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# Screening of free-living bacteria from the rhizosphere of Jute for their multiple plant growth promoting and antagonistic activity against phytopathogens

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## Abstract

Present investigation was undertaken to screen the PGPR isolates from the rhizosphere of Jute for their plant growth promoting and antagonistic activities in the view of an alternative way to chemical fertilizer and hazardous fungicides. A total 76 isolates were isolated from different parts of northern West Bengal and screened for their antagonistic activity against *Macrophomiona phaseolina*. Most promising five isolates were selected for further study and screened for other plant growth promoting and lytic enzyme producing abilities. Out of these, two isolates were Gram positive and rest three Gram negative. All five isolates exhibited several plant growth promoting activities. All five isolates showed IAA and ammonia production whereas four out five showed phosphate solubilization activity. Three PGPR strains exhibited siderophore production and only one isolate showed cyanide production ability. Among the lytic enzymes, chitinase was produced by three isolates. Among them B-3 showed highest degree of chitinase production. Protease was also produced by four strains but amylase and β-1,4-glucanase activity showed by only one isolate, Acti-6. Two isolates B-3 and Acti-6 showed considerable amount of antagonistic activity against three phytopathogens *Macrophomina phaseolina, Fusarium oxysporum,* and *F. semitectum* suggesting that Acti-6 and B-3 showed several attributes to be the potent strains of PGPR and can be used as biofertilizer as well as biocontrol agents.

**Keywords:** PGPR, Jute, Antagonistic activity, Phytopathogen

## Introduction

Improper use of chemical fertilizers and pesticides in search of high crop yield and quality is present practice in agriculture after the green revolution. But this approach is costly and imposes threat to the environment and human health (Xu *et al.* 2014). For the last few years scientists are searching an alternative and sustainable way to overcome this problem. Application of plant growth promoting bacteria may be the way. Plant growth promoting rhizobacteria (PGPR) are soil bacteria those lives in vicinity of root system can produce beneficial effect on plant health. The PGPR can promote the plant growth either directly or indirectly but the exact mechanisms by which they can act beneficially on plant growth have not been fully elucidated. The direct mechanisms of plant growth promotion involve the synthesis of substances by the bacterium which facilitates the uptake of nutrients from the environment (Glick *et al.*, 1999). The direct growth promoting activities are as follows i) nitrogen fixation ii) solubilization of phosphorus iii) production of phytohormones such as auxins, cytokinins, gibberellins and iv) lowering of ethylene concentration (Kloepper *et al.*, 1989; Glick, 1995; Glick *et al.*, 1999). The indirect mechanism of plant growth promotion by PGPR include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal compound and fungal cell wall lysing enzymes such as cellulase, protease, antibiotics and cyanide iv) competition for sites on roots and induced systemic resistance (Kumar *et al.* 2012, Kavamura *et al.*, 2013, Xu *et al.* 2014).In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Alcaligens, Enterobacter, Arthobacter, Bacillus, Burkholderia,* and *Serratia* have been reported to enhance plant growth (Kloepper *et al.*, 1989; Glick, 1995).

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Jute (*Corchorus olitorius* L. and *C. capsularis* L.) is one of the important commercial fiber crop of India and Bangladesh. In India jute mostly grown in its eastern part with area of 0.91 million hectares and production 11.82 million bales (one bale = 180 kgs.). Recently, jute has attracted the concentration due to its eco-friendliness. Jute crop suffers from several fungal, bacterial and viral diseases but the most devastating one is the stem rot of jute, caused by *Macrophomina phaseolina* (Tassi) Goid, affecting both quality and yield (De, 2014). Hence, the present study was intended to isolate and characterize PGPR strains from the rhizosphere of Jute having PGP and antagonistic traits so that they can be exploited as potential bioinoculants.

## Materials and Methods

*Collection of soil samples from Jute rhizosphere*

The present investigation was under taken with an objective to select promising native PGPR strains which can promote the growth as well as can induce resistance against *Macrophomina phaseolina*, the most devastating pathogen of Jute (*Chorchorus sp.*). For this purpose, soil samples were collected from different agricultural fields of the northern part of the West Bengal, India. The samples were placed in plastic bags and stored at 4°C in the Laboratory, Department of Botany, University of North Bengal for further study.

*Isolation and characterization of bacteria*

Bacteria were isolated from the rhizosphere of healthy jute plants (*C. olitorius*). Ten grams of soil particles loosely adhering to the roots were collected. The soil suspension was prepared by dissolving the soil sample in 100 ml of sterile distilled water using magnetic stirrer for 1 h. Then the upper light brown colored layer was pippeted out and serial dilutions were made. Appropriate dilutions were spread over the nutrient agar medium and incubated at 37° C for 24-48 hrs. Colonies with different morphological appearances were selected from the countable plates and sub cultured in nutrient agar slant for their further use. For the long term use bacterial strains were maintained in 50% glycerol at -20° C. A total 76 bacterial isolates were isolated and used in present study.

The selected bacterial isolates were examined for their morphological features in terms of colour, shape, size, surface and gram staining etc.

*Screening for multiple plant growth promoting activities*

All 76 rhizospheric isolates were first screened for their antagonistic activity against *M. phaseolina* and promising isolates were further analysed for their other plant growth promoting activities.

Phosphate solubilizing activity

The phosphate solubilising test was done in the solid medium. Pikovskaya's medium was used for screening of phosphate solubilization (Pikovskaya, 1948). Agar plates were prepared and the bacterial strains were individually spot inoculated at the center of the plates followed by incubation for 5 - 6 days. The plates were observed for clear zone around the colony and the diameter of the clearing zone was recorded.

IAA production

IAA production by bacterial isolates was determined following the methods of Gordon and Weber (1951). Luria Bertani broth medium amended with 0.1 mM tryptophan was inoculated with the isolated bacteria. They were incubated for 24 h at 30°C on rotary shaker and the cultures were centrifuged at10,000 g for 15 min. Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.* (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M $H_2SO_4$ was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature.

Siderophore Production

The selected isolates were characterized for siderophore production following standard method (Schwyn and Neiland, 1987) using blue

indicator dye, chrome azurol S (CAS). For preparing CAS agar, 1 L, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). With constant stirring this solution was added to 72.9 mg hexa-decytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petri plates. The plates were inoculated with the bacteria and incubated at 30°C for 10-15 days till any change in the color of the medium was observed.

HCN production

Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on Luria Bertani agar medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed air-tight with parafilm and incubated at 30°C for 48 h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as an indication of HCN production (Bakker and Schippers, 1987).

Production of Ammonia

The ability of bacterial isolates for the production of ammonia were tested according to Cappuccino *et al.* (1992). For the production of ammonia bacterial isolates were grown in 10 ml peptone water in each tube separately and incubated for 48-72 h at 30 ± 2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production.

*Extra cellular enzyme activities*

Catalase activity

Catalase activity of selected isolates were performed by adding 3-4 drops of hydrogen peroxide ($H_2O_2$) to 48 h old bacterial colonies. The appearance of effervescence indicated catalase activity (Schaad NW, 1992).

Chitinase production

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 10 g colloidal chitin with 20 g of agar in M9 medium ($Na_2HPO_4$ 0.65 g, $KH_2PO_4$ 1.50 g, NaCl 0.25 g, $NH_4Cl$ 0.50 g, $MgSO_4$ 0.12 g, $CaCl_2$ 0.005 g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 28°C for 7-10 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production (Robert and Selitrennikoff; 1988).

Protease activity

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. digest of casein 5.0, Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30°C and plates were examined for development of clear zones around colonies (Walsh *et al.* 1995).

Amylase activity

The bacterial isolates were spot inoculated on starch agar (Beef extract 3.0, Peptone5.0, soluble starch 2.0, Agar 15.0, Distilled water 1 lit.) medium plates and incubated at 30°C for 48 h. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Production of colourless zone surrounding colonies was considered positive for the production of amylase (Shaw *et al.*1995).

β-1,4-glucanase activity

Isolates were inoculated by spotting on the plates having cellulose powder as a sole source of carbon and incubated at 30 ± 2° C for 3-5 days. These plates were examined

for development of clear zones around colonies (Rangel-Castro *et al.*2002).

*Antagonistic activities against phyto-pathogens*

All five isolates were tested for their antifungal activities against *Macrophomina phaseolina*, *Fusarium oxysporum*, and *F. semitectum* on PDA plates. Isolates were inoculated on the surface of agar plate 2 cm away from fungal disc. Antagonist activity was observed after incubation at 28 ± $1^0$ C up to 7 days.

## Results

*Isolation and characterization of bacteria*

A total of 76 bacteria were isolated from various jute growing fields of the northern part of West Bengal. These isolates were evaluated for their antagonistic and plant growth-promoting traits. Out of 76 the best five potential bacterial strains showing antagonistic and PGP activities were selected for characterization.

The morphological characteristics of Acti-2, Acti-3, Acti-6, B-3 and PKV+ varied widely. Acti-2, Acti-3 and PKV +produced smooth, shiny, convex colony with entire margin whereas Acti-6 and B-3 produced waxy, non-elevated colony with undulated margin (Table 1).

*Screening for multiple plant growth promoting activities*

*Phosphate* solubilizing activity

All the isolates except B-3 showed ability for phosphate solubilization on Pikovskaya medium with different efficacy. Out 4 strains Acti-3 showed maximum degree of phosphate solubilization. The phosphate solubilizing activity of the isolates indicates that they are able to secrete the organic acids that chelate the cations, converting insoluble phosphate into soluble form and thus available for plants.

IAA production

All the five strains showed development of brown to pink colour indicating their capability of IAA production. IAA is the most physiologically active form of auxin. Recent investigations on auxin synthesizing rhizobacteria demonstrated that the rhizobacteria can synthesize IAA from tryptophan by different pathways, although the general mechanism of auxin synthesis was basically concentrated on the tryptophan-independent pathways (Spaepen *et al.* 2007). Among the jute rhizospheric isolates PKV+ showed highest IAA production followed by Acti-3, Acti-2 and Acti-6.

Siderophore Production

Siderophore production was determined blue CAS agar medium. Formation of yellow to orange zone around the colonies of isolates Acti-6, Acti-2 and B-3 was observed, indicating their capacity to chelate iron from the surrounding

Table.1 Morphological and Microscopic characters of PGPR isolates

| Isolate Code | Shape | Gram stain | Colour | Surface | Margin | Pigmentation |
|---|---|---|---|---|---|---|
| Acti-2 | Bacilli | Negative | White | Smooth, Shiney | Entire | Non-pigmented |
| Acti-3 | Bacilli | Negative | White | Smooth, Shiney | Entire | Non-pigmented |
| Acti-6 | Bacilli | Positive | Off white | Rough, waxy appearance | Irregular | Non-pigmented |
| PKV+ | Bacilli | Negative | White | Smooth, Shiney | Entire | Non-pigmented |
| B-3 | Bacilli | Positive | White | Rough, waxy appearance | Irregular | Non-pigmented |

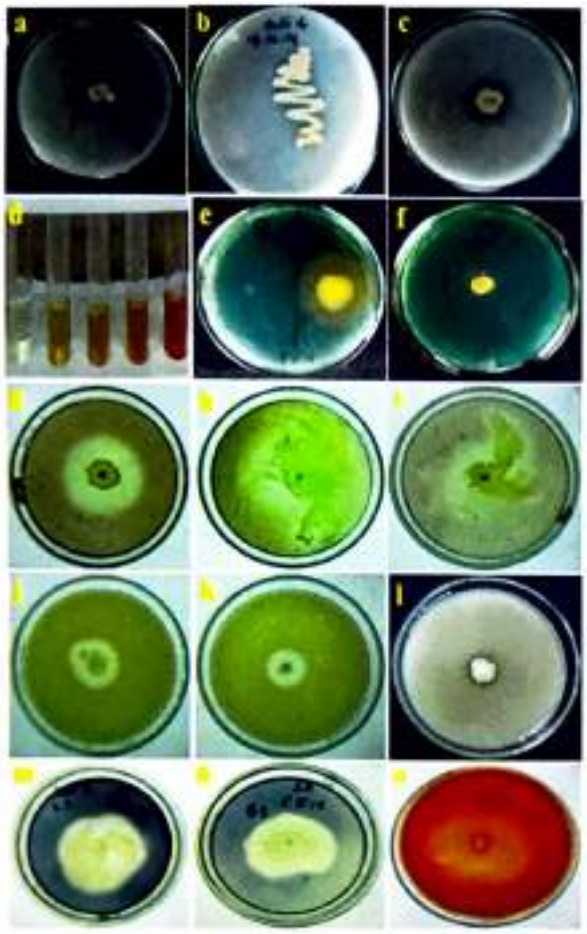

Fig. 1 Phosphate solubilization-Acti-3 (a), Acti-6 (b), PKV+ (c); IAA production-control, B-3, Acti-6, Acti-3, Acti-2 & PKV+ (d); siderophore production-Acti-2 (e) & Acti-6 (f); Chitinase production-B-3(g), Acti-6 (h) & Acti-2 (i); Protease production-Acti-6 (j), B-3 (K) & Acti-3 (l); Amylase production-Acti-6 (m) & B-(n); β- 1, 4-glucanase-Acti-6.

medium and thereby depriving the pathogens.

HCN production

Ability for hydrogen cyanide synthesis was observed for selected five isolates. Among the isolates, only Acti-6 showed the HCN production ability.

Production of Ammonia

The production of ammonia observed in all the five isolates. Ammonia production by the plant growth promoting bacteria helps to influence plant growth indirectly.

*Extra cellular enzyme activities*

Chitinase production

The spot inoculated CDA plates were incubated at 28°C for 7-10 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production. The plates of Acti-6, Acti-2, B-3 showed halo zone around the colonies indicating their capability of secretion extracellular chitinase. It was observed that no extracellular chitinase was secreted by Acti-3 and PKV+ even when grown on chitin amended media.

Protease activity

Proteolytic enzyme production was detected as formation of a clear zone around the colony on skim milk agar. All the isolates showed variable degree of protease activity except PKV+. Among the isolates Acti-6 showed high production of extracellular protease.

Amylase activity

Amylase activity was determined by spot inoculation of isolates on starch agar plates. After 72 to 96 hrs. of incubation the plates were flooded with Iodine solution for1min and the appearance of clear zone surrounding the colony indicates positive for starch hydrolysis test. Among the isolates Acti-6 and B-3 showed amylase activity

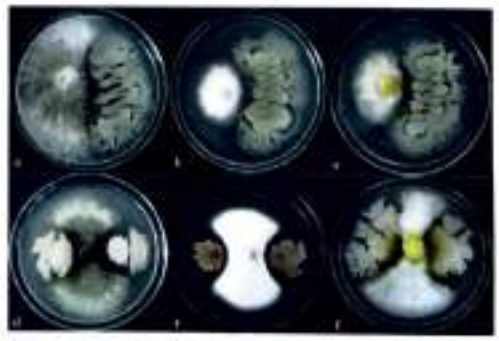

Fig. 2 Antagonistic activity of Acti-6 (a,b,c) and B-3 (d,e,f) against *M. phaseolina* (a), *F. oxysporium* (b) & *F. semitectum*.

Table.2 Plant growth promoting characteristics and lytic enzyme production traits of PGPR strains

| Isolate code | | | | | | Lytic Enzyme Production | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Phosphate solubilization | IAA Production | Siderophore Production | HCN Production | Ammonia Production | Chitinase | Protease | Amylase | β- 1, 4-glucanase activity |
| Acti-2 | ++ | ++ | + | - | + | +++ | + | - | - |
| Acti-3 | +++ | ++ | - | - | + | - | + | - | - |
| Acti-6 | + | + | + | + | + | ++ | +++ | + | + |
| PKV+ | +++ | +++ | - | - | + | - | - | - | - |
| B-3 | - | + | + | - | + | +++ | ++ | + | - |

β- 1, 4-glucanase activity

β- 1, 4-glucanase activity of the isolates were determined in minimal medium plates using cellulose as only carbon source. Only Acti-6 showed a halo zone around the colony after the addition of congo red indicating secretion of extracellular β- 1, 4-glucanase. Plant growth promoting traits and extracellular enzyme secretion by the isolates have been summarized in Table 2 and Figure 1.

*Antagonism against pathogens*

Antagonistic activity of the bacterial isolates were evaluated in terms of inhibition zone diameter as an indicator of the reduction in growth of 3 fungal pathogens. Among the isolates, Acti-6 and B-3 showed antagonistic activity against *M. phaseolina, Fusarium oxysporium* and *F. semitectum* considerably (Fig. 2).

## Discussion

Many types of microorganisms are known to inhabit soil and plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability (Geetha *et al.* 2014). Plant growth promoting rhizobacteria (PGPR) perform important functions in promoting plant growth and sustaining plant health. Direct plant growth promotion by microbes is based on improved nutrient acquisition by solubilizing insoluble phosphate and hormonal stimulation (Walia *et al.* 2014). Varied mechanisms are involved in the suppression of plant pathogens which are often indirectly connected with plant growth. Beneficial plant–microbe interactions have led to development of microbial inoculants for use in agricultural biotechnology (Berg 2009).

In the present study, a total of 76 isolates were isolated from the rhizospheric soil of jute (*C. olitorius*). All the isolates were screened for their antagonistic activity against *M. phaseolina,* most devastating pathogen of jute and other plants. Out of 76 five isolates (Acti-2, Acti-3, Acti-6, B-3 and PKV$^+$) showed varied degree of antagonism against *M. phaseolina* and the isolates were screened for their other *in vitro* plant growth promoting activities. All the isolates except B-3 showed ability for phosphate solubilization on Pikovskaya medium. Among the PGPR strains Acti-3 showed maximum degree of phosphate solubilization, which is due the production of organic acid, thus converting insoluble phosphate into soluble form and available for plants. The mechanism of plant growth promotion by PGPR includes the production of plant hormones. In present study isolates were screened for the production of IAA, which is most active form of auxin. All the five strain showed development of brown to pink colour indicating their capability of IAA production. Isolates Acti-6, Acti-2 and B-3 also showed the siderophore producing capacity. The production of siderophore like iron chelating

compound is an important criterion for plant growth promoting rhizobacteria. They chelate the iron from the surrounding medium and soil, rendering it unavailable to pathogens. Rhizobacteria can inhibit phytopathogens by the production of hydrogen cyanide (Bloemberg and Lugtenberg 2001). HCN is known to inhibit electron transport, and the energy supply to the cell is disrupted leading to the death of the organisms. It also inhibits the proper functioning of different enzymes such as of cytochrome oxidase (Gehring *et al.*1993). Acti-6 showed HCN production ability and thereby it can be considered as a potent antagonist against plant pathogens. Isolates were also screened for protease activity. Out of five isolates, 4 showed protease activity. Very high protease activity was exhibited by Acti-6. Siddiqui *et al.* (2005) reported the biocontrol activity of *Pseudomonas fluorescens* CHA0 by extracellular protease. Three PGPR isolates, Acti-6, Acti-2 and B-3 showed significant amount of chitinase production. Several workers reported biological control of plant pathogen by chitinase producing microorganisms (Raaijmakers *et al.*2006; Kamal *et al.* 2008). Acti-6 and B-3 showed antagonistic activity against *M. phaseolina, Fusarium oxysporium* and *F. semitectum* considerably.

The current study has been undertaken as a search of an alternative to chemical fertilizer and fungicide. A group of promising rhizobacterial isolates was screened through *in vitro* and their plant growth promoting properties. Based on our results we concluded that Acti-6 and B-3 showed several attributes to be the potent strains of PGPR and can be used as biofertilizer as well as biocontrol agents. Future research in this direction is required to harness their potential as bio-inoculants for sustainable agriculture.

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