

Characterization of rhizospheric and endophytic plant growth promoting rhizobacteria isolated from cherry (*Prunus Avium* L) and their effect on the growth of cherry seedlings

DEEPSHIKHA THAKUR, RAJESH KAUSHAL* and VINEET SHYAM*

Department of Organic Agriculture, Amity University, Noida 201313 Uttar Pradesh, India

*Department of Basic Sciences, Dr YS Parmar University of Horticulture and Forestry
Nauni, Solan 173230 Himachal Pradesh, India

Email for correspondence: deepshikhathakur86@gmail.com

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ABSTRACT

The attempts were made to isolate and characterize the rhizospheric and endophytic microorganisms of *Prunus avium* L from various regions of district Shimla, Himachal Pradesh. The isolated bacterial strains were characterized for plant growth promoting activities such as nitrogen fixation, phosphorous solubilization, siderophore production, antifungal activity and HCN production. Out of 14 purified isolates the three isolates (VS₂, VS₆, and VS₉) possessing maximum plant growth promoting attributes were selected for net house studies. The optimization of physiological conditions showed that the bacterial isolates were best grown at temperature 35°C and pH 7. Seedling bacterization significantly increased nutrition (NPK) uptake and various plant parameters over uninoculated plants. These three strains have considerable colonization and competitive ability thus proving to be ecofriendly, economical and more sustainable option in reducing the excessive use of expensive fertilizers.

Keywords: Cherry; PGPR; Endophytic; optimization; growth; seedlings

INTRODUCTION

Prunus avium L (sweet cherry) is a member of Rosaceae family, native of southcentral Europe and Asia Minor (Westwood and Wann 1966), can grow on a variety of soils and is influenced by myriad of abiotic and biotic factors. Indiscriminate use of chemical inputs to control pests and diseases or to provide nutrition to the crop have several negative effects (de Weger et al 1987).

The need of ecofriendly, low cost and renewable sources of nutrients and biological antagonists to increase crop productivity and to sustain soil health has led to a search for supplementation substitution for these inputs with organics/bioproduces (Georgakopoulos et al 1994).

Bacteria are common inhabitants of both the surfaces and the internal tissues of most plants and may have diverse effects on host plant development

(Kumar et al 2016). Root colonizing bacteria (rhizobacteria) that exert beneficial effect on plant development have been defined as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). It is important to screen the diversity of PGPR associated with the cherry plant in their natural habitat to have efficient inoculum for commercial application. Keeping this in view the present studies were aimed at to select and characterize efficient endophytic/rhizospheric microorganisms associated with *P avium* with the objectives of isolation, enumeration and characterization of PGPR from rhizosphere and endorhizosphere of sweet cherry and to study the efficacy of PGPR isolates in growth promotion of plants under net house conditions.

MATERIAL and METHODS

Isolation and enumeration of the microorganisms

Isolation of microorganisms was carried out from the rhizosphere soil and root samples collected

from four sites viz Kotkhai, Baghi, Nankhari and Kotgarh in district Shimla, Himachal Pradesh by the standard pour plate technique (Subba Rao 1999). For endophytic microorganisms root sample was surface-sterilized by 0.2 per cent mercuric chloride (HgCl_2) for two minutes followed by repeated washing in sterilized distilled water.

Screening of the bacterial isolates

Standard methods were adopted to screen the bacterial isolates for various plant growth promoting activities like P-solubilization (Pikovskaya 1948), siderophore (Schwyn and Neilands 1987), HCN (Bakker and Schippers 1987) and auxin production (Gorden and Palleg 1957), growth on N-free medium and antagonism against fungal pathogens (Vincent 1947).

Characterization of bacterial isolates

Identification of bacterial isolates: Selected bacterial isolates were identified on the basis of morphological, cultural and biochemical characteristics by criteria of Bergey's manual of systematic bacteriology (Claus and Berkeley 1986).

Effect of temperature and pH on growth: One hundred ml of nutrient broth was inoculated with 10 per cent of 72 h-old bacterial suspension (OD 1.0 at 540 nm). The suspension was then subjected to constant temperatures (25, 30, 35, 40 and 42°C) for 96 hours. Similar procedure was followed for effect of pH and medium was adjusted to various pH levels (4, 5, 6, 7 and 8).

Phosphate solubilization in liquid PVK medium containing TCP (tricalcium phosphate)

Fifty ml of PVK medium containing 0.5 per cent tricalcium phosphate (TCP) was autoclaved and inoculated with 10 per cent of the bacterial suspension (OD 1.0 at 540 nm) and incubated at $28 \pm 2^\circ\text{C}$ under shaking conditions for 72 h. After 72 h contents were centrifuged at 15000 rpm for 20 min at 4°C. The culture supernatant was used for determination of the soluble phosphate as per the method described by Bray and Kartz (1945).

Quantitative estimation of indole-3-acetic acid (auxins) and siderophores

Standard methods were used for quantitative estimation of IAA (Gorden and Palleg 1957) and siderophores (Schwyn and Neilands 1987).

Net house studies

On the basis of plant growth promoting efficiencies out of 14 screened isolates 3 isolates VS_2 , VS_6 and VS_9 were finally selected for net house studies. The experiment was conducted in completely randomized design replicated five times.

Treatment of plant with inoculum and planting

The cherry seedlings were given root dip treatment in liquid culture of selected bacterial isolates for 1 hour and thereafter planted in pots. Soil obtained from a furrow slice (0-15 cm depth) was sieved through 2 mm sieve and mixed with sand and farmyard manure (FYM) in a ratio of 1:1:1. The mixture was then filled in the pots, moistened to one third of its maximum water holding capacity and seedlings were planted in the pots.

Physico-chemical properties of potting mixture

Freshly-prepared potting mixture was analyzed for important physico-chemical properties and available nutrient status. Soil pH was determined in 1:2 soil water suspension using digital glass electrode pH meter. Electrical conductivity, organic carbon, bulk density, particle density, pore space, maximum water holding capacity, total nitrogen and available phosphorus and potassium were determined by using standard AOAC methods.

Plant parameters and nutrient analysis

Cherry seedlings were observed for plant parameters such as shoot and root characteristics such as shoot and root length and dry weight. The oven-dried samples of plant were ground and sieved (40 mesh) for estimation of total NPK content. Nitrogen was estimated in Kjeltac Auto 1030 Analyzer (Tecator AB, Sweden). Phosphorus was determined by Vanado molybdo-phosphoric method and Potassium by flame-photometer (Jackson 1973).

The data recorded under laboratory and net house conditions for various parameters were subjected to statistical analysis as per method outlined by Gomez and Gomez (1976).

RESULTS

Isolation and screening of bacterial isolates for multifarious plant growth promoting activities

The details of all the selected isolates, their colony morphology, locations, varieties of cherry and approximate age of the plants from which they were isolated are given in Table 1.

Table 1. Description of the PGPR isolates from rhizosphere and roots of cherry plants

Isolate	Colony morphology				Location	Altitude (m)	Latitude and longitude	Commercial variety	Age of plant (years)
	Form	Elevation	Margin	Surface	Shape				
VS ₁	Irregular	Flat	Entire	Smooth	Rods	1930	31°19'0" N, 77°29'0"E	Stella	5
VS ₂	Circular	Raised	Undulate	Rough	Rods	1930	31°19'0" N, 77°29'0"E	Stella	5
VS ₃	Circular	Raised	Entire	Smooth	Rods	2250	31°17'30"N, 77°32'53"E	Black Heart	6
VS ₄	Irregular	Raised	Undulate	Smooth	Rods	2250	31°17'30"N, 77°32'53"E	Black Heart	6
VS ₅	Circular	Convex	Entire	Smooth	Rods	2500	31°15'0" N, 77°32'6"E	Black Heart	3
VS ₆	Irregular	Flat	Erose	Rough	Rods	2300	31°18'41" N, 77°35'4"E	Red heart	5
VS ₇	Irregular	Raised	Undulate	Smooth	Rods	2300	31°18'41" N, 77°35'4"E	Red heart	5
VS ₈ *	Irregular	Flat	Entire	Rough	Rods	2250	31°17'30"N, 77°32'53"E	Black Heart	6
VS ₉ *	Irregular	Flat	Erose	Rough	Rods	2500	31°15'0" N, 77°32'6"E	Black Heart	3
VS ₁₀ *	Circular	Undulate	Undulate	Rough	Rods	2500	31°15'0" N, 77°32'6"E	Black Heart	3
VS ₁₁ *	Irregular	Convex	Erose	Smooth	Rods	1930	31°19'0" N, 77°29'0"E	Stella	5
VS ₁₂ *	Circular	Flat	Lobate	Rough	Rods	1930	31°19'0" N, 77°29'0"E	Stella	5
VS ₁₃ *	Irregular	Convex	Entire	Smooth	Rods	1930	31°19'0" N, 77°29'0"E	Stella	5
VS ₁₄	Irregular	Umbonate	Entire	Smooth	Rods	2300	31°18'41" N, 77°35'4"E	Red heart	5

*Endophytic

Table 2. Screening of selected bacterial isolates for multifarious plant growth promoting activities

Isolate	Phosphate Solubilization ¹	Growth on nitrogen-free medium ²	Antifungal activity against fungal pathogen ³		IAA production ⁴	Siderophore production ⁵	HCN production
			<i>F. oxysporum</i>	<i>P. aphanidermatum</i>			
VS ₁	-	+	-	-	-	-	-
VS ₂	++	++	+++	+++	+++	++	-
VS ₃	+	-	-	-	-	+	+
VS ₄	++	++	-	-	-	-	-
VS ₅	+	++	-	-	-	+	-
VS ₆	++	++	+++	++	++	+++	+
VS ₇	+	+	++	++	-	+	+
VS ₈ [*]	-	+	++	++	+	++	-
VS ₉ [*]	+++	+++	+++	++	+++	+++	-
VS ₁₀ [*]	+	+	++	++	-	++	-
VS ₁₁ [*]	+	-	+	-	-	-	-
VS ₁₂ [*]	++	++	-	-	-	-	-
VS ₁₃ [*]	-	++	-	-	-	-	-
VS ₁₄ [*]	+	+	-	-	-	-	-

*Endophytic

¹P-solubilization in vitro= +: <5 mm wide halo zone, ++: 5-10 mm wide halo zone, +++: >10 mm wide halo zone

²Growth on nitrogen free medium= +: <3 mm colony diameter, ++: 3-6 mm colony diameter, +++: >6 mm colony diameter

³Antibiosis by agar streak assay= +: <70% inhibition, ++: 70-80% inhibition, +++: >80% inhibition

⁴IAA production= +: <15 µg/ml, ++: 15-25 µg/ml, +++: >25 µg/ml

⁵Siderophore activity= +: <5 mm yellowish-orange zone, ++: 5-10 mm yellowish-orange zone, +++: >10 mm yellowish-orange zone

The selected isolates showed multifarious plant growth promoting potentials (Table 2). Out of the fourteen screened bacterial isolates, three best (VS₂, VS₆ and VS₉) isolates were selected for further studies.

Physiological properties of selected bacterial isolates

The optimum temperature of growth was found to be 35°C for all the three isolates (Fig 1). The rate of growth increased with increase in temperature from 25 to 42°C. The bacterial isolates (VS₂, VS₆ and VS₉) were able to grow over a wide range of pH optimum being 7 (Fig 2). Growth at pH 9 was not seen in case of any of the three selected bacterial isolates.

Plant growth promoting activities of selected bacterial isolates

The results represented in Table 3 reveal that VS₆ had highest phosphate solubilizing efficiency (172.22) whereas the maximum phosphate solubilization through quantitative estimation was shown by VS₉ (664.33 µg/ml). VS₆ bacterial isolate showed maximum inhibition of growth against *Fusarium oxysporum* (90.57%), VS₂ against *Rhizoctonia solani* (82.93%) and VS₉ against *Pythium* (86.00%). VS₉ produced significantly higher concentration of IAA (29.67 µg/ml) after 72 hour of incubation followed by VS₆ and VS₂. Quantitative estimation of siderophore using chrome-azurol-S (CAS) liquid assay revealed that bacterial isolate VS₂ produced maximum (68.07%) siderophore unit at 72 hour of incubation.

Net house experiments (Table 4)

Initial status of soil: The initial status of the soil revealed that soil was normal (optimum for most crops) with pH 6.76, EC (0.47 ds/m), organic carbon (1.21 g/kg), bulk density (1.15 mg/m³), particle density (2.33 mg/m³) and pore space (51.32%) with good water holding capacity (36.47%). The available N, P and K were 320.60, 233.74 and 126.00 kg/ha respectively.

Plant parameters: Among three isolates maximum per cent increase in shoot length (60.30), root length (83.73), shoot dry weight (81.06) and root dry weight (89.25) was shown by VS₉ inoculation.

It is evident from Fig 3 that the treated seedlings showed a significant increase in plant parameters such as number of leaves and nodes and leaf area as compared to control after the completion of the trial.

NPK content: The maximum per cent increase in nitrogen (18.96), phosphorus (15.63) and potassium (19.00) content in shoot biomass was observed in seedlings treated with bacterial isolate VS₉.

Soil status at the end of experiment

The data represented in Table 5 indicate that the bacterial isolates did not influence the soil pH significantly over untreated control. The EC value was found maximum (0.47 dS/m) in soil inoculated with VS₂. Soil treated with isolate VS₂ had significantly higher organic carbon (1.20 g/kg) than uninoculated (1.13 g/kg) soil and maximum bulk density (1.19 mg/m³). But particle density was maximum (2.35 mg/m³) in soil treated with bacterial isolate VS₉.

The pore space was maximum in the soil with bacterial isolate VS₉ (53.73%) while maximum water holding capacity (38.11%) was recorded in soil inoculated with isolate VS₂.

The maximum nitrogen (343.40 kg/ha), phosphorus (149.20 kg/ha) and potassium (248.00 kg/ha) was recorded in soil inoculated with isolates VS₉, VS₆ and VS₉ respectively (Table 4). It can be concluded (Fig 4) that there was a significant increase in NPK content of soil over initial value.

DISCUSSION

Out of all purified isolates VS₂, VS₆ and VS₉ were found to possess maximum plant growth promoting attributes and had good prospects to be used as biofertilizers for quality production and growth of cherry seedlings. The results are supported by the similar other studies which have demonstrated the various plant growth promoting properties of isolates such as P-solubilization and siderophore (Neilands 1981), HCN (Voisard et al 1989), auxin production (Beneduzi et al 2008) and growth on N-free medium and antagonism against fungal pathogens (Giacomodonato et al 2001). The beneficial role of plant growth promoting bacteria in plant growth by different mechanisms has also been reported (Ashrafuzzaman et al 2009, Rostamikia 2016).

Inoculation of crop plants with plant growth promoting rhizobacteria is a contemporary agricultural practice used to improve crop yield. The seedling treatment with selected bacterial cultures under net house conditions showed increase in shoot length, shoot

Table 3. Plant growth promoting activities of selected bacterial isolates

Isolate	P-solubilization		Antifungal activity (% growth inhibition)			Indole-3-acetic acid (µg/ml)	Siderophore activity	
	P-solubilization efficiency (%)	P-solubilization in liquid medium (µg/ml)	<i>F oxysporum</i>	<i>R solani</i>	<i>P aphanidermatum</i>		Zone size (mm)	Siderophore unit (%)
VS ₂	147.22	447	83.83	82.93	83.87	25.5	8.00	68.07
VS ₆	172.22	567.67	90.57	79.13	82.10	23.00	12.67	19.42
VS ₉	140.65	664.33	88.80	78.33	86.00	29.67	14.67	33.03
CD _{0.05}	0.39	102.50	1.08	1.98	0.70	3.43	3.59	12.11

Table 4. Per cent increase in shoot and root parameters and NPK content of shoot under net house conditions

Isolate	% increase in shoot and root parameters over control				% increase in NPK in shoot biomass over control			
	Shoot		Root		N		P	
	length	dry weight	length	dry weight				
VS ₂	40.02	50.60	19.83	36.25	15.09	6.25	12.06	
VS ₆	54.24	67.75	64.50	71.62	16.81	12.50	16.08	
VS ₉	60.30	81.06	83.73	89.25	18.96	15.63	19.00	
CD _{0.05}	10.13	17.80	16.31	12.56	0.17	0.17	0.29	

Table 5. Effect of liquid-based culture of selected bacterial isolates on physico-chemical properties and NPK content of soil

Isolate	Physico-chemical properties of soil						NPK content of soil (kg/ha)		
	pH	Electric conductivity (dS/m)	Organic carbon (g/kg)	Bulk density (mg/m ³)	Particle density (mg/m ³)	Pore space (%)	Maximum water holding capacity (%)	N	P
Control	6.79	0.45	1.13	1.17	2.30	53.47	36.43	328.10	133.50
VS ₂	6.85	0.47	1.20	1.19	2.33	50.93	38.11	338.50	141.60
VS ₆	6.87	0.45	1.07	1.16	2.31	51.30	36.96	336.10	149.20
VS ₉	6.74	0.44	1.13	1.18	2.35	53.73	36.50	343.40	139.50
CD _{0.05}	0.18	0.04	0.05	0.04	0.09	0.86	0.56	2.22	1.61
									2.67

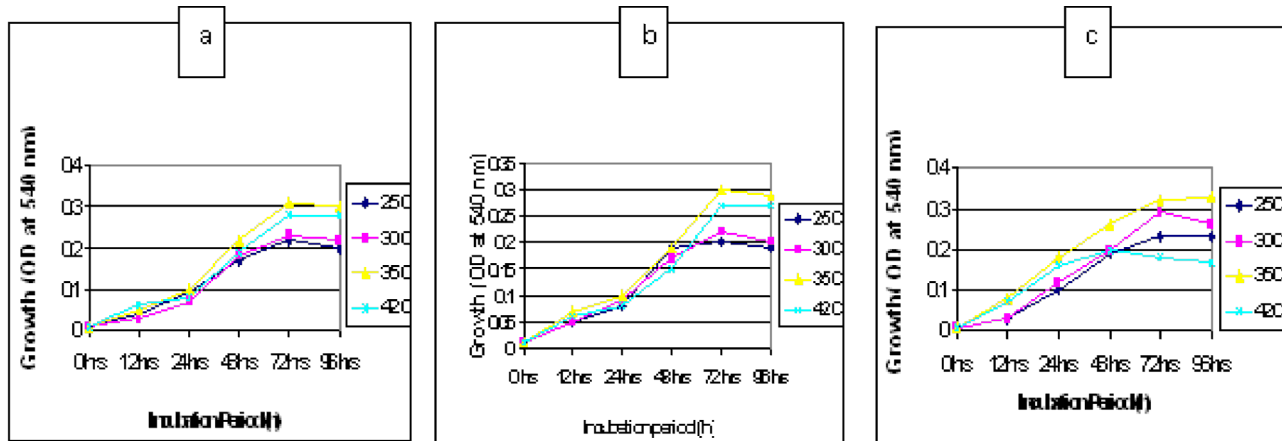


Fig 1. Effect of different temperatures on the growth of selected isolates of (a) VS₂ (b) VS₆ and (c) VS₉

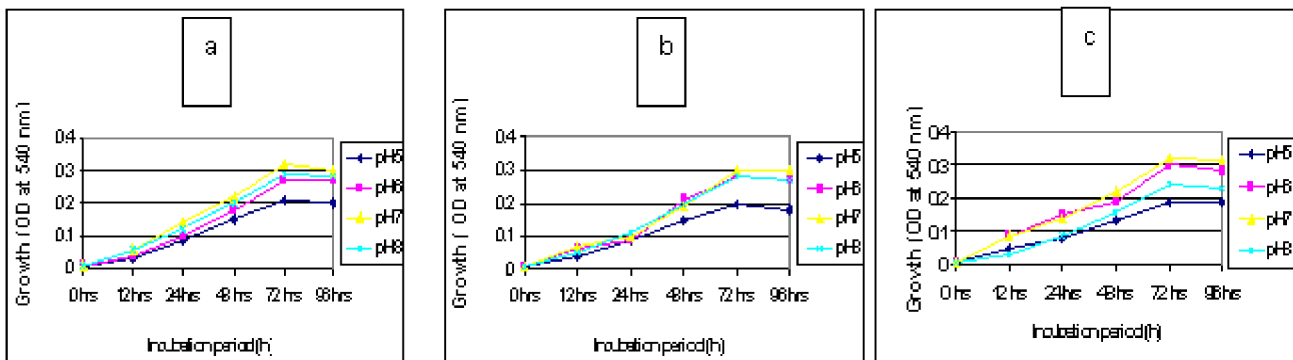


Fig 2. Effect of different pH values on the growth of selected isolates (a) VS₂ (b) VS₆ and (c) VS₉

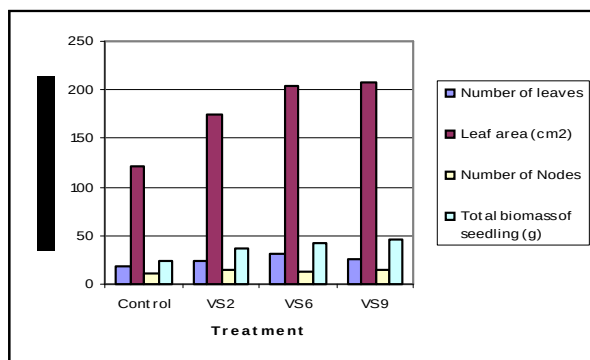


Fig 3. Increase in various plant parameters

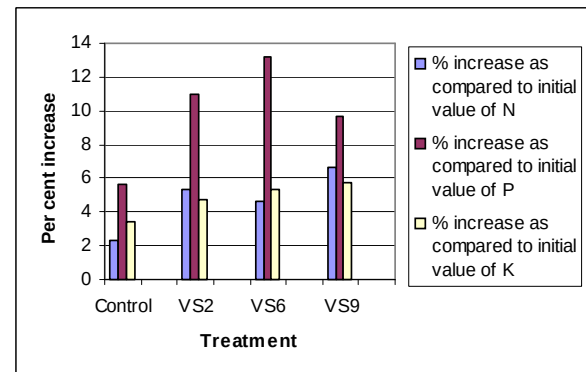


Fig 4. Increase in NPK of soil over initial value

dry weight, root length and root dry weight over uninoculated control. These results are in accordance with those of Singh et al (2008) who also reported that the plants treated with *Bacillus subtilis* BN1 isolate showed stimulatory effects on all plant vegetative parameters. Increase in the growth of shoot and root as compared to uninoculated control may be attributed to the production of plant growth regulators by bacterial isolates (Janzen et al 1992).

The inoculation of seedlings with different bacterial cultures not only improved their growth but also increased the uptake of nitrogen, phosphorus and potassium significantly. This increase may be attributed to nitrogen fixation, phosphate solubilization and increase in root length and volume of soil forage by roots. It was notable that larger the total rhizosphere population more was the accumulation of nitrogen,

phosphorus and potassium content in plants. Certain plant growth promoting rhizobacteria affect the development and function of roots by improving uptake of minerals such as nitrate and potassium and water uptake (Tilak et al 2005) and phosphate through organic acid secretion and phosphatases production (Kim et al 1997, Richardson 2001). In earlier studies it has been found that phosphorus uptake increases significantly (13-23%) in rice when inoculated with plant growth promoting rhizobial isolate (Biswas et al 2000). PGPR have been referred to have great impact on nitrogen nutrition by increasing NO_3^- uptake capacity indirectly as a consequence of stimulated lateral root development and possibly directly by stimulating NO_3^- transport systems (Mantelin and Touraine 2003).

There was no significant difference in pH, EC, OC, bulk density, particle density and water holding capacity of soil from the initial value. The available nitrogen and available phosphorus were in medium whereas potassium was in high range. Available NPK content of soil treated with bacterial isolates increased by 2.29-6.64, 5.62-13.62 and 3.45-5.75 per cent respectively over control. These results are in conformity with those of Talatam (2001). Zhang et al (2006) showed that different environmental parameters, content of soil organic carbon, total nitrogen and altitude could affect the diversity of soil flora including nitrogen-fixing bacteria.

CONCLUSION

The studies revealed that all three selected PGPR isolates were consistent in improving the different growth parameters of sweet cherry seedlings which may imply that isolates could be used as PGPR for enhancing the growth and yield of the crop in the field conditions. This process not only compensates the higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to the soil.

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