

Original Research Article

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Isolation and Characterization of a Promising Plant Growth Promoting Rhizobacterium from *Trichosanthes dioica* Roxb. Rhizosphere

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ABSTRACT

A total number of 10 rhizospheric bacteria were isolated from root environment of some healthy plants of a drastic diseased field with massive blighted symptoms of *Trichosanthes dioica* of Akilpur of Hooghly district, West Bengal (22.94° E 88.12° N) where soil is alluvial in nature. Among the isolates, one strain TR9 showed remarkable plant growth promoting activity. Cell free culture filtrate of TR9 produced clear zones of inhibition (diameter ranging from 1.67cm to 2.67cm) against different plant pathogenic fungi viz. *Colletotrichum acutatum*, *Alternaria alternata* and *Helminthosporium compactum*. It is quite capable of lowering the pH of its environment and also can solubilise insoluble phosphate sources. Siderophore production by this strain gives a positive result. Biochemical characterization indicated the ability of the strain to utilize a broad range of carbon sources, catalase positivity and also to reduce the nitrate and nitrite. It is highly sensitive to 4-quinolone derivative antibiotics viz. ciprofloxacin, norfloxacin and resistant to beta lactams like ampicillin, cephalothin, cephalixin. Light compound microscopy showed it to be a Gram negative rod shaped one which was re established by scanning electron microscopy. All these characteristic features indicate the strain to be a species of *Burkholderia*.

Keywords

PGPR, Anti fungal, Scanning electron microscopy, *Burkholderia*.

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Introduction

Plants-microbes relationship is a common but important phenomenon. Diverse microorganisms, varying from pathogens to beneficial, continuously interact with higher plants in soil ecosystem and influence the development of plant growth in the soil (Ahmad *et al.*, 2008; Taghavi *et al.*, 2009). In the rhizosphere of crop plants it is crucial in contributing protection as well as supplementation of nutrients to the crop plants by their microbial associates. Among the different plant growth promoting characters, ability to solubilize insoluble

phosphatic materials in the soil, production of siderophore for iron chelation, ability to kill pathogens to crop plants makes a rhizospheric bacterial strain more potent PGPR. Efficient insoluble phosphate solubilizing microorganisms have been isolated from various rhizosphere with PGP activity (Ghosh *et al.*, 2016; Rodriguez and Fraga, 1999). Exploration by characterizing all those PGP activities may lead to find a promising PGPR strain for field application. As phytopathogens reduce crop productivity at various extents, control of such pathogens become

necessary. Proper and tricky use of these rhizospheric bacteria can be a sharp wing of biological control. Many rhizobacteria are proven to be useful in inhibiting plant pathogens efficiently (Plant Pathology, Agrios, 2005; Ghosh *et al.*, 2015; Ghosh *et al.*, 2016). In our present endeavor we also wanted to isolate a potent PGPR strain from alluvial soil zone of lower Gangetic plain, where the soil is highly active because of elite agricultural practice and phosphate requirement in the soil is in highest demand.

Materials and Methods

Isolation of the organism

Rhizospheric soil of a very healthy *Trichosanthes dioica* plant from a heavily diseased field with massive blighted symptoms was collected from Akilpur of Hooghly district, West Bengal, India. The soil sample was quickly brought to laboratory. One gram of soil was mixed in 10 ml of sterile distilled water very well to make a suspension. Different dilutions of the soil suspension were spread on Pikovskaya agar medium containing in soluble tri calcium phosphate as sole phosphate source. Plates were incubated at $28\pm 2^{\circ}\text{C}$ for 72-96 hrs for the growth of the soil microbes. The composition of Pikovskaya agar medium was like (gm/L): dextrose 10; Ca_3PO_4 5; $(\text{NH}_4)_2\text{SO}_4$ 0.5; NaCl 0.2; MgSO_4 0.1; KCl 0.2; yeast extract 0.5; $\text{MnSO}_4, 7\text{H}_2\text{O}$ 0.002; $\text{FeSO}_4, 7\text{H}_2\text{O}$ 0.002; agar agar 20, H_2O 1000 ml; pH 7.0 ± 0.2 . Bacterial colonies, forming clear solubilizing appearance round it on insoluble phosphate containing Pikovskaya agar plate, were selected, purified and maintained for further studies.

Antifungal studies

Antifungal activity of the selected strain TR9 strain was evaluated against three plant pathogenic fungi *viz.* *Colletotrichum*

acutatum MTCC2074, *Alternaria alternata* VBAV007 and *Helminthosporium compactum* MTCC351 using cell free supernatant of TR9 by agar well diffusion (Fernández *et al.*, 1992) method and dual culture over lay method (Magnusson and Schnürer, 2001).

Agar well diffusion method: The rhizospheric strain TR9 was cultured in nutrient broth at $28\pm 2^{\circ}\text{C}$ and aliquots were collected at every 24hrs interval for five days. The aliquots were centrifuged to collect the supernatants, 50 μl of which was further used in the wells on malt agar plates seeded with three fungal spores. The plates were incubated for next 72-96hrs and observed.

Dual culture overlay method: The bacterial strain was streaked on sterile nutrient agar medium plates and were allowed to grow for 24 hrs at $28 \pm 2^{\circ}\text{C}$. Seven ml of malt extract soft agar (0.7% agar) medium containing 100 μl of fungal mycelia culture were overlaid on the plates. Plates were further incubated for next 72-96 hrs at the same incubation condition.

Phosphate solubilization

TR9 was screened upon creation of prominent clear solubilizing zone on Pikovskaya agar plate. The quantitative assay of phosphate solubilization was performed using Pikovskaya broth with insoluble tri calcium phosphate as sole phosphate source. TR9 was inoculated to 100 ml sterile Pikovskaya broth with 0.5% tri calcium phosphate (w/v) at $28\pm 2^{\circ}\text{C}$ with mild shaking condition (100rpm) for 120 hrs. After regular interval of 24hrs daily aliquots were collected. Uninoculated Pikovskaya broth was treated as control. The amount of soluble phosphate was measured by the method of Chen (Chen *et al.*, 1956) after centrifuging the culture filtrate at 10000 rpm for 10 minutes. The experiment was performed in three sets separately.

Measurement of pH

The pH of the cultures was measured at different days of incubation in Pikovskya broth containing insoluble phosphate with the help of a digital pH meter (Systronics Digital pH meter 802). The experiment was performed with a triplet set.

Siderophore production assay

The qualitative test for production of siderophore by TR9 was performed on CAS (Chrome Azurol S) agar plate (Ames-Gottfred *et al.*, 1989). Sterile CAS agar plates were prepared following protocol. The bacterial strain was streaked on the plates and incubated for 24-48 hrs at 28±2°C to check if there is any orange coloration.

Biochemical tests

Carbohydrate utilization test

Carbohydrate utilization test was performed with HiCarbo™ kit (KB009A /KB009B1 /KB009C). TR9 was inoculated in nutrient broth and incubated overnight and checked the OD at 620 nm if it was more than 0.5. Uninoculated nutrient broth was set as control one. Culture with OD ≥ 0.5 was taken and inoculated 50µl in each well of the kit. The kit (Part a, Part B and Part C) were incubated at 30±2°C for 18-24 hrs.

Catalase test

Catalase test was performed to take some amount of freshly cultured colonies on a clean dry glass slide with the help of a loop 1-2 drops of 3% H₂O₂ was added to it and checked if any bubble formation occurred.

Nitrate reduction test

Nitrate reduction test was done using ready prepared nitrate broth from HiMedia. The

bacteria was inoculated into this broth and observed after incubation of 32±2°C for 18-24hrs.

Antibiotic sensitivity assay

Antibiotic sensitivity assay was performed using Himedia Octodiscs. From a freshly cultured medium, 100 µl was taken and evenly spread over nutrient agar plates. Each of the seven octodiscs holding different antibiotics at different concentrations was placed on the plates. It was incubated at 28±2°C for 24hrs.

Scanning electron microscopic study

Morphology study was performed via scanning electron microscopy. The specimen was prepared as follows: overnight cultured cells were harvested by centrifugation at 10000 rpm for 10 minutes. Bacterial cell pellets were collected and washed twice saline and prefixed with a mixture of 3% glutaraldehyde and 5% DMSO in 0.05 M acetate buffer, pH 5.0, for 30 minutes. After centrifuging at 10000 rpm for 10 minutes cell pellets were harvested and washed twice with 0.1M sodium acetate buffer, pH 5. Cells were collected centrifuging at 10000 rpm for 10 minutes and applied over a thin slide, heat fixed and were dehydrated through a series of ethyl alcohol starting with 30% through 40, 50, 60, 70, 80, 90, 95% and finally with 100% with 10 minutes of dehydration in each grade (Mandal *et al.*, 2013). Slide was mounted on stab and gold coated (Coater IB-2, Gike Engineering, Japan) before observing in microscope (HITACHI S-530, Japan).

Results and Discussion

Isolation of a potent phosphate solubilizing bacteria

There is a vast possibility to have some plant growth supporting agent from a healthy plant

of a diseased field. Thus ten phosphate solubilizing bacteria were isolated from such a healthy *Trichosanthes dioica* rhizospheric soil. Among those isolates, one strain, TR9 was selected for further studies considering its remarkable phosphate solubilizing ability (Fig. 1).

Characterization of the isolated strain

The strain was characterized based on morphological and biochemical parameters. For morphological characterization Gram staining and SEM study was done. Gram staining revealed the strain to be Gram negative rod shaped which was supported by the SEM study (Fig. 2) also. The strain

responded positively to nitrate and catalase tests. TR9 can utilize dextrose, maltose, fructose, mannose and showed positive response for esculin hydrolysis, citrate utilization and malonate utilization (Table 1). The strain was resistant to beta lactum antibiotics like ampicilin, cephalothin, cephalixin but sensitive to tetracycline, kanamycin, streptomycin, chloramphenicol, norfloxacin, gentamycin, tobramycin, trimethoprim and others (Table 2). Based on the different biochemical and morphological characteristics and matching the data obtained with Bergeys’ Manual of Systematic Bacteriology (Vermis *et al.*, 2002) the strain was preliminary identified to be a member of the genus *Burkholderia*.

Table.1 Different carbohydrate utilization by TR9

Carbohydrates	Utilization by TR9	Carbohydrates	Utilization by TR9
Lactose	+	L-Arabinose	+
Xylose	+	Mannose	+
Maltose	+	Rhamnose	+
Fructose	+	Cellobiose	+
Galactose	+	Sorbitol	
Dextrose	+	Citrate utilization	+
Sucrose	+	Malonate utilization	+
ONPG	-	Esculin hydrolysis	+

‘+’ indicates utilized, ‘-’ not utilized

Figure.1 Phosphate solubilization by TR9

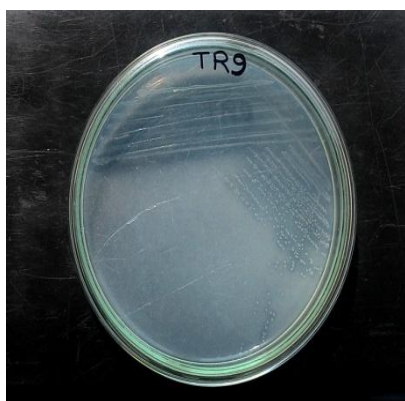


Table.2 Antibiotic sensitivity profile of TR9

Antimicrobial agent	Sensitivity	Antimicrobial agent	Sensitivity	Antimicrobial agent	Sensitivity
Tetracycline	Sensitive	Gentamycin	Sensitive	Penicillin-G	Resistant
Amikacin	Sensitive	Norfloxacin	Sensitive	Ampicilin	Resistant
Carbenicilin	Sensitive	Tobramycin	Sensitive	Cephalothin	Resistant
Ciprofloxacin	Sensitive	Clindamycin	Resistant	Colistin	Resistant
Co-Trimazine	Sensitive	Vancomycin	Sensitive	Sulphatriad	Sensitive
Kanamycin	Sensitive	Cloxacilin	Resistant	Ticarcilin	Sensitive
Nitrofurantoin	Resistant	Cefaloridine	Sensitive	Cefalexin	Resistant
Streptomycin	Resistant	Linkomycin	Resistant	Erythromycin	Resistant
Chloramphenicol	Sensitive	Methicilin	Resistant	Oxacilin	Sensitive
Sulphamethoxazole	Resistant	Oleadomycin	Sensitive	Amoxicilin	Resistant

Table.3 Anti fungal activity of TR9 by dual culture overlay method

Plant pathogenic fungal strains	Diameter of zones of inhibition (cm)				
	Day 1	Day 2	Day 3	Day 4	Day 5
<i>Colletotrichum acutatum</i> MTCC2074	1.27	1.38	1.7	1.96	2.67
<i>Alternaria alternata</i> VBAV007	1.58	1.6	1.62	1.66	1.67
<i>Helminthosporium compactum</i> MTCC351	1.9	1.97	1.98	2.0	2.47

Figure.2 Scanning electron micrograph of TR9

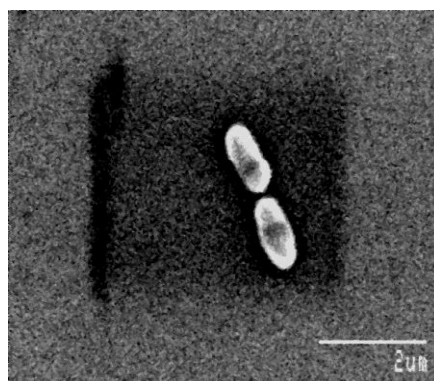


Figure.3 Antifungal activity of TR9 by dual culture overlay method against A) *Colletotrichum acutatum* MTCC2074 B) *Alternaria alternata* VBAV007



Figure.4 Phosphate solubilizing kinetics with pH drops by TR9

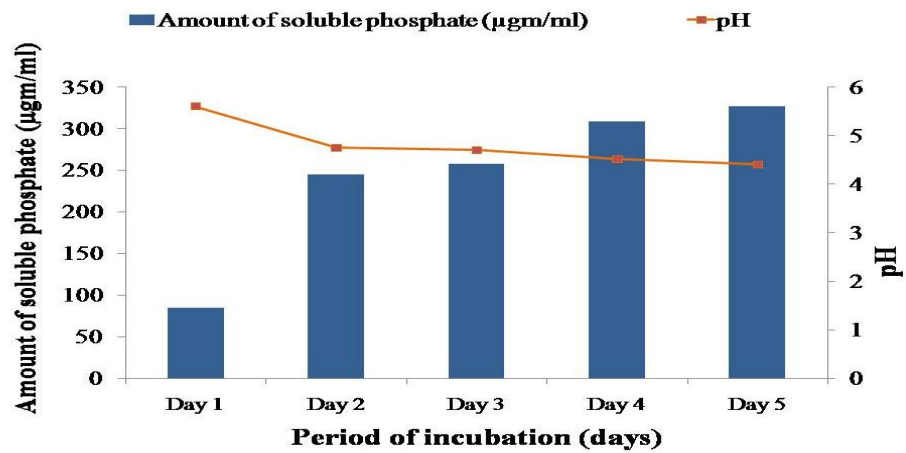
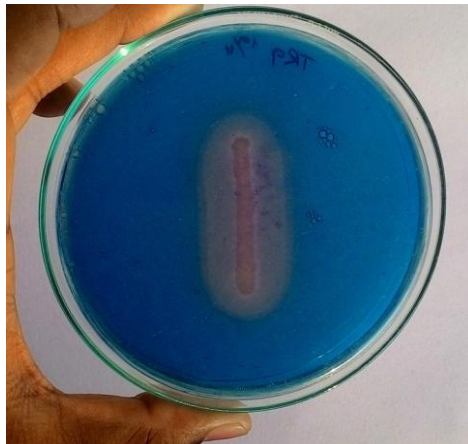


Figure.5 Siderophore production by TR9



Antifungal activity of the strain

TR9 showed a clear antagonistic activity against *Colletotrichum acutatum*, *Alternaria alternata* and *Helminthosporium compactum* when tested via dual culture overlay method (Fig. 3) against different plant pathogenic fungi. It produced a very clear zone of inhibition suppressing the fungal growth surrounding it. The results obtained are very promising in terms of biological control of phytopathogens. In agar well diffusion method increasing of diameter of zones of inhibition found with 5th day of culture filtrate.

Maximum inhibition was found in case of *Helminthosporium compactum* when it was treated with 5th day culture of TR9 where the diameter of zone of inhibition was 2.67 cm. On the other hand least inhibition by the strain was found for 5th day CFS against *Alternaria alternata* which was 1.66cm. Day wise antifungal data is tabulated in table 3. It is previously reported that *Burkholderia cenocepacia* can successfully control the leaf spot of *Aloe vera* caused by *Alternaria alternata* (Ghosh *et al.*, 2016) and also can reduce the incidence of fruit rot of jackfruit by *Rhizopus stolonifer* (Ghosh *et al.*, 2015). Not only from the view of PGP activities, TR9 can be helpful in disease control too.

Kinetics of phosphate solubilization and siderophore production

The strain is quite capable to solubilize insoluble phosphate of Pikovskaya broth containing insoluble tricalcium phosphate and produced 327 µg/ml soluble P at 5th day of incubation while pH of the culture drops to 4.41. A rapid increase of amount soluble phosphate was found in between 24 to 48 hrs which is 85 µg/ml to 245µg/ml and it is also reflected in lowering the pH from 5.6 to 4.75 (Fig. 4). Solubilization of phosphate may be

due to the production of organic acids like gluconic and 2-ketogluconic acids which chelates the metal cations from insoluble phosphates (Khan *et al.*, 2009). This supports the gradual decrease of pH. Formation of a clear bright orange coloration on blue CAS agar plate surrounding the growth of the bacteria indicates the ability of siderophore production by the strain (Fig. 5). As availability of ferric iron is crucial to proper functioning of the terminal oxidation pathway and ultimately the efficiency of the rhizobacteria, siderophore production by the selected strain TR9 thus makes it an efficient one.

In conclusion microbiology and agriculture are very tightly connected to each other. From the root tip to the top of shoot or from the view of pathogen to bio-control agent presence of microbes flash everywhere. Having some bacterial strain with different anti pathogenic property and plant growth promoting activity is a good sign from beneficial aspects. The strain TR9 which has been isolated from the *Trichosanthes dioica* rhizosphere has shown both nutrient mobilizing property as well as pathogen killing property which can be prospective bio-fertilizer inoculums in agricultural application after designing proper formulation.

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