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SCREENING AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH CHERRY (*PRUNUS AVIUM* L.)

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ABSTRACT

A total of 20 bacterial isolates (9 rhizospheric and 11 endophytic) were isolated from cherry of Shimla district of Himachal Pradesh and characterized for multifarious plant growth promoting traits like P-solubilization, siderophore, IAA, HCN, ammonia production, nitrogen fixation and antagonistic activity against *Rhizoctonia solani, Fusarium oxysporum* and *Pythium aphanidermatum*. On the basis of their efficacy towards plant growth promoting traits, two isolates viz. RT2 and RK1 were selected which showed production of 69.32 and 50.70 μg/ml of IAA, 175.84 and 138.24% of siderophore and solubilised 160.33 and 112.67 μg/ml of insoluble phosphorous, respectively. Both isolates were found to be HCN and ammonia producers and was also able to grow on nitrogen-free medium. RT2 isolate exhibited antagonism against *Rhizoctonia solani* (33.43%), *Fusarium oxysporum* (38.18%) and *Pythium aphanidermatum* (41.89%) whereas RK1 showed antagonism against *Fusarium oxysporum* (50.33%) and *Pythium aphanidermatum* (40.33%) only. Morphological, physiological and biochemical characterization placed RT2 and RK1 under the genus *Bacillus*. Therefore, results suggested that RT2 and RK1 can be used as biofertilizers/bioprotectants for enhanced productivity and protection of cherry under high hills conditions of Himachal Pradesh.

INTRODUCTION

In the last few years, there has been a tremendous increase in our knowledge about the use of microbial mediated approaches such as plant growth promoting rhizobacteria (PGPR) (Rana *et al*., 2015) and arbuscular mycorrhizal fungi (AMF) (Kirti *et al*., 2016) to improve plant growth through enhanced supply of essential nutrients and sustain soil health. PGPR are recognized as eco-friendly, non-hazardous, non-bulky and economically suitable substitute for modern agriculture practices for plant productivity and protection because the chaotic use of chemical or synthetic compounds have created environmental pollution, health hazards and also induced phytotoxicity. PGPR colonize plant root interiors, migrate to the different plant parts exclusively in the intercellular space and exert beneficial effects on plant growth either indirectly or directly; indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms; while direct promotion of plant growth by PGPR involves either providing plants with a compound synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment (Dutta *et al*., 2014). PGPR includes the genera *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Rhizobium* and *Serratia* (Dursan et al., 2008). The predominant PGPR's belong to genera *Bacillus* and *Pseudomonas* because of their association with soil organic matter, nutritional diversity and rapid growth rate (Joshi and Bhatt, 2011). Since there are no commercial PGPR formulations for cherry, therefore effective strategies for selection of superior indigenous strains of PGPR are needs of the hour. Thus the current study was undertaken to isolate, screen and characterize bacterial isolates from cherry orchards in Shimla District of Himachal Pradesh with special allusion to their plant growth promoting abilities.

MATERIALS AND METHODS

Collection of soil and root samples

The rhizospheric soil and root samples of cherry plants were collected from two locations i.e. Jubbal and Kotgarh having four sites viz. Dhochi, Dhar, Kandiyali and Thanedhar of Shimla district of Himachal Pradesh. The samples were placed in plastic bags and brought to Soil Microbiology Laboratory, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan for further isolation and characterization work.

Isolation and enumeration of microbial population

Bacterial population persisting in the rhizospheric soil samples and roots was determined on nutrient agar and soil extract medium by the standard serial dilution and plate count

technique (Subba Rao, 1999). The isolated colonies that developed on enriched NA medium (master plate) after incubation of 24 to 48 h were replica plated onto Pikovskaya's agar and Jensen medium. The microbial count was expressed as colony forming unit per gram of soil (cfu g^{-1} soil).

Screening and characterization of PGPR isolates

The screening of the bacterial isolates for various plant growth promoting activities were performed by adopting the standard methods. Estimation of P-solubilization in liquid PVK medium containing tri-calcium phosphate (TCP) was determined as described by Bray and Kurtz (1945). Phosphate solubilizing activity and growth on nitrogen free medium was estimated by the method of Pikovskaya (1948) and Jensen (1987), respectively. The ability of bacterial isolates to produce siderophore, IAA and HCN was assessed by Schwyn and Neilands (1987), Gordon and Paleg (1957) and Bakker and Schippers (1987), respectively. For the detection of ammonia production, the method of Lata and Saxena (2003) was used. The biocontrol potential of the bacterial isolates against test fungal pathogen (*Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium aphanidermatum*) was ascertained by agar streak plate method and per cent growth inhibition was calculated as described by Vincent (1947). Morphological and biochemical characterization of the isolates was performed as per the criteria of Bergey's Manual of Systematic Bacteriology (Claus and Berkley, 1986).

Statistical analysis

The data recorded for various parameters under laboratory conditions were statistically analyzed as described by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Isolation and enumeration of microbial population

Microbial population colonizing the rhizosphere of cherry on four different media *viz*. Nutrient agar (NA), Pikovaskaya's agar (PVK), Jensen medium (JM) and Soil extract medium (SEM) is presented in Table 1. The maximum rhizospheric microbial count (153.83×10^5cfu/g) was observed on SEM at Dhochi site of Jubbal location and minimum (33.83×10^5cfu/g) was recorded on JM at Dhar site of same location. The interaction among location and aspect also showed significant effect on NA, SEM and JM with the maximum (181.00×10^5 cfu/g soil) microbial population at Dhochi site having Northern aspect, and the minimum (31.67×10^5 cfu/g soil) at Dhochi site of Jubbal location on the same aspect. However, the interaction effects of location and aspect on PVK medium were non-significant. The total endorhizobacterial population on NA medium harboured the maximum (144.00×10^1 cfu/g root) microbial population at Dhochi site of Jubbal location and the minimum (34×10^1 cfu/g root) counts were recorded at Thanedar site of Kotgarh location on Jensen medium. Interaction among location and aspect also showed significant effect on all the mediums with the maximum (150.33×10^1 cfu/g root) counts on NA medium at Dhochi site of Jubbal location having Southern aspect and the minimum (24.33×10^1 cfu/g root) on Jensen medium at Kandiyali site of Kotgarh location having Northern aspect (Table 2.). The variation in microbial population in the rhizosphere at all the sampling sites may be due to positive influence of root exudates, age of plant, variety/cultivar type, time of sampling, physico-chemical properties of soil, and environmental conditions of study area (Wieland *et al*., 2011). The results are further in confirmation with the findings of Shishido *et al*. (1999) who reported the greatest variation in microbial population with respect to location/plant parts.

Screening and characterization of PGPR isolates

All the bacterial isolates exhibited variation for different plant growth promoting traits (Table 3.). The P-solubilization efficiency (PSE) on agar plate had great variation with value ranging from 97.09 to 154.17 per cent. P-solubilization efficiency (PSE) was maximum (154.17%) for RK1 isolate in solid PVK medium, however, P-solubilization in liquid PVK medium was maximum (160.33 μg/ml) for RT2 isolate after 72 hr of incubation. Phosphorus (P) is one of the major essential macronutrient required for biological growth and development of the plants (Mehta *et al*., 2010). Most of the phosphorus present in soil is in form of insoluble phosphates and hence unavailable to plants. PGPR are able to solubilize precipitated phosphates and enhance phosphate availability to the plants. Similar release of phosphate has been reported by Chauhan *et al*. (2015) in the range of 89 to 257 mg/ml by *Bacillus* spp

Quantitatively, maximum (175.84% siderophore unit) was produced by isolate RT2 and minimum (47.88% siderophore unit) after 72 h of incubation corresponded to isolate SK3. Siderophore production is a biocontrol mechanism belonging

Table 1: Enumeration of rhizospheric bacterial population (cfu g^{-1} soil) from cherry (*Prunus avium* L.) at various locations of Shimla district of Himachal Pradesh

LOCATION		Rhizospheric bacterial population (10^5cfu/g soil)											
		Nutrient Agar (NA)			Pikovskaya's Medium (PVK)			Jensen Medium (JM)			Soil extract Medium (SEM)		
		Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean
Jubbal	Dhochi	112.33	102.33	107.33	99.33	92.33	95.83	31.67	42.33	37	181	126.67	153.83
	Dhar	66.33	58.67	62.5	60.33	37.33	48.83	30.33	37.33	33.83	59.67	70.33	65
Kotgarh	Kandiyali	172	132.33	152.17	94.33	84.33	89.33	54.67	35.67	45.17	150.33	130.67	140.5
	Thanedhar	86.67	85.67	86.17	77.67	70.83	73.5	57.33	60.33	58.83	86.67	72.67	79.67
	Mean		109.33	94.75		82.92	70.83		43.5	43.92		119.42	100.08
$CD_{0.05}$	L		5.41			7.39			6.05			4.73	
	A		3.83			5.23			NS			3.34	
	L×A		7.65*			NS			8.56*			6.69*	

*Interaction is significant at 5% level of significance

Table 2: Enumeration of endophytic bacterial population (cfu g^{-1} root) from cherry (*Prunus avium* L.) at various locations of Shimla district of Himachal Pradesh

Location		Endophytic bacterial population (10^1cfu/g root) Nutrient Agar (NA)			Pikovskaya's Medium (PVK)			Jensen Medium (JM)			Soil extract Medium (SEM)		
		Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean	Northern Aspect	Southern Aspect	Mean
Jubbal	Dhochi	137.67	150.33	144	58	61.33	59.67	40.33	31.67	36	130.33	110.33	120.33
	Dhar	89.67	73	81.33	48.67	35.67	42.17	42.67	51	46.83	83	81	82
Kotgarh	Kandiyali	59.33	67	63.17	39.33	46	42.67	24.33	32.67	28.5	70.33	72	71.17
	Thanedhar	65	99	82	59	69	64	31	37	34	100	71.67	85.83
	Mean		87.92	97.33		51.25	53		34.58	38.08		95.92	83.75
$CD_{0.05}$	L		4.31			2.46			3.59			4.09	
	A		3.05			1.74			2.54			2.89	
	L×A		6.10*			3.48*			5.07*			5.78*	

*Interaction is significant at 5% level of significance

Table 3 : Screening of selected bacterial isolates for multifarious plant growth promoting traits

Bacterial isolates	% P-solubilization efficiency	P-solubilization (µg/ml)	% siderophore unit	IAA production (µg/ml)	Nitrogen -free medium	HCN production	Ammonia production
RK1	154.17	112.67	138.24	50.7	+	+	+
SK3	110.01	130	47.88	35.13	+	+	+
RK3	97.09	112.67	146.97	40.83	+	+	+
RD2	108.33	105.33	102.74	46.84	+	+	+
RT2	141.28	160.33	175.84	69.32	+	+	+
$CD_{0.05}$	40.33	6.11	41.47	0.25	-	-	-

(+) indicates positivity of test; (-) indicates negativity of test

Table 4: Growth inhibition (%) of test fungus by selected bacterial isolates of cherry (*Prunus avium* L.)

Bacterial isolates	Per cent inhibition *Rhizoctonia solani*	*Fusarium oxysporum*	*Pythium aphanidermatum*
RK1	00.00 (00.00)	50.33 (45.17)	40.33 (39.41)*
SK3	26.44 (30.93)	00.00 (00.00)	00.00 (00.00)*
RK3	31.40 (34.07)	00.00 (00.00)	41.37 (40.01)*
RD2	00.00 (00.00)	00.00 (00.00)	33.18 (35.16)*
RT2	33.43 (35.31)	38.18 (38.15)	41.89 (40.32)*
$CD_{0.05}$	1.01	0.46	0.38

*Values in parentheses are arc sine transformed values

Table 5: Morphological and biochemical characteristics of selected bacterial isolates of cherry

Characteristics	Bacterial Isolates RK1	SK3	RK3	RD2	RT2
Morphological					
Form	Irregular	Irregular	Irregular	Circular	Irregular
Elevation	Flat	Raised	Raised	Flat	Flat
Margin	Undulate	Erose	Curled	Lobate	Undulate
Surface	Rough	Rough	Smooth	Rough	Rough
Gram's test	+	+	+	+	+
Shape	Rods	Rods	Rods	Rods	Rods
Endospore formation	+	+	+	+	+
Biochemical					
Catalase test	+	+	+	+	+
Starch hydrolysis	-	+	-	-	+
Casein hydrolysis	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
Indole test	-	-	-	-	-
Fermentation of Glucose	+	+	+	+	+
Citrate utilization test	+	+	+	-	+
Hydrogen sulphide production	-	-	-	-	-
Methyl red test	+	+	+	+	+
Voges Proskauer test	-	-	-	-	-

(+) indicates positivity of test; (-) indicates negativity of test

to PGPR's group under iron limiting conditions (Singh and Varma, 2015). The results are in confirmation with the findings of Kaushal and Kaushal (2013) who have reported siderophore production by *Bacillus* spp. ranging from 19.40 to 51.36 per cent. The IAA production ranged from 35.13 to 69.32 µg/ml. IAA regulates cell division, elongation, differentiation and

pattern formation in plants (Sahasrabudhe, 2011). Our results are in agreement with those of Mandyal *et al*. (2012) and Sharma *et al*. (2015) who have reported IAA production on Luria bertani broth by *Bacillus* spp. ranging from 12 to 25 μg/ml and 20 to 95 μg/ml, respectively.

All isolates were able to grow on nitrogen-free medium. Biological nitrogen fixation is considered as one of the major mechanisms which benefit plants. The ability of *Bacillus* spp. to grow on nitrogen -free medium has been reported by Kaushal *et al*. (2011). All the five isolates were positive for HCN production under *in vitro* conditions. HCN is a secondary metabolite synthesized by PGPR to strengthen the host's disease resistance mechanism (Ramette *et al*., 2003). Kachhap et al. (2015) reported similar production of HCN by *Pseudomonas* spp., *Enterobacter* spp., *Azotobacter* spp. and *Acetobacter* spp. All five bacterial isolates were also found to be ammonia producers. Similar ammonia production by *Bacillus* spp. has been reported by Pradhan and Mishra (2015).

All five bacterial isolates exhibited variation in antifungal activity against the test fungal pathogens (Table 4). Three isolates (SK3, RK3 and RT2) showed inhibition against *Rhizoctonia solani*, two isolates (RK1 and RT2) against *Fusarium oxysporum* and four isolates (RK1, RK3, RD2 and RT2) showed inhibition against *Pythium aphanidermatum*. However, only RT2 exhibited antagonism against all test fungal pathogens *i.e. Rhizoctonia solani* (33.43%), *Fusarium oxysporum* (38.18%) and *Pythium aphanidermatum* (41.89%) whereas, RK1 showed antagonism against *Fusarium oxysporum* (50.33%) and *Pythium aphanidermatum* (40.33%). The formation of zone may be due to secretion of antifungal substance by the bacterial cell that might have diffused in the medium hence, inhibited the fungal growth. Our results are in corroboration with those of Mehta *et al*. (2014) who have reported ability of *Bacillus methylotrophicus* CKAM to induce resistance in apple against *Pythium aphanidermatum* (62.5%) and Idris *et al*. (2007) who have also reported inhibitory effects against *Fusarium oxysporum* in the range of 7.06 to 66.33% by various *Bacillus* strains. Sharma *et al*. (2014) reported per cent inhibition against *Rhizoctonia* spp. in the range of 7.27-53.84 by *Pseudomonas* strain.

On the basis of predominant growth and multifarious plant growth promoting activities on different media five efficient PGPR isolates *viz*. RK1, SK3, RK3, RD2 and RT2 were selected and subjected to morphological, physiological and biochemical tests. Morphological and physiological characteristics for each isolate are listed in Table 5. The selected five isolates showed variation in colony, elevation, margin and surface. The isolates grew at a wider pH (4-8) and temperature range (10-45ºC) respectively, however all the isolates showed optimum growth at 35ºC and 7.0 pH. Majority of the bacterial isolates were able to produce catalase enzyme, ferment glucose and also utilized citrate. On the basis of morphological and biochemical characteristics, the isolates were identified as *Bacillus* spp. as per the criteria of Bergey's Manual of Systematic Bacteriology. Similar morphological and biochemical characteristics of *Bacillus* have been reported

by Ghani *et al*. (2013). The sustainable exploitation of these PGPR will act as bio-stimulation, bioregulators, biofertilizers and bioprotectants to produce healthy plants and quality fruits with minimal usages of chemical inputs.

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