ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 01, pp.12258-12263, January, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

PLANT GROWTH PROMOTING CHARACTERS OF *GLUCONACETOBACTER DIAZOTROPHICUS AZOSPIRILLUM BRASILENCE* AND PHOSPHOBACTERIA (*BACILLUS MEGATHERIUM*)

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ARTICLE INFO

Article History:
Received 29th October, 2014
Received in revised form
15th November, 2014
Accepted 28th December, 2014
Published online 31st January, 2015

Key words:

PGPB – Nitrogen fixation,
Phosphate solubilization,
Siderophore, *Azospirillum*,
Gluconacetobacter, Phosphobacteria.

ABSTRACT

PGPB – Plant Growth Promoting Bacteria are the group of beneficial bacteria promote crop growth and yield by exerting many beneficial aspects like N fixation, P solubilization, growth promoting substances production and siderophore production. In the present research about 13 isolates were isolated and identified as *Azospirillum brasilence*, *Gluconacetobacter diazotrophicus* and phosphobacteria (*Bacillus megatherium*) and these isolates were studied for its PGPB nature to provide N & P nutrients and biocontrol nature through siderophore production. The findings of the present investigation clearly showed the interesting future of tested organisms. *Gluconacetobacter diazotrophicus* showed maximum efficiency on N fixation and P solubilization, *Azospirillum* showed efficiency and recorded maximum value for IAA synthesis and phosphobacteria (*Bacillus megatherium*) recorded significant value in siderophore production. Based on the present findings while inoculating these organisms to the crop plants, it is better to inoculate all three PGPB in the form of consortium for better results and further efficiency of these organisms must be exploited on crop plants in environmental conditions.

INTRODUCTION

The rhizosphere concept was originally developed by Lorenz Hiltner in the year 1904; so many researchers extensively studied and reported that the soil environment attached to the root region as a hot spot of variety of microbial abundance and activities due to the presence of rich amount of nutrients in the form root exudates and rhizodeposits. Plant growth-promoting rhizobacteria (PGPR) presently known as PGPB can stimulate plant growth, yield, reduces pathogen infection, as well as reduce biotic or abiotic stresses and these Plant beneficial microorganisms are in the interest of application in agriculture either as biofertilizers or as biopesticides as well as used in phytoremediation. The rhizosphere is the area in and around root zone and its adhered soil particles known to attract majority of PGPB bacterial organisms through root exudates and rhizodeposits and fraction of humus which are rich in nutrients, vitamins and minerals. Rhizosphere and rhizoplane colonization has been described to be linked to root exudation and the carbon fixed by plant photosynthesis known to be partly translocated into the root zone and released as root exudates which are rich in various carbohydrates, amino acids, organic acids, as well as other compounds and these compounds provide a source of nutrients for root – associated bacterial organisms, root exudates normally released in to the rhizosphere and microorganisms were known to be chemo attracted and move towards root exudates, then PGPB organisms can colonize and multiply both in the rhizosphere and the rhizoplane.

PGPB- plant growth promoting bacteria are vital groups of bacterial organisms known for many beneficial characters *viz*: degradation of organic matter, nitrogen fixation, and phosphorus solubilization, production of growth promoting substances like IAA, auxin, gibberellins, cytokines and ethylene, production of siderophore, solubilization of zinc, iron and protecting the rhizosphere area by eliciting root metabolic activities and through the suppression and also eliminating phytopathogens. The well known PGPB include bacterial organisms belonging to the genera *Azospirillum, Azotobacterr, Gluconacetobacter diazotrophicus, Pseudomonas, Bacillus, Phosphobacteria* etc. They are able to exert positive influence on plants by various mechanisms. PGPB directly affect the metabolism of the plant by providing substances that are usually in short supply. Plant growth – promoting rhizobacteria (PGPB) can stimulate plant growth, increase yield and reduce the occurrence, disease incidence of pathogenic microorganisms and also influence to overcome biotic or abiotic stresses, without conferring pathogenicity.

Review

Sugarcane rhizosphere is known to harbor variety of microorganisms in which PGPB organisms also a group of bacterial microorganisms. These PGPB as well as other

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microbes in the rhizosphere are attracted by rich nutrient fluid namely root exudates, once the PGPB organisms invaded in to the rhizosphere then utilizes the rich nutrient substances as food material and leads to continuous multiplication in the rhizosphere and some organisms enters into the internal root region and other parts of the plants namely endophytes. Recent days endophytes also known to influence Plant growth and yield.

Biological nitrogen fixation in tropical regions has been well documented, however, very little is known about the organisms involved in the process. The first observations indicating interactions or associations of diazotrophs with non leguminous plants were those of *Beijerinckia* sp in the rhizosphere of sugarcane and the specific association of *Azotobacter paspali* with *Paspalum notatum*. More recently several *Azospirillum* species have been found free living in the soil or associated with many grasses and cereals (Dobereiner *et al.,* 1993). They may increase plant yield by enhancing root growth, root hair formation, nitrate reduction and or N_2 fixation. The occurrence of diazotrophic bacteria in association with sugarcane has been reported earlier by Patriquin *et al.* (1980), Ruschel and Ruschel (1981) and Cavalcante and Dobereiner (1988).

The worldwide occurrence of endophytic *Gluconacetobacter diazotrophicus* in sugarcane plants suggests a beneficial association between a diazotroph and a grass species that proved *G. diazotrophicus* to be a superior PGPB organism known better N fixation, P solubilization production of growth promoting substances, Zinc solubilization and biocontrol in sugarcane. In many previous studies of grass associated diazotrophic bacteria, the observed plant growth promotion has occurred via transfer of fixed N from the diazotroph to sugarcane (Sevilla *et al.,* 2000; Stella and Prabudoss 2010). Sathyan and Thangaraju (2003) reported that the nitrogen fixing efficiency of *G. diazotrophicus* from sweet potato crop. The relative N fixing efficiency of *G. diazotrophicus* was studied sugarcane Pazhaniraja and Prabudoss 2014.

Production of plant growth stimulating Auxin, Indole acetic acid (IAA), is now regarded to be the major factor responsible for the ability of *Azospirillum brasilense* to promote plant growth. Wheat root proliferation and nutrient uptake was enhanced by inoculation with wild type *A. braislense* but not by mutants, deficient in IAA biosynthesis (Barbieri *et al.,* 1991; Barbieri and Galli, 1994). *Azoarcus* sp strain BH 71 (Reinhold-Hurek *et al.,* 1993) originally isolated from surface sterilized roots of kallar grass in Pakistan could colonize the interior portion of rice after inoculation of plants (Hurek *et al.,* 1994). Chincholker *et al* 2000 reported that hydraxamate type of siderophore is produced by both antagonistic fungi and bacteria. These iron chelating agents causes growth inhibition, decrease in nucleic acid synthesis, sporulation, etc.

MATERIALS AND METHODS

Screening of isolates for nitrogen fixation

The nitrogen fixing capacity of the test organisms was evaluated by using Acetylene Reduction Activity (ARA) following the standard procedure (Bergensen, 1980). Twenty five ml of LGI and Nfb medium were prepared in 100 ml vials. The vials were inoculated with 25 µl of *G. diazotrophicus* and *Azospirillum* isolates and incubated under static condition in an incubator 28 ± 1°C. After 5 days of growth the cotton plugs were replaced by suba-seal septa and tightened with aluminium cap. The air in the vial was replaced with nitrogen gas. Ten per cent (v/v) of the inert gas was removed and ten per cent pure acetylene gas was injected. The vials were incubated for 24 h at room temperature. After incubation, 1 ml of gas sample was withdrawn and injected into the gas chromatograph (Systronics 4010, India) fitted with porapak Q column (6″ × 1/8″) and FID detector. The column temperature was maintained at 80°C. Nitrogen gas was used as carrier gas at the flow rate of 20ml min^{-1}.

The acetylene reduction activity of the strains was calculated using the formula:

Sample peak length of ethylene (mm) × Attenuation × Volume of gas phase of flask × 0.0006
Incubation time (h) × Volume of gas simple injected into gas chromatograph (ml)

The acetylene reduction activity of the sample was expressed as n moles of ethylene formed mg of protein h^{-1}. At the end of experimental period the cell protein content of the cultures were determined following the method described by Lowry *et al.* (1951).

Estimation of phosphate solubilizing capacity of isolates
Preparation of reagent

Reagent I : Chlorostannous acid
2.5g stannous chloride (S_nCl_2. $2H_2O$) was dissolved in 10 ml of warm concentrated HCl, volume was made upto 100 ml with distilled water

Reagent II : Chloromolybdic acid
15.0g of Ammonium molybdate was dissolved in 400ml warm distilled water and added with 342 ml of 12N HCl and cooled and volume was made upto one litre with distilled water.

Quantitative test

The *G. diazotrophicus, Azospirillum and Phosphobacteria* isolates were inoculated in Pikovskaya's broth with known amount ($5.0gl^{-1}$) of insoluble phosphorus i.e. Tricalcium phosphate and incubated at 30°C on shaker for 7 days. Then the culture broth was centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to a test tube without disturbing the residues settled at the bottom of the centrifuge tube. A quantity of 0.1 ml of the aliquot was taken in a 50 ml volumetric flask and added with 10 ml of reagent I. After 0.25 ml of reagent II was added and immediately the volume was made up to 50 ml with distilled water. The blue colour developed was measured at 600 nm in spectrophotometer. The corresponding amount of soluble phosphorus was calculated using the standard curve of potassium dihydrogen phosphate

and expressed in µg PO_4 / 0.5 mg insoluble P/mg sucrose utilized.

Calorimetric estimation of IAA

One ml of *G. diazotrophicus, Azospirillum and Phosphobacteria* culture at exponential stage was inoculated in acetic LGI broth (with and without precursor tryptophan) and incubated at room temperature. All the flasks were wrapped with black paper to avoid photo-inactivation of the biologically active compounds. After one week the culture filtrate was adjusted to pH 2.8 with 1N HCl. Then it was extracted with equal volume of peroxide free cold (4°C) diethyl ether in a separating funnel covered with black paper, by keeping for 4 h at 4°C with intermittent shaking. Discarding the aqueous phase, the extraction was repeated for 2-3 times. The organic phases were pooled and evaporated to dryness in the dark. After dissolving the residue in 2.0 ml of methanol, a quantity of 0.5 ml was added to 1.5 ml of distilled water and 4.0 ml of Salpher's reagent (1.0 ml of 0.5N ferric chloride in 50.0ml of 35 per cent perchloric acid) and incubated in darkness for 1 hour at 28°C. The intensity of pink colour developed was read in Spectrophotometer at 535 nm. The quality of 1AA was expressed in µg/ml by referring to a standard graph of 1AA.

Siderophore production by *G. diazotrophicus, Azospirillum and* Phosphobacteria *and* estimation of catecholate siderophore production (Modi *et al*., 1985)

The isolates of *Gluconacetobacter diazotrophicus, Azospirillum and Phosphobacteria* were grown in the 50 ml of LGI liquid medium and Nfb medium incubated in an orbital shaking incubator (180 rpm) for 24 hours at 280c. After incubation, each culture was centrifuged at 10,000 rpm for 20 min and the cell free culture supernatant was used for siderophore estimation. Salicylate acid (siderophore) production by the isolates was estimated on ethyl acetate extracts of culture supernatant using a modification of the ferric-chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts were prepared by extracting 20 ml of supernatant twice an equal volume of solvent at pH2. For the assay one volume of the reagent was added to one volume of the sample and the absorbance was determined at 560 nm for salicylates with sodium salicylic as standard (1.0 mol of sodium salicylate gave an absorbance of 0.05).

Estimation of hydroxamate siderophore production (Schwyn and Neil ands, 1987)

The cell free culture supernatant were prepared with the help of respective cultures and used for hydroxamate siderophore estimation. To 0.5 ml of culture supernatant 0.5 ml of 6m H_2SO_4 was added and the mixture was auto calved in a glass tube containing 1 ml of sulphanilic acid (1% m/v) in 30% acetic acid (v/v) and 0.5 ml of 1.3% iodine in 30% acetic acid (v/v) were added. The excess of iodine was destroyed by the addition of 1 ml of 2% (w/v) Na_3AsO_4 solution. A solution of x-naphthylamine (0.3% in 30% acetic acid 1 ml) was then added and the total volume made upto 10 ml distilled water. After 30 min, the absorbance at 526 nm was measured in hydroxylamine HCl was used as a standard and 1 micromole of compound gave an absorbance of 0.1.

RESULTS AND DISCUSSION

In the present research about13 isolates were isolated from the rhizosphere soil samples of sugarcane. All the isolates were purified, characterized and identified as *Gluconacetobacter diazotrophicus, Azospirillum brasilence,* and Phosphobacteria (*Bacillus megatherium*) and are finally examined for PGPB nature. The isolates of the above bacterial organism except Phosphobacteria showed potentiality on nitrogen fixation, among the isolates the best diazotrophs namely *Gluconacetobacter diazotrophicus* and *Azospirillum* recorded on par values on nitrogen fixation. Phosphobacteria and *Gluconacetobacter diazotrophicus* showed phosphate solubilizing efficiency and among the isolates *Gluconacetobacter diazotrophicus* showed maximum efficiency on phosphate solubilization and it was followed by *Bacillus megatherium*. Other isolates were not recorded much significant on nitrogen fixing efficiency and phosphate solubilization. In the present research interestingly all the isolates produced IAA from the precursor tryptophan in the case of other growth promoting substances production only *Azospirillum* produced significance amount compared with other isolates.

Screening of isolates for nitrogen fixation and phosphate solubilizing capacity

All the thirteen isolates recorded nitrogenase activity. The maximum of 376.00 n moles of C_2H_4/hr/mg cell protein was showed by *G. diazotrophicus* PGPBG3 followed by *G. diazotrophicus* PGPBG1 (370.00 n moles C_2H_4/hr/mg cell protein). Nitrogenase activity all the isolates recorded appreciable values on phosphate solubilizing capacity and showed values not less than 0.60µg PO_4/0.5 mg insoluble P/mg sucrose utilized. The *G. diazotrophicus* isolate PGPBG3 showed maximum phosphate solubilizing capacity (0.75 µg PO_4/0.5 mg insoluble P/mg sucrose utilized) among the thirteen isolates. This was followed by *G. diazotrophicus* PGPBG1, PGPBG2 and PGPBP3, are recorded on par values and recorded (0.70 µg PO_4/0.5 mg insoluble P/mg sucrose utilized).

Screening of isolates for Nitrogen fixing efficiency of sugarcane rhizosphere soil

S.No.	Name of the isolates	Nitrogenase activity (n moles C_2H_4/ h / mg of cell protein) (n moles C_2H_4/hr/mg cell protein)
1	PGPBG1	370.00
2	PGPBG2	366.00
3	PGPBG3	376.00
4	PGPBA1	360.00
5	PGPBA2	358.00
6	PGPBA3	363.00
7	PGPBA4	355.00
8	PGPBA5	336.17
9	PGPBP1	-
10		-
10	PGPBP2	-
11	PGPBP3	-
12	PGPBP4	-
13	PGPBP5	-

Screening of isolates for Phosphate solubilizing efficiency of sugarcane rhizosphere soil

S.No.	Name of the isolates	Amount of PO_4 solubilized (µg/0.5mg insoluble P/mg of sucrose utilized)
1	PGPBG1	0.70
2	PGPBG2	0.68
3	PGPBG3	0.75
4	PGPBA1	-
5	PGPBA2	-
6	PGPBA3	-
7	PGPBA4	-
8	PGPBA5	-
9	PGPBP1	0.64
10	PGPBP2	0.62
11	PGPBP3	0.67
12	PGPBP4	0.61
13	PGPBP5	0.55

The nitrogenase activity of PGPB bacterial organisms namely *G. diazotrophicus* and *Azospirillum brasilence* strains were first proved by Cavalcante and Dobereiner (1988) in a report indicating 240 n moles C_2H_4 h^{-1} mg cell $protein^{-1}$. In the present study, all the isolates showed appreciable amounts of nitrogenase activity ranging from 320.00 to 386.20 n moles $C_2H_4h^{-1}$ mg cell $protein^{-1}$. The variation may be due to collection of samples from different locations and from different soil types. The same was already reported by Caballero-Mellado and Martinez-Romera (1994); Oliveira *et al.* (2009). Interestingly phosphobacteria fails to show any amount of nitrogen fixation.

Reis *et al.* (1994) reported an increase in nitrogenase activity (350 and 420 n moles $C_2H_4h^{-1}$ mg cell $protein^{-1}$ respectively) of two strains *viz*., due to supplementation with cane juice. In the present study, the *G. diazotrophicus* PGPBG3 and PGPBG1 strains recorded above 380 n moles of C_2H_4 h^{-1} mg cell $protein^{-1}$ in acetic LGI semisolid medium without any supplementation and Azospirillum isolates recorded nitrogen fixation in the range of 336.17 to 358.22. In general, the nitrogenase activity was more in *Gluconacetobacter* strains when compared with *Azospirillum* strains, it indicates the superiority of the local isolates. Interestingly, all the isolates of *Gluconacetobacter diazotrophicus* and phosphobacteria recorded appreciable amount of phosphorous solubilization. The results of the experiment revealed one more additional benefit from *G. diazotrophicus* inoculation. The results were in accordance with the reports of Mowade and Bhattacharya (2000); Saravanan *et al*. (2007); Intore *et al.* (2009) who reported the P-solubilising ability of *G. diazotrophicus*.

Screening of different isolates for IAA of sugarcane rhizosphere soil

S.No.	Isolates	IAA (µg ml^{-1})
1	PGPBG1	2.14
2	PGPBG2	2.10
3	PGPBG3	2.16
4	PGPBA1	2.28
5	PGPBA2	2.23
6	PGPBA3	2.34
7	PGPBA4	2.21
8	PGPBA5	2.18
9	PGPBP1	1.95
10	PGPBP2	1.90
11	PGPBP3	2.00
12	PGPBP4	1.85
13	PGPBP5	0.83

Screening of PGPB isolates for IAA production

Soil samples were collected from the rhizosphere of sugarcane at random from the below mentioned places *viz*., Vilagam, Sivapuri, Vallampadugai, Erumpur and Killai, Cuddalore District, Tamilnadu. Strains were isolated from sugarcane rhizosphere soil collected from different locations were screened for their efficiency on IAA production. The quantity of IAA produced ranged from 0.83 to 2.34 µg ml^{-1} of the culture medium. The isolate PGPBA3 was found to be the most efficient on IAA production.

Plant growth and development in sugarcane was mainly taking place with the help of plant growth promoting hormones, because auxins, cytokinins and gibberellins commonly play a role in bacterial-plant interactions. All the strains of *G. diazotrophicus, Azospirillum brasilence* and *Bacillus megatherium* were shown to produce high amounts of IAA in culture medium. In the present study, all the isolates recorded good amount of IAA in calorimetric estimation. Suman *et al.* (2001) reported that three folds increase in IAA production by the addition of tryptophan in medium. The results of present study were in accordance with the findings of Intorne *et al.* (2009). Indeed, recent studies against that any beneficial effects it may have on plant growth are more likely to be via mechanisms other than N_2 fixation, such as production of Indole acetic acid (IAA) (Fuentes – Ramirez *et al.,* 1993; Sevilla *et al.,* 1998; Bastian *et al.,* 2000; James *et al.,* 2001; Madhaiyan *et al.,* 2005).

Screening of PGPB isolates for siderophore production

Among the 13 isolates from PGPBP3 showed higher production of both catecholate (12.1 mg/litre) and hydroxamate type (15.5mg/litre) of siderophore, while the PGPBA5 isolates showed lower production of both catecholate (9.0 mg/litre) and hydroxamate (9.2 mg/litre) type. The isolates from different are varied in their siderophore production capacity. The isolates from bud were better than root and rhizosphere isolates, where as leaf and stem isolates were comparatively poor in siderophore production.

Screening of different isolates for Siderophore production

S.No.	Isolates	Siderophore production (mg/litre)	
		Catecholate Type	Hydroxamate Type
1	PGPBG1	12.0	15.0
2	PGPBG2	11.9	14.9
3	PGPBG3	12.1	15.5
4	PGPBA1	9.6	10.5
5	PGPBA2	9.4	10.3
6	PGPBA3	9.7	10.7
7	PGPBA4	9.2	9.7
8	PGPBA5	9.0	9.2
9	PGPBP1	11.0	13.7
10	PGPBP2	10.3	13.1
11	PGPBP3	11.5	14.0
12	PGPBP4	10.0	12.6
13	PGPBP5	9.9	10.9

Siderophores is an iron chelating agent and many beneficial bacterial organisms known for the production of siderophore like *Pseudomonas*, *Bacillus, Azotobacter* etc. these kinds of iron chelating agents creating competition for iron between

microorganisms. Siderophore production under iron stress conditions confers upon these antagonistic organisms as an added advantage, resulting in exclusion of pathogens due to iron starvation. The fungal siderophores have lower affinity (O'Sullivan and O'Gara, 1992). In the present investigation all the isolates able to produce siderophore in varying amount whereas *Bacillus megatherium* produced and recorded maximum values in both types of siderophore production.

Future work

Further the efficiency of these PGPB organisms should be exploited under field condition as a effective bio inoculants to crop plants.

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