INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 5, p. 405-412, 2017

RESEARCH PAPER OPEN ACCESS

Screening and characterization of Rhizobacteria antagonistic to *Pseudomonas syringae* causing bacterial canker of stone fruits in Punjab and KPK

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Key words: Rhizobacteria, *Pseudomonas syringae*, Stone fruits, Bacterial canker, Screening

http://dx.doi.org/10.12692/ijb/10.5.405-412 Article published on May 30, 2017

Abstract

Several bacteria colonize plant roots but few of them promote plant growth directly or indirectly are termed as plant growth-promoting rhizobacteria (PGPR). Current study was conducted to isolate rhizobacterial isolates antagonistic to *Pseudomonas syringae* causing bacterial canker of the stone fruits(apricot, peach and plum) growing in the areas of Punjab and Khyber Pakhtoonkhwa (KPK) provinces of Pakistan. A total of 80 (later pooled to get 16) soil samples were collected from the rhizosphere of apricot, peach and plum and 185 isolates were obtained. Out of 185 rhizobacterial isolates, 10 isolates showed greater potential (> 20 mm zone of inhibition) during *in vitro* antagonistic activity against *P. syringae*, and were selected for their morphological and biochemical characterization. Among antagonistic isolates, 6 responded negatively and 4 positively to Gram's straining. All the antagonistic isolates possessed one or more growth promoting character(s) such as siderophore production, phosphate solubilization, ammonia production and hydrogen cyanide (HCN) production. Six isolates were positive to fluorescence production. Enzymatic activity was exhibited by 8 isolates, during their catalase and lipase activity. Therefore present study suggested that isolates having antagonistic potential as well as plant growth promoting characteristics can be used as bio-protectant/bio-fertilizer to enhance plant growth in stone fruits.

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Introduction

Rhizosphere is a narrow zone of soil surrounding the root system (Walker *et al.,* 2003) where complex interactions take place and involves number of microbial communities existing at the same time. The diversity of the rhizospheric and nodule bacteria is very high (György *et al.,* 2010). Plant growth-promoting rhizobacteria (PGPR) promote plant growth directly by biological nitrogen fixation (Verma *et al.*, 2013), hormones production such as auxins (Cassan *et al.*, 2009), phosphate solubilization (Krey *et al.*, 2013), or indirectly by suppressing plant pathogens (Wang *et al.*, 2009). The suppression of pathogen can be observed through various mechanisms (e.g. competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins) and through inducing plant defense mechanisms (Weyens *et al.,* 2009). There are number of studies where rhizobacteria have shown potential to inhibit several important economic pathogens. According to Oliveira *et al.* (2003) antagonistic bacterial inoculation in sugarcane (*Saccharum* spp.) results in leading to increased biological nitrogen fixation, high biomass index and productivity. Soybean (*Glycine max* (L.) Merr.), with bacteria is competent against sporulation of pathogenic fungi (Assumpcao *et al.*, 2009).

Mohammadi *et al.* (2010) reported that *Pseudomonas syringae* is causing the major problem of bacterial canker of stone fruits in many parts of the world. It causes the severe economic losses by attacking all important commercially grown *Prunus* spp., *P. syringae* pv. *syringae* is causing a devastating disease decline in apricot, peach, plum as well as in cherries by reducing its production worldwide (Gilbert *et al.*, 2009). *P. agglomerans* has been reported as a biological control agent against several fungal and bacterial diseases, provides an effective control against P. syringae (Holt, 1994).

Though there is lot of work on antagonists in food crops but the information about rhizobacteria of stone fruits is limited and particularly in Pakistan, there is no data to this specific disease. The present study was aimed to search out for rhizobacterial isolates that could inhibit the bacterial canker pathogen (*P. syringae*) and their preliminary identification on biochemical basis was done. Further, the potential antagonists were also tested for possessing plant growth promoting traits.

Material and methods

A systematic survey of stone fruit growing areas of Punjab (Rawalpindi, Islamabad, Murree, Pind Noshehri and Attock) and Khyber Pakhtoonkhwa (KPK) (Abbottabad, Haripur and Mansehra) was carried out to collect the soil samples. Soil samples were collected by removing the top 3 cm of soil from the rhizosphere of stone fruits. A total of 80 samples were collected from 8 areas of Punjab and KPK and the samples were pooled to get 16 samples (5 samples from each area were pooled to give 1 sample). Soil samples were stored in polythene bags at 4°C (for maximum 1 month) for further studies.

Isolation and purification of rhizobacteria from soil samples

For isolation of rhizobacteria, 0.5g of rhizospheric soil was added in 4.5ml of sterile water and mixture was shaken by using vortex mixer. Serial ten-fold dilutions were prepared from the mixture and 0.1ml aliquot from dilutions (10^{-7}, 10^{-8} and 10^{-9}) was placed onto nutrient agar (NA) and King's B medium (KBM) plates enriched with 100µg/ml of cycloheximide. Plates were incubated for 48h at 28±2°C. Colonies with distinct morphological appearance were purified by dilution streaking and purified isolates were cultured on NA slants at 4°C for further use in experiments.

In Vitro Evaluation of Antagonistic Potential of Rhizobacterial isolates

A bacterial suspension (10^{8}cfu/ml) of the pure culture of *P. syringae* was added in NA after autoclaving and three paper discs were placed equidistantly on solidified NA plates. The rhizobacteria isolates were applied to the paper discs. The plates were incubated for 24-48h at 28°C.

Biochemical characterization of Rhizobacterial Isolates

Siderophore production

Siderophore production was observed by using Chrome Azurol S medium (CAS-medium) (Husen, 2003). Each isolate was streaked on the surface of CAS medium and incubated at room temperature for 3-5 days. Yellow orange halo zone formation around the colony was observed as a positive result of siderophore production.

Phosphate solubilization

Bacterial isolates were streaked on petri plates containing Pikovaskya's agar medium. Then the plates were kept in an incubator for 7 days at 28° C (Pikovaskya, 1948). After 7 days plates were examined for halo zone and data were recorded.

Hydrogen cyanide (HCN) Production

HCN production was assessed by streaking the rhizobacterial isolates on King's B agar medium supplemented with glycine. A sterile filter paper soaked in 0.5% (w/v) picric acid was kept in the underside of each Petri plate lid. Petri plates were then properly sealed with Para film and were incubated at 30°C for 48h. A color change of the filter paper from deep yellow to reddish-brown color was an indication of positive response to HCN production (Baker *et al.,* 1987).

Fluorescence production

Bacterial isolates were streaked on King's B agar followed by incubation at 28°C for 48h. After incubation the plates were examined under UV light for production of fluorescence.

Production of Ammonia

Fresh cultures of bacterial isolates were inoculated in 10 ml peptone water in different tubes separately and incubated for 48h at 28°C on incubated shaker. Nesseler's reagent (0.5ml) was added in each tube separately. Development of brown to yellow color was an indication of ammonia production (Cappucino *et.al,.* 1992).

Indole-3-acetic acid

Bacteria cultures were grown in Luria broth amended with L-tryptophan (1µg ml^{-1}) for 72h. After incubation, centrifugation at 10,000g for 10min was done and the supernatant was collected. One ml of culture filtrate was put to react with 2ml of Salkowski's reagent at 28°C for 30min. The appearance of pink color was an indication of the synthesis of IAA.

Evaluation of Enzymatic activity of Rhizobacterial Isolates

Catalase activity

Two drops of hydrogen peroxide were added to the bacterial culture and the development of bubbles was an indication of positive response of catalase activity.

Lipase activity

Bacterial cultures were grown on NA plates supplemented with egg yolk and were kept in incubation for 48h. After incubation, aqueous solution of copper sulphate ($CuSO_4$) was added to the NA medium and then keptfor 10-15min. The excess reagent was poured off. Appearance of greenish blue colour around the colony was considered as the confirmation of lipase activity (Omidvari, 2008).

Results

A total of 185 rhizobacterial isolates were obtained from the rhizosphere of different varieties of stone fruits from different areas of Punjab and KPK. These isolates were maintained at 4°C for further use and the potential antagonists were preserved at -80°C.

In Vitro Evaluation of Antagonistic Potential of Rhizobacterial isolates

Tenrhizobacterial isolates obtained from the rhizosphere of peach, plum and apricot exhibited remarkable *in vitro* activity against *P. syringae*. Though there were many isolates antagonistic to *P. syringae* but only those isolates were selected having zone of inhibition greater than 20mm. Among the 10 bacterial isolates, zone of inhibition diameter ranged between 20mm to 37mm. Isolates Rh-3 and Rh-8 exhibited highest inhibition with 37mm and 35mm diameter of inhibition respectively.

Moderate inhibition activity was shown by the isolates Rh-1, Rh-2,Rh-5, Rh-6 and Rh-10. A comparatively less inhibition activity was shown by the isolates Rh-4, Rh-7 and Rh-9 (Table 1).

Microscopic and Morphological characterization of PGPR Isolates

The morphological and microscopic characteristics of PGPR isolates varied greatly. Among the 10 selected PGPR isolates, 4 were bacilli, 3 were rod and 3 were spherical shaped in morphology. All the isolates were motile and response to the Gram's staining test was that 4 isolates were positive while 6 were negative. All the isolates ranged between the colony size from 0.2-1.7mm. The microscopic and morphological details of selected isolates are given (Table 2).

Biochemical Characterization and Enzymatic activity of PGPR Isolates

Siderophore production

When the PGPR isolates were grown on CAS agar medium, a yellow halo zone was formed around the colony of all isolates observed after 3 days of incubation which indicated that all the isolates were siderophore producers (Table 3).

Phosphate solubilisation

After growing the isolates on Pikovaskya's medium for 7 days, it was observed that all the isolates showed a positive response to phosphate solubilisation which was evident from the formation of halo zone around the colony (Table 3).

HCN Production

Six isolates showed change in color of filter paper to reddish brown designating them as HCN producers while the remaining 4 isolates showed no change in color of filter paper (Table 3).

Fluorescence production

Fluorescence production was observed by 6 isolates when observed under UV light (Table 3).

Production of Ammonia

Ammonia production test showed a positive response among the all isolates. A change in colour from brown to yellow was observed in all 10 isolates (Table 3).

Indole-3-acetic acid (IAA)

In our study, 7 isolates were able to produce IAA without addition of tryptophan while the remaining 3 isolates were unable to produce IAA even with tryptophan (Table 3).

Enzymatic Activity of PGPR Isolates

Catalase activity

It was observed that all the isolates showed catalase activity except the isolates Rh-6 and Rh-8 (Table 3).

Lipase Activity

The appearance of greenish blue color around the bacterial colony was observed in 8 out of 10 isolates which were considered positive to lipase test (Table 3).

Table 1. Selected antagonistic rhizobacterial isolates from the rhizosphere of different stone fruits.

S. No.	Isolates	Zone of Inhibition (mm)	Location of sampling	Variety
1	Rh-1	34ab	Madrotha, Attock	Florida king, Peach
2	Rh-2	32b	Khan Pur,Haripur	Early grand, Peach
3	Rh-3	37a	Pind Hasham Khan, Haripur	Shireen, Peach
4	Rh-4	22d	Balakot, Abbottabad	Red flesh early, Apricot
5	Rh-5	27cd	Birote, Abbottabad	Fazle manani, Plum
6	Rh-6	29c	Nara, Abbottabad	Nari, Apricot
7	Rh-7	23d	Hilkot, Mansehra	Swat selection, Apricot
8	Rh-8	35ab	Ghora gali, Murree	Travet, Apricot
9	Rh-9	21d	Pind Noshehri	Ruby red, Plum
10	Rh-10	28c	NARC, Islamabad	Florida king 6A, Peach

Table 2. Microscopic and Morphological characterization of Selected PGPR Isolates.

S. NO	Isolates	Morphology	Motility	Gram Reaction	Colony Size mm	Colour	Pigmentation	Colony Edges
1	Rh-1	Bacilli	Motile	+ive	1.1-1.3	Off white	-	Rough
2	Rh-2	Bacilli	Motile	+ive	0.9-1.2	Off white	-	Rough
3	Rh-3	Rod	Motile	-ive	0.9-1.1	Yellowish	+	Smooth
4	Rh-4	Spherical	Motile	-ive	0.2-0.5	White	+	Smooth
5	Rh-5	Rod	Motile	-ive	1.4-1.6	White	+	Smooth
6	Rh-6	Bacilli	Motile	+ive	1.1-1.3	Creamy	-	Rough
7	Rh-7	Spherical	Motile	-ive	0.8-1.0	White	+	Smooth
8	Rh-8	Bacilli	Motile	+ive	1.2-1.4	Creamy	-	Rough
9	Rh-9	Spherical	Motile	-ive	1.5-1.7	White	+	Smooth
10	Rh-10	Rod	Motile	-ive	0.7-0.9	Yellowish	+	Smooth

Table 3. Biochemical Characterization and Enzymatic activity of selected PGPR Isolates.

S. No	Isolates	Siderophore	Phosphate solubilisation	HCN	Fluorescence	Ammonia	IAA	Catalase	Lipase
1	Rh-1	+	+	-	_	+	+	+	_
2	Rh-2	+	+	+	_	+	+	+	+
3	Rh-3	+	+	+	+	+	+	+	+
4	Rh-4	+	+	+	+	+	+	+	+
5	Rh-5	+	+	+	+	+	+	+	+
6	Rh-6	+	+	_		+	-	-	-
7	Rh-7	+	+	_	+	+	-	+	+
8	Rh-8	+	+	_	-	+	+	_	+
9	Rh-9	+	+	+	+	+	_	+	+
10	Rh-10	+	+	+	+	+	+	+	+

Discussion

Plant growth promoting rhizobacteria have been characterized as a group of bacteria which help the plants in improving its growth and yield by the involvement of two different mechanisms either directly or indirectly. PGPR have been reported to improve plant growth either through direct stimulation by the synthesis of phyto hormones (Xie *et al.,* 1996) or by decreasing the effect of pathogens (Weller *et al.,* 2002). In present study, a total of 185 rhizobacteria were isolated and out of 185 rhizobacterial isolates 10 exhibited significant antagonistic potential during *in vitro* activity against *P. syringae* were selected for further characterization. A remarkable inhibition activity was exhibited by the different isolates of plant growth promoting rhizobacteria against *P. syringae* ranging between 20mm to 37mm as Kremer and Kennedy (1996) reported that a major group of bacteria with the potential for biocontrol is the *Psuedomonas* and *Bacillus* genus is reported to be very effective in the biocontrol of many bacterial diseases (Kim *et al.,* 2003). On the basis of microscopic observations it was recorded that out of 10 rhizobacterial isolates, 6 were gram negative and 4 were gram positive bacteria. In current study, 7 out of 10 isolates produced IAA (Table 3). IAA helps in increase the plant growth by improving the uptake of minerals and water of plant system (Rosenblueth *et al.,* 2006). Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen, this low availability of phosphorous to plants is because the majority of soil P is found in insoluble forms, while the plants absorb it only in two soluble forms, the monobasic (H_2PO_4) and the diabasic (HPO_4) ions (Bhattacharyya and Jha, 2012). Almost all the selected 10 isolates were able to show positive response to phosphate solubilisation is another evidence of being phosphate solubilizing bacteria (Table 3).

Our results are in line with the findings of Zaidi *et al.* (2009), who reported that Phosphate-solubilizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with P from sources otherwise poorly available by various mechanisms. In environment, iron occurs principally as Fe^{3+}, that is present in insoluble form and this form of iron makes it unavailable to both plants and microorganisms (Rajkumar *et al.,* 2008). Bacteria that acquire iron by producing low molecular weight iron chelating compounds are siderophores, In both Gram-negative and Gram-positive rhizobacteria, iron present in (Fe3+) is reduced to Fe2+ which is further released into the cell from the siderophore (Rajkumar *et al.,* 2010). In our experiment all the isolates were able to produce siderophore (Table 3). When the enzymatic activity was assessed, 8 out of 10 isolates were able to produce catalase activity and 8 isolates were also able to show lipase activity Our results showed a great harmony with the findings of Buchenaier (1998). The potential mechanism of plant growth promoting rhizobacteria to provide a protective effect on the root and suppression of pathogens are through the help of plant hormones like IAA (Loper and Schroth, 1986) and other plant growth promoting substance like Siderophore (Leong, 1986).

To summarize the results of present studies, PGPR have variety of different characteristics that make them distinguishable from the other bacterial species. As no previous studies on the isolation of rhizobacteria from the rhizosphere of stone fruits are reported so it is also investigated through results that PGPR isolated from the rhizosphere of stone fruits are able to promote plant growth as they involve different direct and indirect mechanisms, necessary for plant growth.

Acknowledgement

We acknowledge the sincere thanks to Higher Education Commission, Pakistan (Project No. HEC-2286) for providing the funds to conduct this study.

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