promotion and their ability to elicit 'induced systemic tolerance' against abiotic stresses has been documented. A study done by Damodaran *et al.*, (2013), 16 rhizobacteria through isolated natural selection from saline sodic soils, and characterized them using morphological and biochemical parameters and out of them two stress tolerant PGPR traits are found. Plant growth promoting bacteria that have activity of 1-aminoacyclopropane-1-carboxylate (ACC) deaminase active was isolated from soil, were show positive results in enhancing plant growth on presence of high salt concentration.

Tomato seedling treated with isolated bacteria significantly increased the fresh and dry weight in the presence of up to 172mM NaCl salt. The bacterium reduced the production of ethylene by tomato seedlings, which was otherwise stimulated when seedlings were challenged with increasing salt concentrations. In the presence of salt the bacterium increased the water use efficiency (WUE). This may suggest that the bacterium acts to alleviate the salt suppression of photosynthesis (Mayak *et al.*, 2004).

Microbial interactions as symbiotic or parasitic, other types of associations between plants and microorganisms are often overlooked. Endophytic bacteria colonize inner host tissues. Sometimes in high numbers, without damaging the host or eliciting strong defense responses. The molecular basis of endophytic interactions is still not well understood. Better understanding of community dynamics, signaling and functions in endophyte-plant association made by culture-independent methods for community analysis and functional genomic as well as comparative genomic analyses (Hurek and Hurek, 2011). Application of plant growth promoting rhizobacteria (PGPR) in the field has been limited by a lack of knowledge of ecological factors that determine their survival and activity in the plant rhizosphere. To be effective, PGPR must maintain a critical population density of active cells.

Inoculation with PGPR strains can temporarily enhance the population size, but inoculants often have poor survival and compete with indigenous bacteria for available growth substrates (Martínez-Viveros *et al.*, 2010). *Rhizobium* spp. which fix N_2, from the atmosphere and from root nodules on legumes, were the first biofertilizer identified and have been used commercial an inoculants for legume for over 100 years (Kannaiyan, 2002).

Materials and Methods

Soil sample collection

The experimental material for the present study consists of soil samples. Collection of soil sample was done from rhizosphere of plant, *Chlorophytum borivilianum* along with their open areas. The soil bound to the roots was collected from the plant and kept in ice-box. Similarly the soils from adjoining area devoid of plantation were also collected at a depth just below 30cm. All the soil samples were preserved at -20ºC. Each of the collected soil samples were homogenously and separately mixed for further study.

Isolation and identification of bacteria

Isolation

One gram of each soil sample was taken and dispersed in 10ml of 0.85% saline water and vortexed for 5 minutes. After that serial

dilution was carried out in order to isolate single colonies.

Appropriate dilutions were plated on nutrient agar plates, by taking 100µl of suspension after brief vortexing. After that inoculated plates were incubated. Then 20–25 colonies per plate were selected and re streaked. Purity of the cultures obtained by many streaking were checked by staining and biochemical test.

Identification

Partial identification of bacterial colonies were done by microscopic observation and biochemical testes like gram's staining (Ying, 2008), colony color, H_2S production (Clarke, 1953), motility and methyl red test etc.

PGPR activity of isolated bacteria

PGPR activity of isolated bacteria estimated *in vitro* using biochemical tests.

Test for N-fixation activity from bacterial isolates

For the identification of nitrogen fixing microbes, nitrogen fixation activity tested on media containing malic acid, K_2HPO_4, KOH, $MgSO_4$, NaCl, $CaCl_2$ $FeSO_4$, $NaMoO_4$, $MnSO_4$ and agar—used as solidifying agent for media.

N-fixation activity was tested on both liquid and solid media. 0.5% bromothimol blue was use as pH indicator.

Test for phosphate solubilizing activity of bacterial isolates

Bacterial strains were evaluated for their ability to solubilize inorganic phosphate. Agar medium containing calcium phosphate as the inorganic form of phosphate was utilized in this assay. Each isolated culture was streaked on the plates; five per plate, and the plates were incubated at 27°C for 3 days. A zone of clearing around the colonies after 3 days was scored as positive for phosphate solubilization. The experiment was performed twice with five replicates for each bacterial strain.

Test for Indole Acetic Acid (IAA) production

For the IAA production test firstly bacterial isolates were cultured on Luria broth medium containing tryptophan for 24 hr at 27^oC at 100–150rpm on shaker. Then different tubes containing growth of isolated microbes was centrifuged at 10,000rpm for 10 min. Then supernatants were separated in new tubes, now 1 ml of each supernatant of particular isolates were mixed with 1.5 ml of Salkowski's reagent and kept for 30 minutes. Standard solutions of 10ng/ml, 25ng/ml, 100ng/ml, 200ng/ml and 400ng/ml were used to estimate the IAA producing activity.

Results and Discussion

Isolation of bacteria

Total 30 bacteria were screened for PGPR activity in which 10 bacteria were selected on the basis of their particular activity. Plant growth promoting rhizobacteria were assayed for their ability to produce indole like IAA, nitrogen fixation activity and phosphate solubilizing activity.

These isolates were tested for their PGPR activity in *Chlorophytum borivilianum* with respect to control conditions.

Identification of bacterial isolates

10 bacteria were selected on the basis their particular activity was identified on the basis of gram's staining (Figure 1&2), colony

color, H_2S production, motility and methyl red test – for compatibility. Isolate names are given randomly during work (Table 1).

Estimation of N-fixation activity from bacterial isolates

Firstly isolated rizobacteria were screened for their ability to fix nitrogen in the solid and liquid medium in which S9 isolate gave maximum O.D. than other isolates (S-29, A3, C1, D2, B1-01-64, PGPR20, D3 and A5) who had also shown nitrogen fixation activity (Table 2 & Figure 3).

Phosphate solubilizing activity of bacterial isolates

Phosphate solubilizing activity was estimated using disc diffusion assay. Out of 30 bacteria only D3 showed activity with 61.71 mm diameter (Fig. 4).

Estimation of IAA production from bacterial isolates

Totally 30 bacteria were screened for PGPR activity in which only A3 showed IAA production. Amount of IAA production was calculated using known IAA standards A3 gives OD 1.493 i.e. 100ng/ µl ((Table 3 & Figure 5).

In conclusion, the above results support the hypothesis that isolated bacteria utilizes either one of the growth promoting mechanisms (N-fixation, Phosphate solubilization and IAA production) or a combination of actions to increase the plant growth and are the good alternative for chemical hazardous fertilizers and also crop production cost reducing agent.

Further research in developing mutants of N-fixation, phosphate solubilization or IAA production activity would help in elucidating the major direct mechanism of action of isolates in promoting the growth of plants under laboratory and field conditions. This would help in developing a potential inoculant for use in agriculture in the future. Future work in being enclosed on RAPD analysis of theses isolates for knowing their molecular basis.

Table.1 Identification of 10 bacterial isolates

S.No.	Test Sample	Gram's staining	Colony color	H_2S production	Motility	Methyl red test
1.	S-29	+	Light brown	+	-	+
2.	A3	-	Yellowish	-	+	+
3.	D3	-	Yellowish	-	+	-
4.	C1	+	Pale color	+	-	-
5.	B1-01-64	+	Light brown	+	+	+
6.	D2	+	Pale color	+	+	-
7.	Pgpr 20	+	Pale color	+	-	+
8.	S-26	+	Yellowish	-	-	+
9.	A5	-	yellowish	-	+	+
10.	S-9	-	Light brown	+	+	-

Table.2 N_2-fixation activities of bacterial isolates

S.No.	Test Sample	N_2 Fixation (Broth)	N_2 Fixation (agar)	Absorbance @540nm
1.	S-29	+	+	0.312
2.	A3	+	-	0.084
3.	D3	-	+	
4.	C1	+	+	1.207
5.	B1-01-64	+	+	0.347
6.	D2	+	+	0.837
7.	Pgpr 20	+	+	1.585
8.	S-26	-	+	
9.	A5	+	+	0.342
10.	S-9	+	+	2.863

Table.3 OD of standards

No.	Standard Concentration	Volume (µl)	Absorbance
1.	10 ng/ µl	20 µl	0.377
2.	25 ng/ µl	45 µl	0.734
3.	100 ng/ µl	200 µl	1.493
4.	200 ng/ µl	400 µl	2.052
5.	400 ng/ µl	1000 µl	2.863

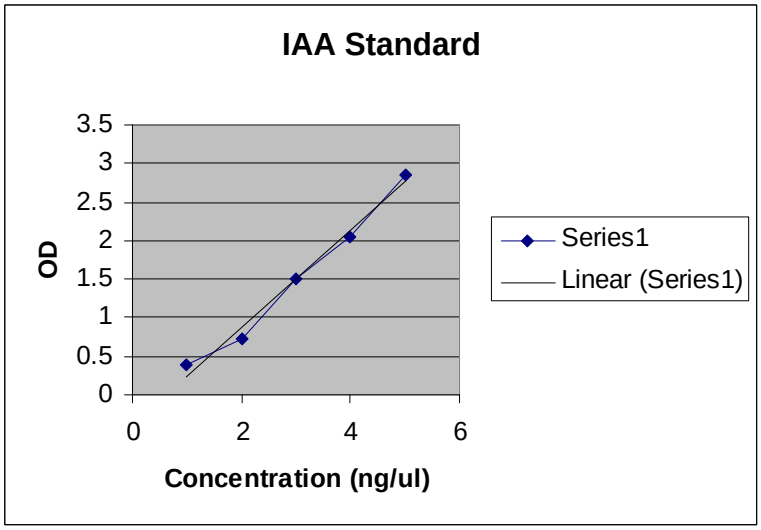


Fig.1 Gram staining of PGPR 43 (G-ve)

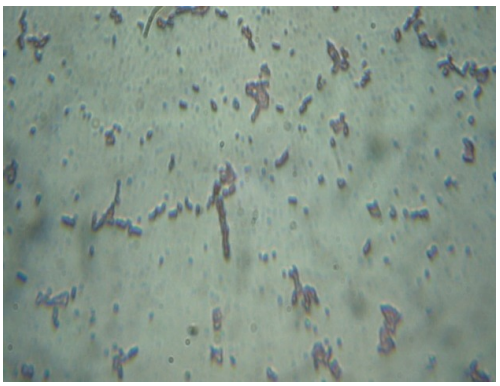


Fig.2 Gram staining of S-29 (G+ve)

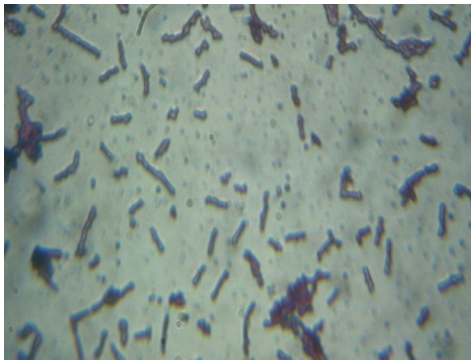
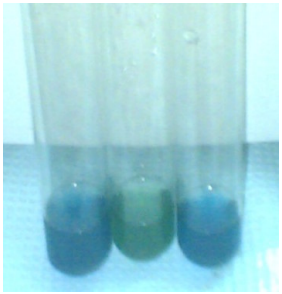


Fig.3 N-fixation activity on liquid and solid media



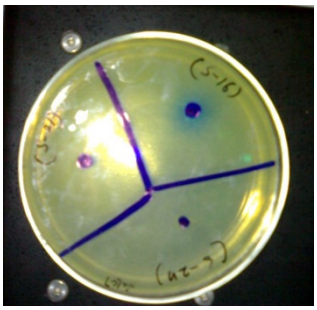


Fig.4 Phosphate solubilizing activity

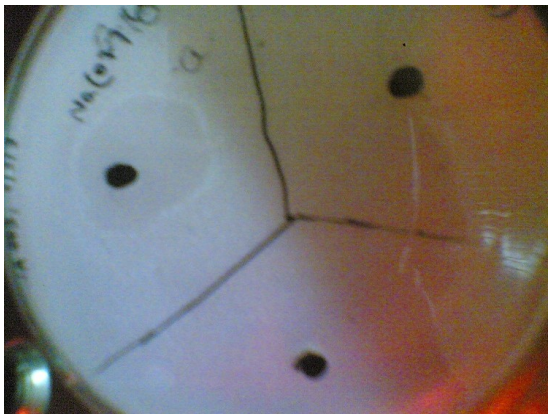


Fig.5 IAA production

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