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Isolation, enumeration and identification of rhizospheric and endophytic *Bacillus* sp. associated with *Prunus avium* L. (Sweet cherry)

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Abstract

The region around the root, the rhizosphere, is relatively rich in nutrients. Consequently, it supports large and active microbial population capable of exerting beneficial, neutral or detrimental effects on plant growth. Such bacteria colonizing plant roots are known as rhizobacteria. Endophytic bacteria live in the plant tissue and are considered potential biocontrol agents. Plant growth promoting rhizobacteria (PGPR) are very small portion of rhizobacteria (2-5%) that colonise plant roots and enhance plant growth by wide varieties of mechanisms. The most predominant rhizosphere colonizing bacteria belongs to *Pseudomonas* and *Bacillus* species because of their association with soil organic matter, nutritional diversity and rapid growth rate. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. Hence, we have isolated, enumerated and identified *Bacillus* sp. from rhizosphere and roots of *Prunus avium* L. (sweet cherry) for the formulation of PGPR inoculum. Isolation of the microorganisms was carried out from the rhizosphere and roots of the sweet cheery collected from different locations of Distt. Shimla, Himachal Pradesh, India. In total fourteen isolates were selected, out of which seven belonged to rhizosphere and rest seven were endophytic. The isolates were morphologically, physiologically and biochemically characterized by specific tests. According to characterization the bacterial isolates were found to be of *Bacillus* sp. which further can be investigated for their plant growth promoting activities for the preparation of an effective inoculum for the cherry plants.

Introduction

In the present era, harmful microorganisms affecting plant health is one of the foremost and constant threats to food production and ecosystem stability. Indiscriminate use of chemical inputs to control pests and diseases or to provide nutrition to the crop have several negative effects i.e. development of pathogen resistance to the applied agents and their non-target environmental impacts (De Weger *et al.*, 1987). Furthermore, the growing costs of pesticides/ fertilizers particularly in less affluent regions of the world and consumer demand for organic food has led to a search for supplementation substitution for these inputs with organic or bio-degradable products (Georgakopoulos *et al.*, 1994).

Root colonizing bacteria (rhizobacteria) that exert beneficial effect on plant development has been defined as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). These microorganisms may enhance plant growth via direct influence (increased solubilization and uptake of nutrients or production of plant growth regulators), or by indirect mechanism (suppression of growth of plant pathogen by producing antibiotics, siderophores, extracellular enzymes of by inducing systemic resistance) (Glick *et al.,* 1994; Kloepper *et al.,* 1989).

As the crop production has intensified over the past few decades, producers have become more and more dependent on chemical inputs/ synthetic inputs as a relatively reliable method of crop production. This indiscriminate use of chemical inputs has several negative environmental effects. So, in present scenario, one of the most acceptable and environmentally conscious approaches to solve these problems is to manipulate crop rhizosphere population by inoculating beneficial bacteria (Nelson, 2004).

Considering the above problems in the present time of organic farming, there is a need of ecofriendly, low cost renewable sources of nutrients and biological antagonists to increase the crop productivity and to sustain soil health. In the absence of commercial PGPR inoculant for cherry plants, it is important to screen the diversity of PGPR associated with the cherry plant in its natural habitat to have efficient inoculum for commercial application. Keeping this in view the present study is aimed to isolate, enumerate and identify rhizospheric and endophytic *Bacillus* sp. associated with *Prunus avium* L. (sweet cherry).

Material and method

Isolation and enumeration of the microorganisms

Collection of samples

Isolation of the microorganisms was carried out from the rhizosphic soil and root samples of the *Prunus avium* L. (sweet cherry) collected from the main zone and it's cultivation by selection four sites namely

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Kotkhai, Baghi, Nankhari, Kotgarh in the Distt. Shimla, Himachal Pradesh, India.

Isolation of microorganism from rhizosphere soil

One gram of the rhizosphere soil was placed in 9 ml of sterilized distilled water under aseptic conditions. The soil suspension was diluted in 10 fold series and the microbial count was determined by the standard spread plate technique (Subba Rao, 1999).

Isolation of endorhizobacteria

The 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. The surface sterility of roots were crosschecked by incubating the surface sterilized roots in nutrient agar medium overnight. One gram of surface sterilized root sample was placed in 9 ml of sterilized distilled water and was crushed to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in 10 fold series and bacterial count was determined by standard spread plate technique (Subba Rao, 1999).

Identification of selected bacterial antagonist

On the basis of morphological, cultural and biochemical characteristics by criteria of Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) the identification of bacterial isolates were performed.

Characterization of bacterial antagonist

Effect of temperature on growth

100 ml of nutrient broth was taken in a conical flask and was inoculated with 10 per cent of 72 h old bacterial suspension and the growth curves were drawn by growing the culture at various temperatures (4ºC, 25ºC, 30ºC, 35ºC, 45ºC).

Effect of pH on growth

100 ml of nutrient broth was taken in a conical flask. The medium was adjusted to various pH (4,5, 6, 7and 8) using 1 N NaOH or 1 N HCl as the case may be. Each tubes were inoculated with 10 per cent of 72 h old bacterial cell suspension and the growth of the isolate at different pH was studied.

Results

Population densities associated with rhizosphere soil of Cherry seedlings

The results presented in table 1 reveals that the rhizosphere soil of seedlings collected from all the four sites had bacteria capable of growth on nutrient agar (NA), Nitrogen free medium (Jensen's medium), soil extract medium and Pikovskaya's (PVK) medium. The colony forming units (cfu) for rhizosphere soils were rather different for the different locations and varied with the medium used for the enumeration. Soil extract medium supported the growth of large population of bacteria followed by Nutrient agar medium, Pikovskaya's medium and then Nitrogen free medium.

Table 1. *Enumeration of bacterial population in rhizosphere of cherry plants*

Locations	Rhizosphere soil bacterial population(10^4 × cfu/g soil)			
	Nutrient agar medium	Nitrogen free Jensen's medium	Soil Extract Medium	Number of colonies on PVK medium
Kotkhai	204.00	72.00	274.00	85.33
Baghi	220.33	52.33	264.33	85.00
Nankhari	286.67	73.67	295.00	103.00
Kotgrah	185.33	69.00	258.33	105.67

Endophytic bacterial population by surface sterilization and dilution technique

The results (Table 2) reveals that the roots of plants collected from all the sites harboured bacteria capable of growth on different media. The colony forming units (cfu) for roots were also different for different locations and varied with the medium used for the enumeration. The endorhizobacterial population was more on nutrient agar medium followed by Soil extract medium, Pikovskaya's medium and Nitrogen free medium.

Table 2. *Enumeration of endophytic bacterial population associated with cherry plants*

Locations	Rhizosphere soil bacterial population(10^4 × cfu/g soil)			
	Nutrient agar medium	Nitrogen free Jensen's medium	Soil Extract Medium	Number of colonies on PVK medium
Kotkhai	67.33	34.33	55.33	43.00
Baghi	63.33	42.67	42.00	47.33
Nankhari	95.67	43.33	47.67	53.67
Kotgrah	105.00	46.00	58.00	54.33

Morphological, physiological and biochemical characteristics of selected bacterial isolates

Morphological properties of bacterial isolates

The results (Table 3) indicate colony morphology, Gram's reaction and cell shape of selected bacterial isolates. All bacterial isolates showed positive gram's reaction except VS_1, VS_5 and VS_7. It was also noted that the selected bacterial isolates were rod shaped.

Table 3. *Morphological characteristics of selected bacterial isolates from cherry plants*

Isolates	Form	Elevation	Margin	Surface	Gram's reaction	Shape
VS_1	Irregular	Flat	Entire	Smooth	-	Rods
VS_2	Circular	Raised	Undulate	Rough	+	Rods
VS_3	Circular	Raised	Entire	Smooth	+	Rods
VS_4	Irregular	Raised	Undulate	Smooth	+	Rods
VS_5	Circular	Convex	Entire	Smooth	-	Rods
VS_6	Irregular	Flat	Erose	Rough	+	Rods
VS_7	Irregular	Raised	Undulate	Smooth	-	Rods
*VS_8	Irregular	Flat	Entire	Rough	+	Rods
*VS_9	Irregular	Flat	Erose	Rough	+	Rods
*VS_{10}	Circular	Undulate	Undulate	Rough	+	Rods
*VS_{11}	Irregular	Convex	Erose	Smooth	+	Rods
*VS_{12}	Circular	Flat	Lobate	Rough	+	Rods
*VS_{13}	Irregular	Convex	Entire	Smooth	+	Rods
*VS_{14}	Irregular	Umbonate	Entire	Smooth	+	Rods

* Endophytic

Biochemical and physiological properties of bacterial isolates

Table 4 represents biochemical characteristics of the bacterial isolates. All the bacterial isolates showed positive results for catalase test, oxidase test, fermentation metabolism of glucose and gelatine liquification test. Where as denitrification test was shown positive by all the isolates except $VS_{10,}$ VS_{11} and $VS_{13.}$

The results in table 4 shows that the selected bacterial isolates were grown at different temperature (4^0C, 25^0C, 30^0C, 35^0C, and 45^0C). The isolates grew well in temperature range of 25^0C - 35^0C. The growth was static at 5^0C and 45^0C. The optimum temperature of growth was found to be 35^0C. The rate of growth increased with increase in temperature from 20^0C to 35^0C and revealed by the decrease in growth during the increase in inoculation period from 24 to 72 hours.

The bacterial isolates were able to grow over a wide range of pH. Bacterial growth increases gradually with increase in pH from 6-8 up to 72 hours of incubation. Growth at pH 9 was not seen in any case of all the selected bacterial isolates.

Table 4. *Biochemical and physiological characterization of selected bacterial isolates*

Isola-tes	Catalase	Oxidase	Fermentation metabolism of glucose	Gelatin Liquific-ation test	Denitrific-ation	Growth at temperature					Growth at pH				
						4°C	25°C	30°C	35°C	45°C	4	6	7	8	9
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

Discussion

Globally sweet cherries are produced commercially in 65 countries with a production of 2.3 million metric tons (MMT), (Anonymous, 2004). In India, cherries are commercially grown in the states of Jammu and Kashmir and Himachal Pradesh. In Himachal Pradesh 2000 hectares of land is under cherry cultivation and the production is 438 tons (Anonymous, 2009).

Indiscriminate use of chemical fertilizers and use of pesticides against various soil borne disease of cherry adds to the cost of production and environmental pollution. Therefore, plant growth promoting rhizobacteria (PGPR), which can supply various nutrients such as NPK, plant regulators (IAA)

and biological control of soil borne diseases via siderophore and antibiotic production, may have great potential to improve the production.

The beneficial effects of the microorganisms have been studied for several crops (Lazarovits and Nowak, 1997). However, such studies on root associated bacteria have not been conducted with sweet cherry in Himachal Pradesh. Therefore, an attempt has been made to isolate, enumerate and identify *Bacillus* sp. associated with cherry seedlings.

The data represent the variation in the microbial population both in rhizosphere and roots of sweet cherry. The total microbial population in general was more in rhizosphere as compared to roots. The results are in line with those of Shishido *et al.,* (1999) who has also reported greatest variation in microbial population with respect to location/plant parts for isolation purpose. The variation in the population of both rhizosphere soil bacteria and endophytes may be attributed to source, location, age of plant, variety/ cultivar type, time of sampling, physico-chemical properties of soil and environment conditions.

The rhizosphere and phyllosphere of the plants under natural conditions harbours a large and varied population of the microorganisms. It has been demonstrated that bacteria belonging to genera *Bacillus* and *Pseudomonas*, not only proliferate rhizosphere, but also reside inside the root tissue of agriculturally important crop species (Hallmann *et al.,* 1997). The endophytic bacterium actually resides within apoplastic spaces inside the host plant and there are only few evidence of endophytes occupying intracellular spaces (An *et al.*, 2001).

As evident from the table 4, the isolated microorganisms from rhizospheric soil and roots are rod shaped, are positive for catalase, oxidase, fermentation metabolism of glucose and gelatine liquification tests. Rao (1999) also identified the *Bacillus* isolates on the basis of similar biochemical tests. Based on the colony morphology, microscopic observations, cultural, biochemical and physiological properties, the isolates were identified as belonging to genus *Bacillus sp*

Conclusion

The rhizosphere of the plants under natural conditions harbours a large and varied population of the microorganisms and bacteria belonging to genera *Bacillus* and *Pseudomonas*, not only proliferate in the rhizosphere, but also reside inside the root tissue of agriculturally important crop species.

An important factor to be considered when screening new isolates is their activity in the range of environments in which they would be expected to be used, in particular. For this reason it is important to study native strains isolated in the same region where they may be used as inoculants.

Taking this in to account, the effort was made to search for native strains from the rhizosphere of cherry for preparation of the better inoculums for the cherry crop.

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