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ANTAGONISTIC ACTIVITY AND MOLECULAR CHARACTERIZATION OF ISOLATED *PSEUDOMONAS AERUGINOSA* AGAINST *PSEUDOMONAS SYRINGAE* CAUSING BACTERIAL CANKER DISEASES IN APRICOT

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Abstract

Phytopathogenic bacteria are causing severe losses in crops, orchards and vegetables. Bacteria isolated from the rhizosphere of plants, acting as biological control agent, play an important role in suppression of disease caused by phytopathogenic bacteria. The purpose of this study is to do the molecular characterization of *Pseudomonas aeruginosa* isolated from the rhizospheric soil of apricot trees and assessing its antagonistic potential against bacterial canker disease caused by *Pseudomonas syringae*. During *In vitro* activity Rh37 produced best results against all the virulent isolates of *P. syringae*. Rh37 as stongest antagonistic isolate was selected and referred to molecular characterization. During greenhouse evaluation of antagonists, rhizobacteria when applied resulted into maximum disease suppression and enhanced plant growth characters (Shoot height, shoot fresh weight and shoot dry weight). On molecular characterization, Rh37 was identified as *Pseudomonas aeruginosa.* The result of this study suggests *P. aeruginosa* as a good biocontrol agent against *P. syringae* causing bacterial canker disease.

Key words: Bacterial Canker, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, Apricot.

Introduction

Apricot is one of the significant, highly nutritive, attractive and major fruit specie of cold areas of Pakistan. The cultivation and production of this fruit is done on the highly elevated areas (3000 meters) of Pakistan (Anon., 2015). In the Himalayan regions forest trees give yield of 47.5 kg in each year (Parmar & Kaushal, 1982). Northern areas like Malakand division of KPK and Gilgit Baltistan and high elevated regions of Balochistan province are main growing areas of apricot production (Jasra & Rafi, 2002).

Two species of *Pseudomonas* like *Pseudomonas syringae* pv. *Morsprunorum* and *Pseudomonas syringe* pv. *Syringae* which are pathogenic and cause disease of stone fruits including peach also known as (*Prunus persica*), others include apricot (*Prunus armeniaca.*), plum (*Prunus domestica*) and cherry (*Prunus cerasus*). Canker and die-back are the severe diseases in European countries caused by *Pseudomonas* sp. (Klement, 1977). These two species have various host ranges like *P. syringae* pv. *morsprunorum* is pathogenic to plum and cherry, other species like *P. syringae* pv. *syringae*is pathogenic to peaches and apricots (Kennelly *et al*., 2007). Disease prevalence is enhanced in areas with chronic cold and frost climate conditions, which could be further enhanced by *P. syringae* strains that are ice nucleation active. Severity of diseases is reduced in Mediterranean zones although these species of *Pseudomonas* exist in those areas which cause weak epidemics (Kennelly *et al*., 2007).

Rhizospheric microorganisms are now used as bio-control agents in order to control the pathogenic micro-organisms and this is efficient way to control the plant diseases rather than other control measures. Rhizobacteria are found in abundance in the root areas. Rhizobacteria secrete the chemicals which are also beneficial to plant roots as well as to control the plant pathogens. The use of PGPR, biological control provides many sources of resistance because PGPR competes with plant pathogens for food, space and niche by secreting several chemicals which effects the growth of *Pseudomonas* spp. Chemicals which are secreted includes cell wall degrading enzyme, IAA production, HCN production and siderophores (Kloepper, 1994).

Materials and Methods

The laboratory work was done in the Plant Bacteriology lab of Department of Plant Pathology of PMAS-Arid Agriculture University Rawalpindi, Pakistan and the Tropical Research and Education Center, University of Florida, USA.

Survey and sampling: An extensive survey of apricot growing areas of Punjab and Khyber Pakhtoon Khwa was conducted (Table 1). Random Soil samples were collected randomly from the rhizosphere of apricot plants. From each orchard five soil samples were taken, properly labeled and were brought to the laboratory of bacteriology in Department of Plant Pathology, Pir Mehr Ali Shah-Arid Agriculture University Rawalpindi, Pakistan for further processing and characterization.

Bacterial pathogen *Pseudomonas syringae*: Bacterial pathogen *Pseudomonas syringae* most virulent isolates were acquired from the Plant Bacteriology Laboratory of PMAS UAAR, which were isolated from the infected leaves and fruits of apricot, having bacterial canker disease. *P. syringae* was confirmed by using pathogenicity test and hypersensitive response.

Table 1. List of areas and locations of Punjab and Khyber Pakhtoon Khwa surveyed during 2014-15 and 2015-16.

Province	District/ Area	Sub areas
Punjab	Rawalpindi	Murree, Gujar Khan, ChakShehzad, NARC
	Attock	Dherikot, ShakarDara, Qaziabad, Madrotha, Thekrian
	Chakwal	Chakwal, BARI
Khyber Pakhtoon Khwa	Peshawar	Peshawar
	Haripur	Jattipind, Nara amaz, Tofkian, Pind Hashim Khan, Khan Pur
	Abbotabad	Singi Mera, Bagnotar, Balakot, Malkot, Nambal, Sajikot, Nara
	Mansehra	Baffa, Battal, Hilkot, Jaloo, Shinkiari, Dhodal, Bandi, Malik Pura
	Swat	Shergarh, Sakhakot, By pass thanra, Barikot, Mingora, Thakhtband bypass, Sherpalam, baghderi, matta
	Naran Kaghan	Naran Kaghan

Isolation of rhizobacteria: Serial dilution technique was performed for isolation of *Pseudomonas* spp. First of all 1g soil was mixed into test tube having 9 ml water. Then test tube was shaken on vortex mixer for half minute to get homogenous suspension. One ml of suspension from first tube was mixed into next test tube having 9 ml water and mixed it on vertex mixer. After that dilution series was performed technique up to 10^{-7} dilutions. Test tubes having dilutions of (10^{-2}, 10^{-5} and 10^{-7} correspondingly) were used and inoculated the suspensions in plates having King, s broth media. After that pure culture were formed by streak plate method (King *et al*., 1954).

Morphological characterization: Rhizobacterial isolates were grown on Nutrient Agar, King's Broth and Azotobacter Agar Media for 24 hours at 28ºC and were morphological characterized on the basis of colony colour, colony shape, colony margin, colony elevation and media used. Temporary slides were prepared by taking a drop of water and isolates colony was picked by wire loop and smeared in water drop. After that slide was examined under microscope.

***In vitro*evaluation of antagonistic rhizobacteria:** To check the efficacy of rhizobacterial isolates *in vitro*, dual culture plate technique was used. To perform this technique, first of all during media preparation, bacterial suspension of pathogenic bacteria *Pseudomonas syringae* of 5×10^7 colony forming units in per ml was added to nutrient agar and king's broth media recipes. After that media was poured to various petriplates. After that rhizobacterial isolates were taken and suspension of each rhizobacteria was made up to 5×10^7 cfu/ml by mixing in distilled water. After that few drops of rhizobacteria were poured with help of micropipette in to poisoned media plates with equal concentrations. For control petriplates were simply inoculated with distilled water for negative control. After that plates were covered with parafilm and kept in incubator for 48-72 hours at 28°C. After 2-3 days plates were examined by measuring the diameter of rhizobacteria growth to check which rhizobacteria is effective against *Pseudomonas syringae* (Dhingra & Sinclair, 1995).

Evaluation of antagonistic potential of rhizobacteria in greenhouse: The isolates exhibiting antagonistic response during *In vitro* evaluation were selected for further studies under green house conditions. The selected isolates were applied individually and in combination with and without *P. syringae*.

Preparation of inoculum: Inoculum of all the bacterial isolates (including rhizobacteria and *P. syringae*) was prepared in nutrient broth (NB). Individual isolate was inoculated to the NB medium and incubated on shaker at 28ºC and 200 rpm overnight. The cultures were centrifuged at 10,000 rpm for 10 min and the cells were suspended in 0.85% saline solution (prepared in SDW) and final concentration was adjusted to 10^8 cfu/ml at OD6oo using spectrophotometer.

Treatments: Rhizobacterial suspension was foliar sprayed on apricot plants prior to spray with *P. syringae* suspension. After 1 week of treatment with rhizobacterial isolates, *P. syringae* suspension was prepared in saline solution and was foliar sprayed on the leaves. Plants were placed under humid chamber in moist conditions for overnight and then were placed under controlled conditions. The non-treated control plants were dipped in tap water and were planted in infested soil. Disease and growth data were recorded after 4 weeks.

Following treatments were used;

To = Control
T1 = Rh1
T2 = Rh1+ Ps
T3 = Rh23
T4 = Rh23+Ps
T5 = Rh37
T6 = Rh37+ Ps
T7 = Rh42
T8 = Rh42+Ps
T9 = Rh49
T10 = Rh49+Ps

Evaluation of disease and growth parameters: When each treatment was done after that plant structures like shoot length, weight and disease severity was measured and data was compiled after all experiments.

Biochemical characterization: Biochemical charact-erization of the potent rhizobacterial isolates was done to check their efficacy. Following biochemical tests were performed.

IAA production: For Indole acetic acid production, tryptophan with a concentration of 40 g/ml was mixed with nutrient broth media. After that every rhizobacterial strain was again grown on nutrient broth media and then kept in shaker for 2 days at temperature of 28°C at 2

hundred revolutions per minute. After 2 days culture filtrate were rotated in spinning machine at twelve thousand rpm for ten minutes and 1 ml solution was taken and then mixed with Salkowski,s reagent. Then kept that at room temperature for half an hour.

Production of siderophore: Siderophores production was done by using agar media of CAS blue for bio-control rhizobacterial strains. Pure culture of rhizobacteria was picked by wire loop and then mixed in CAS media. Then plates were covered and kept in incubator at temperature of 30°C for one week (Louden *et al*., 2011).

Phosphorus solubilization: Process of solubilization of phosphorus was done by using National Botanical Research Institute Phosphate abbreviated as NBRIP, Purification of bacteria was done on the culture media and then incubation was done at 30°Cfor one week. Phosphate solubilizer was measured by the formation of halo region between the formations of bacterial growth.

Flourescent pigment production: Rhizobacteiral isolates were grown on Kings Broth media and after 48 hrs plates were observed under UV light.

Molecular characterization of antagonistic rhizobacterial isolate: Molecular characterization was done at Tropical Research and Education Center, University of Florida. DNA isolation was carried out byusing Qiagen's QIA amp DNA minikit. Extraction of Deoxyribose Nucleic Acid was done on one percent gel having agrose solution which was dyed with chemical known as ethidium bromide to measure its reliability and photographs were taken by UV light.

Complete DNA was utilized as a pattern and the wanted portion (16S) was augmented by utilizing a technique known as Polymerase Chain Reaction. The intensification of the *16S* ribosomal RNA trait was done by consuming widespread genes (8F:5′AGTTTGAT CCTGGCTCAG-3′; 1510R: 5′-GGCTACCTT GTT ACG A-3′) as reviewed by (Katsivela *et al*., 1999). Intensified Polymerase Chain Reaction produce of the 16S ribosomal gene were alienatedon 1 percent gel having enzyme agrosein 0.5× (Tris- EDTA) buffer comprising two µl ethidium bomide (20mgml^{-1}).

DNA sequencing of given object was delivered to Macrogen Korea. Strain sequence was compared with the National Center for Biotechnology Information (NCBI) database using Blast N and identified the closest match. Phylogenetic tree was constructed by using Tamura Nei model with 1000 bootstrap by using MEGA-6 Software.

Results

During survey of apricot growing areas, recorded data revealed that bacterial canker has become one of the significant diseases of apricot. In total eleven districts, approximately 300 or chards were surveyed with prevalence 100% ineach area and no or chard was found free of bacterial canker. Composite of randomly collected soil samples were formed for further processing.

Bacterial pathogen *Pseudomonas syringae*: Most virulent isolates of *Pseudomonas syringae* (Ps 3, Ps 9 and Ps 17) were used, which were already available in the Plant Bacteriology Lab of PMAS UAAR.

Isolation ofrhizobacteria: From the single pure colony cultures, isolates were differentiated on the basis of characters like morphology, size, color, structure and from the media that was used for culturing (Table 3). As the result of streak plate method (Fig. 1), a total number of 109 isolates of *Pseudomonas* spp. were obtained from the 11 districts of apricot growing areas of Pakistan (Table 2).

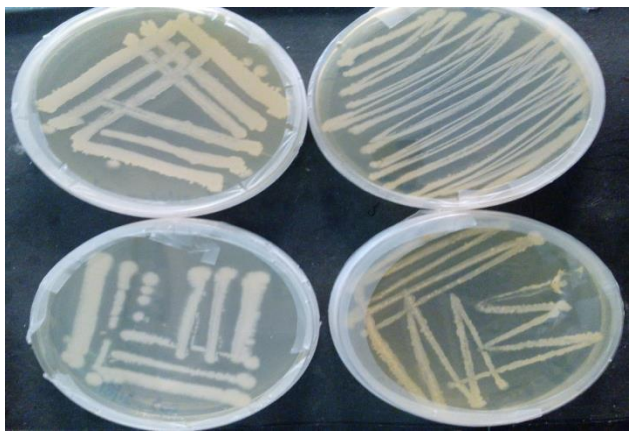

Fig. 1. Streaking ofrhizobacterialisolates.

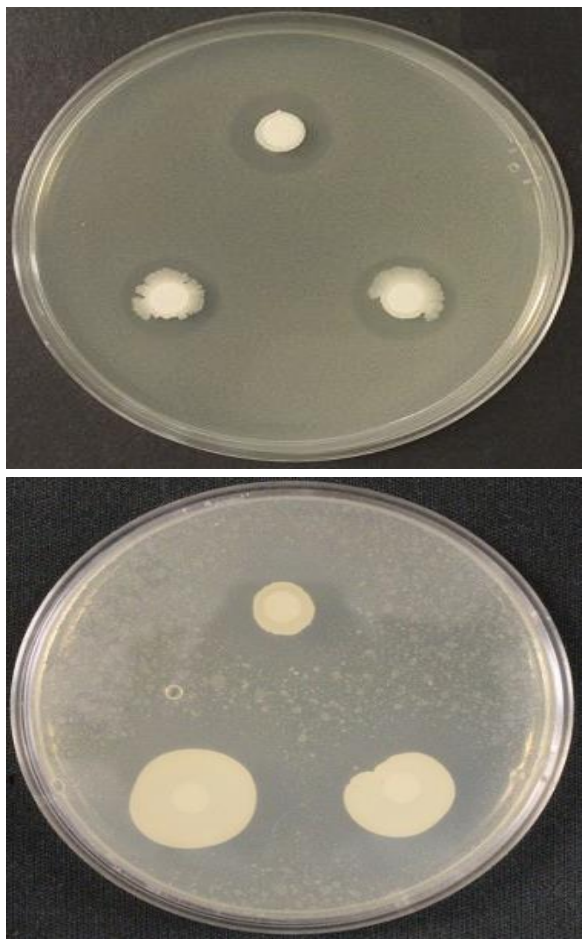

Fig. 2. Zone of inhibition by antagonistic rhizobacteria on left vs. Control on right.

Table 2. Rhizobacterialisolates retrievedfrom apricot growingareas of Punjab and KPK.

Area	*Pseudomonas*
Attock	11
Rawalpindi	21
Chakwal	13
Haripur	8
Abbottabad	9
Mansehra	14
Peshawar	5
Charsadda	5
Swat	11
Naran	9
Kaghan	13
Total	109

Dual culture plate technique: Evaluation of bio-control isolates of rhizobacteria with zone of inhibition or dual culture technique discovered 10 prospective isolates were antagonistic to either isolate of *P. syringae* (Fig. 2). The isolates having zone of inhibition greater than 8mm in radius with either isolate was selected for further studies and is given (Table 4). Rh 37 showed highest zone of inhibition against all the 03 isolates of *P. syringae.*

Greenhouse evaluation ofantagonistic PGPR: In green house evaluation, disease incidence was significantly reduced in rhizobacteria treated plants. An increase in the height of plant shoot, shoot fresh weight and shoot dry weight of apricot was observed (Table 5), when treated with antagonistic rhizobacterial suspension. There was a significant reduction observed on the account of growth parameters, when *P. syringae* was applied individually to apricot plant.

Table 3. Morphological characters of rhizobacterial isolates.

Isolates	Colony colour	Colony shape	Colony margin	Colony elevation	Media used
Rh1	Off white	Irregular	wavy	Flat	Azotobacter Agar
Rh2	White	Irregular	irregular	Flat	Nutrient Agar
Rh4	White, small sticky	Slightly dome shaped	undulated	Raised	Nutrient Agar
Rh5	Light yellow	Circular	wavy	Raised	King B
Rh7	Pale yellow	Irregular	wavy	Raised	King B
Rh21	Creamy white	Round	flat	Flat	Nutrient Agar
Rh23	Small grayish white	Round	wavy	Raised	Nutrient Agar
Rh37	Pale yellow, shiny	Pointed	undulated	Flat	King B
Rh42	Creamy white	Flat	flat	Raised	Nutrient Agar
Rh49	White small	Round	flat	Raised	Nutrient Agar

Table 4. Zone of inhibition of antagonistic isolates against virulent isolates of *P. syringae*.

Rhizobacterial isolates	PS3	PS9	PS17
Rh1	5 ± 1	8 ± 1.2	5 ± 1.2
Rh2	0 ± 0	10 ± 2.5	0 ± 0
Rh4	0 ± 0	9 ± 3.3	8 ± 3.7
Rh5	9 ± 3.3	0 ± 0	0 ± 0
Rh7	7 ± 2.7	10 ± 2.5	0 ± 0
Rh21	9 ± 3.5	0 ± 0	7 ± 3.3
Rh23	6 ± 2.2	4 ± 1.2	4 ± 1.2
Rh37	8 ± 3.5	11 ± 2.8	6 ± 1.3
Rh42	5 ± 2.1	7 ± 1.6	2 ± 1.2
Rh49	8 ± 3.1	2 ± 1.2	2 ± 1.2
Control	0 ± 0	0 ± 0	0 ± 0

Biochemical characterization

IAA production: IAA production was examined among the rhizobacterial isolates, but isolates Rh37 showed high production of indole acetic acid, with concentration of 18.5µg/ml which was examined by the production of color and also the optical density of 550 was determined and highest optical density was examined with this isolate (Table 6).

Production of siderophore: The siderophore production was also examined in the isolates, only 4 isolates showed no capability to eliminate the siderophore production and zero halo region was examined in the bacterial growth on culture media of blue CAS although the other 06 isolates showed ability to chelate the ferric ion in siderophore production which was observed in isolate Rh37 as evident by the halo zone (Table 6).

Phosphorus solubilization: Two of isolates which were Rh5 and Rh37 showed the solubilization of phosphorus, while remaining isolates showed no halo region/ phosphorus solubilization (Table 6).

Fluorescent pigment production: Fluorescent pigment production is the main characteristics of *Pseudomonas* spp. and isolates grown on kings Broth Media when observed under UV light produced fluorescence confirmed that they belong to *Pseudomonas* spp. (Table 6).

Molecular characterization of antagonistic isolate: Rhizobacterial isolate Rh37 exhibiting highest antagonistic activity potential was selected for molecular characterization. Identification of Rhizobacterial isolate was based on phylogenetic analysis. When comparison was done on sequence of 16SrDNA with the other sequences present on website of NCBI, Rh37 showed it shomology with *P. fluorescens* and *P. aeruginosa* (Fig. 3).

Table 5. Effect of treatments on diseaseincidence and plantgrowth parametersof apricot plants undergreenhouse conditions.

Treatments	Disease incidence (%)	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Rh1	00	337.45	462.50	239.54
Rh1 + Ps	21.9	36.10	59.65	37.45
Rh23	0	35.45	57.39	36.25
Rh23 + Ps	17.80	35.41	55.48	34.91
Rh37	0	38.13	65.51	40.93
Rh37 + Ps	10.7	37.85	63.71	38.24
Rh42	0	37.9	64.61	39.43
Rh42 + Ps	14.40	37.45	63.35	36.41
Rh49	0 f	37.33	64.5	37.46
Rh49 + Ps	13.95	36.25	62.61	35.97
Ps	42	27.33	42.98	24.14

Table 6. Plant growthpromotingtraits of antagonists.

Isolates	Siderophore production	P-solubilization	IAA production (µg/ml)	Florescent pigment
Rh1	-	-	4 ± 0.41	-
Rh2	-	-	4.50 ± 0.45	-
Rh4	+	-	4 ± 0.29	+
Rh5	-	+	12 ± 1.0	+
Rh7	++	-	10.5 ± 0.21	-
Rh21	+	-	3.8 ± 1.5	-
Rh23	+	-	8.5 ± 2.8	-
Rh37	++	+	18.5 ± 2.8	+
Rh42	+	_	5.3 ± 0.21	-
Rh49	_	-	3.5 ± 1.21	-
Control	-	-	0	-

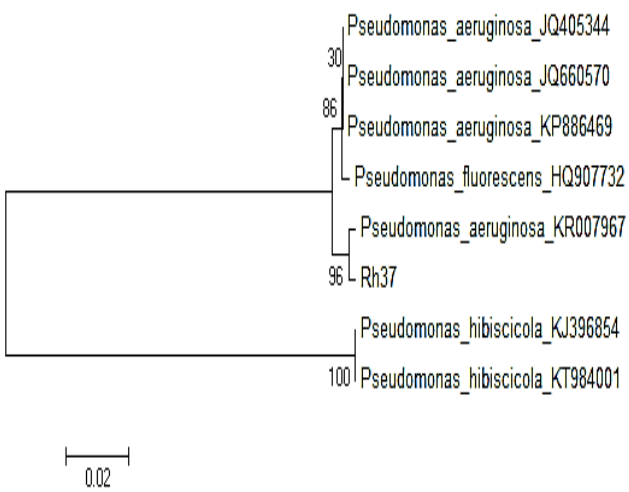

Fig. 3. Neighbor-joining tree based on partial *16S rDNA* gene sequencing showing phylogenetic position of strain Rh37. Cluster analysis was performed having bootstrapvalue1000 using MEGA-6 soft ware. *Psuedomonas hibiscicola* (KJ396854) was used as an out group.

Discussion

P. syringae which cause bacterial canker is an important bacterial pathogen that is still increasing its host range and infecting more and more crops, fruits and vegetables by posing serious threats to the production of fruits. The presence of pathogen without development of symptoms (hidden infections) has let it distribute more widely. This is the reason that the pathogen has developed several races, biovars and possesses huge diversity (Vanneste *et al*., 2013).

Keeping in view the importance of this problem, an extensive survey of major apricot growing areas of Punjab and Khyber Pakhtoon Khwa was conducted in present study. A number of microorganisms such asbacteria and fungi are present inthe roots and soil surface. 109 isolates were isolated from the rhizosphere of apricot. It has been supposed that between the soil surface communities, only 2-5 percent is helpful to plant (Antoun & Prevost, 2006). Between the gram positive bacteria in soil, 95 percent belong to *Bacillus* while other 5 percent comprise of *Arthrobacter* and *Frankia* (Garbeva *et al*., 2003). While considering gram negative bacteria, *Pseudomonas* is the most ubiquitous genus prevailing in the soil surface (Barriuso *et al*., 2008).

Activity of antagonists dominates on other microorganisms because if the pathogenic microorganisms exceed in population, they will cause much damage. Biondi *et al.*, (2009) reported that there is an innovative approach to control the bacterial spot and bacterial canker disorder by using antagonistic bacteria, like *Psuedomonas fluorescence*. Our results from this research are completely approved and in line with his approach of bacterial canker disease control in apricot.

Biocontrol is an effectiveand alternative method for suppressing plant pathogens (Harman, 2000), and this

approach is commercially available now (Velivelli *et al.*, 2014). In the present study attempts to control bacterial canker disease by using plant growth promoting rhizobacterial isolates were made. Disorders caused by bacteria are complicate to manage due to nonpresence of complete immune host, development of pathogens' resistance to antibiotics and the minimum efficiency of copper compounds when conditions suitable for pathogens and environmental concern (Soylu *et al*., 2003). So, the current work is a significant observation in controlling the ailment of canker disease of stone fruits.

On treatment with PGPR strains, a slightly enhancement in the vigor of apricot plant was observed. We observed an enhancement in the plant shoot height and in shoot fresh and dry weight in apricot plants and our results of these treatments were same to results attained by Raj *et al*., (2003).

There are some reports in the study uttering that by discharging many of enzymes, siderophores, antibiotics, by triggering host physiology and defense mechanism, these plant growth promoting rhizobacteria isolates are reducing the canker growth inside the host (Prashar *et al*., 2013). To support our findings, earlier studies suggest that phosphate-solubilizing and siderophore-producing *Bacillus* spp., *Pseudomonas* spp., *Pseudomonas fluorescens* have showed antagonistic activity against several phytopathogens (Han *et al*., 2015).

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