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Plant growth promoting and antagonistic potential of indigenous PGPR from tomato seedlings grown in mid hill regions of Himachal Pradesh

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Abstract
A total of 859 rhizobacteria and 574 endorhizobacterial isolates were obtained from different tomato growing sites of Himachal Pradesh, India. Predominant population of rhizo and endorhizobacteria with respect to sites showed variations and their population growth was observed to be higher on nutrient agar than on Pikovskaya medium. All the isolates were screened for their plant growth promoting attributes and only 17 isolates were found to be efficient phosphate solubilizers with 15 mm or more halo zone formation on PVK medium. These efficient isolates were further screened for their ability to fix atmospheric nitrogen by growing them on nitrogen free medium as well as for their antifungal activity against two fungal pathogens of tomato i.e. *Fusarium oxysporum* and *Rhizoctonia solani*. Out of total, 58.82% were found to be nitrogen fixers while 52.94% bacteria showed inhibition against selected soil borne pathogens. Morphological and biochemical characterization showed colonization of *Bacillus* sp. along with *Pseudomonas* sp., *Enterobacter* sp., *Acinetobacter* sp. and some unidentified P-solubilizing bacteria in the tomato rhizosphere.

Keywords: Rhizobacteria, phosphate solubilization, antifungal activity

Introduction
Tomato (*Lycopersicon esculentum* Mill.) a member of Solanaceae family, is the second most cultivated vegetable crop after potato and India is one of the largest tomato producer of the world with a production of almost 1, 87, 35,900 metric tons (MT) on a cultivated area of 8, 82,000 ha (FAO, 2010) [13]. The area under tomato cultivation in HP is 29,420 ha with production of 6, 27,280 MT. Himachal Pradesh constitutes only 3.35% of the total tomato production in the country (NHB, 2014).

Sustainable tomato production is constrained worldwide by diseases and pests. *Rhizoctonia solani* and *Fusarium oxysporum* are major soil borne fungal pathogens of both green house and field grown tomatoes in the world. *Fusarium*s wilt of tomato caused by *Fusarium oxysporum*. f.sp. *lycopersici* and *Rhizoctonia solani* causing damping off, cankers, root rots, fruit decay, foliage disease, causes serious economic losses (Sasirekha *et al*. 2012) [31].

The endless variety and complexity of many plant diseases caused by fungi have led to the development of a correspondingly large number of fungicides; unfortunately several plant pathogens have developed resistance to certain fungicides (Agrios, 2005) [3]. Furthermore, use of chemicals has its adverse effects on the environment and the non-target organisms. Therefore, development of effective biological tools that may replace/supplement the highly prevalent agrochemicals i.e. chemical fertilizers and pesticides is necessary for advancement in the sustainable agriculture practices. Exploring PGPR for this endeavor has proved to be a promising strategy (Adesemoye *et al*. 2008; Zaidi *et al*. 2009) [2, 42].

Plant growth promoting rhizobacteria (PGPR) offer an environmentally sustainable approach to increase crop production and health. They impart beneficial effects on plants by colonizing roots and stimulating overall plant growth by improving seed germination, plant emergence, root development, mineral nutrition, water utilization and biocontrol of plant pathogens (Egamberdiyeva and Hoflich 2004; Siddiqui 2006) [12, 34]. It is therefore, imperative to isolate and screen indigenous strain of PGPR from tomato seedlings as plant growth promoting and antagonists, which can be used, as bio-inoculants to increase the growth and yield of this crop. In view of this different tomato growing regions falling under mid ranges of Himachal Pradesh, India were mined for isolation of efficient rhizobacterial isolates. The isolated rhizobacterial strains were further screened for their plant growth promoting and biocontrol potential against fungal pathogens of tomato.

Material and Methods

Study area and sample collection

Seven different tomato growing sites of Himachal Pradesh, India falling under mid hill regions were selected viz., Fagu (Rajgarh), Habbni pull, Tickri, Pandha, Gaura, Chail and Nando for the collection of rhizospheric soil and root samples. The composite soil/root samples were placed in plastic bags and stored under refrigerated conditions.

Isolation of rhizobacteria

From tomato rhizosphere, 10 gm of soil (rhizospheric/bulk) was weighed and mixed with 90 ml sterile distilled water in 250 ml conical flask. Flask was shaken vigorously for 5 to 10 min to form homogenous suspension. The soil solution was then allowed to settle for 10-15 min before further processing. The suspension was serially diluted and dilutions were plated (Wollum 1982) [41] on nutrient agar (NA) and Pikovskaya agar medium (PVK), separately with three replicates every time. Plates were incubated at 28±2 ℃ for 24-72 h and counts were expressed in terms of cfu g^{-1}.

Isolation of endorhizobacteria

For isolation of endo-rhizobacteria, roots were washed thoroughly under tap water to remove adherent soil particle and then surface sterilized by immersing them in 80% ethanol for 3-4 min. Roots were then dipped in sterile 0.05 M phosphate buffer, pH 7.0 for 30 min and finally, washed 3 times with distilled water. Roots were then cut into small pieces and macerated in the same buffer using sterilized mortar and pestle (Patel and Desai 2015) [26]. The suspension was serially diluted to 10 folds and bacterial counts were expressed in terms of cfu g^{-1}.

Qualitative phosphate Solubilization

Primary phosphate solubilizing activity of bacterial isolates was tested by streaking on the Pikovskaya's agar plates and plates were incubated at 37 °C for 72 h. The bacterial solubilization of phosphorus was exhibited with yellow coloured zone produced around the isolated bacterial colony (Pikovskaya's 1948) [27].

Quantitative estimation of phosphate solubilization

Quantitative estimation of P solubilization by the potential bacterial isolates was done by inoculating 50 ml of National Botanical Research Institute's Phosphate (NBRIP) broth (Nautiyal 1999) [25], supplemented with 0.5 per cent of tricalcium phosphate (TCP) and incubated for 11 days under shaken conditions at 28 ± 2 °C. The inorganic phosphate content in the supernantant was spectrophotometrically determined by vanado-molybdate yellow color method (Jackson 1973) [17]. The amount of P solubilized was extrapolated from the standard curve of potassium dihydrogen orthophosphate and expressed as µg ml^{-1} over the control.

Ability to grow on nitrogen free medium

A loopful of 24 h old culture of each isolate were streaked on nitrogen free glucose agar medium and incubated at 28 ± 2 °C for 24-28 h. The growth of bacteria on plates indicated nitrogen fixing ability of the isolates (Jensen, 1987) [18].

Antagonistic activity of bacteria against fungal pathogens

Antagonistic activity of bacterial isolates was tested against *Fusarium oxysporum* and *Rhizoctonia solani* using agar streak method (Berg *et al*. 2000) [8]. Rhizobacterial isolates were streaked on one edge of potato dextrose agar (PDA) plates containing 5 mm of mycelial plugs at the centre and growth inhibition was calculated after 5 to 7 days of incubation at 28 ± 2 °C for 5 days by using method described by Vincent, (1947) [39].

Morphological and metabolic characterization of bacterial isolates

The morpho-biochemical characterization of bacterial isolates was done on the basis of colony morphology, microscopic examination and biochemical tests. Characterization was made according to Bergey's Manual of Systematic Bacteriology (Vos *et al*., 2009) [40].

Results

Isolation and enumeration of rhizo and endorhizospheric bacteria

Soil and tomato root samples collected from different sites were subjected to isolation and enumeration on nutrient agar and Pikovskaya's agar medium and results were expressed as cfu g^{-1} of whole soil and root system.

Rhizospheric bacterial population was found to be highest for Pandha (200×10^5 cfu g^{-1} soil) while Chail had the lowest rhizobacterial count (50×10^5 cfu g^{-1} soil) on nutrient agar medium (Table 1). The percentage of phosphate solubilizing bacteria for different sites was ranged between 32-100%, with highest percentage at Chail as well as Nando (100%) and lowest at Gaura (32%). Except Fagu and Gaura, the other five locations had 50% or more P-solubilizing bacterial population.

The endorhizobacterial population was more abundant on nutrient agar than Pikovskaya medium thereby indicating that nutrient agar was more appropriate for isolation of endophytic bacteria than Pikovskaya medium. On nutrient agar, highest endorhizobacterial population (200×10^5 cfu g^{-1}) was obtained at Fagu and lowest at Gaura (19×10^5 cfu g^{-1}). Similarly on Pikovskaya's agar, maximum endorhizobacterial population was obtained from Fagu (150×10^5 cfu g^{-1}) and no endorhizobacteria were obtained from Habbni pull, Tickri and Pandha which may be attributed to the absence of substrate at these locations. Clear halo zone forming P-solubilizing bacteria were found highest at Gaura and Chail (100 %) followed by Fagu (93%) and Nando (88%).

Plant growth promoting characteristics of bacterial isolates

Phosphate-solubilizing bacteria (PSB) have been considered as one of the possible alternatives for mediating inorganic phosphate solubilization and increasing its availability to the plants. Out of 278 P- solubilizing rhizobacteria and 200 endorhizobacteria, only 17 isolates were found to be potential P-solubilizers and showed a halo zone formation of more than 15 mm on PVK agar (data not shown). These 17 efficient P-solubilizing bacteria were further screened for quantitative estimation of P solubilization using NBRIP broth supplemented with 0.5 percent TCP upto 11 days of incubation (Table 3).

The solubilization of TCP by these isolates ranged between 112.21-667.17 µg ml^{-1} (Table 3). A general trend of increase in P-solubilization was observed upto 5-7th days of incubation and thereafter a decrease was observed. Significantly highest P solubilization of 667.17 µg ml^{-1} was shown by isolate TX-II after 7 days of incubation followed by PZ-I and HB-I showing P-solubilization of 601.33 and 557.17 µg ml^{-1} respectively. Minimum P-solubilization of 112.21 µg ml^{-1} was noted in isolate TX-III after 3 days of incubation.

The selected 17 P-solubilizing bacterial strains were screened for their ability to grow on nitrogen free medium and only 10 were able to grow on nitrogen free medium. The selected strains were also screened for *in vitro* antibiosis against fungal pathogens viz., *Fusarium oxysporum* and *Rhizoctonia solani* (Table 4). Out of seventeen, 10 isolates (58.82%) showed inhibitory action against *Fusarium oxysporum* and nine isolates (52.94%) inhibited *Rhizoctonia solani*. Against *Fusarium oxysporum*, highest antagonistic activity was exhibited by strain TX-II (62.00%), whereas against *Rhizoctonia solani*, highest inhibition was given by strain PZ-I (54.44%).

Morpho-biochemical characterization of efficient PSB isolates

Isolates with maximum phosphate soulbization efficiency were characterized tentatively on the basis of morphological and biochemical characteristics (Table 5). The majority of isolates were gram positive, rod shaped, motile and endospore forming bacteria. All these isolates were positive for catalase and negative for MR tests. Bacterial isolates were tentatively identified as species of *Pseudomonas, Bacillus, Enterobacter, Enterococcus, Acinetobacter* as per the criteria of Bergey's Mannual of Systematic Bacteriology. However, few species remained unidentified.

Discussion

Rhizosphere is a war field of interactions between microorganisms and plant roots. This region of soil is much richer in bacteria than the surrounding bulk soil (Hiltner 1904) [15]. Rhizobacteria are considered as the most important community microbiota that increased the plant growth basically by changing the whole microbial community structure and protect the plant roots against various phytopathogens (Benizri *et al*. 2001) [7]. Mid hill tomato growing regions of Himachal Pradesh, India are little explored with respect to the PGPR diversity. So far there have been negligible attempts made to study the plant growth promoting traits and diversity of PGPR associated with tomato grown in mid hill regions of the state.

Therefore in the present investigation, rhizobacteria and endo rhizobacteria were isolated from tomato soil and root samples growing in seven different sites of Solan and Sirmaur Districts of Himachal Pradesh, India. A total of 859 rhizobacteria and 574 endorhizobacteria were isolated on NA agar out of which 278 rhizobacteria and 200 endorhizospheric bacteria were found to be P-solubilizers. Phosphate solubilizing bacteria (PSB) enhance the P availability to plants by mineralizing organic P and solubilizing precipitated phosphates present in the soil (Rodriguez *et al*. 2006) [30]. In the present study a high percentage of P-solubilizing rhizobacteria might be corroborated with rich abundance of insoluble P substrate in the sampling sites (Arjun and Harikrishnan, 2011) [5]. Clear halo zone forming P-solubilizing bacteria were found to be highest at Gaura and Chail followed by Fagu and Nando. Out of 278 P-solubilizing rhizobacteria and 200 endorhizobacteria, only 17 were efficient P-solubilizers and showed a clear halo zone of more than 15 mm on PVK agar. The diameter of halo zone depends upon nature, quantity and diffusion rate of organic acids produced by the microorganisms into the surrounding medium (Vessey, 2003) [38]. These 17 efficient P-solubilizing bacteria were further screened for quantitative estimation of P solubilization using NBRIP broth.

Ranjan *et al.* (2013) [28] also isolated PSBs from different soil samples and selected 12 efficient PSB isolates on their ability to form clear zone on Pikovskaya's agar medium. The P-solubilization was ranged between 218.2-667.1 µg/ml. Our results are in accordance with the Nautiyal *et al*. (2000) [24] who observed TCP solubilization in the range of 200-450 µg ml^{-1} by P-solubilizing bacteria from alkaline soils of tropical India. Decrease in the solubilized P-concentration could be due to the higher microbial uptake rate than its solubilization during the later period of incubation and also due to the depletion of substrate itself (Rodriguez and Fraga 1999) [29]. The re-immobilization of a portion of soluble P as reported by Chun-qiao *et al*. (2009) [11] could also be the reason of decrease in P concentration in the later stage of incubation.

In the present study, the P-solubilizing bacterial isolates also showed inhibition of phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani*. Growth inhibition of phytopathogens may be attributed to production of antibiotics (Kavroulakis *et al*. 2010) [19], extracellular enzymes (Amaresan *et al*. 2012) [4], induced systemic resistance (Liu *et al*. 1996) [21], and biofilm formation (Haggag & Timmusk, 2008) [14]. Similar to our results, Sharma *et al*. (2015; 2016) reported *Bacillus subtilis* strains S_{25} and 2a1 showing inhibition of various phytopathogenic fungi.

Various morphological and biochemical tests are necessary for the precise identification of PGPR to the species level. All isolates represented morphologically different colonies (Table 5). These selected morphotypes represent the most dominant rhizobacterial population. Although applicable to a small number of bacterial species, the use of colony morphotype having marked characteristics and biochemical tests are of ecological relevation to obtain information on the distribution of rhizobacteria in the rhizosphere environment and root tissues of tomato plants. On the basis of morphological and biochemical characteristics most of the isolates were tentatively identified as *Bacillus* sp. followed by *Pseudomonas* sp. The occurance of *Bacillus* sp. is in agreement with the previous reports where *Bacillus* sp. has been frequently isolated from tomato (Suarez *et al*. 2008; Banerjee *et al*. 2010) [36, 6]. The predominance of *Bacillus* is due to its ability to efficiently use the nutrients provided by plants through exudates, including that root exudates exert a selective pressure on the proliferation of specific group of bacteria. Besides this Enterobacter and Acinetobacter were isolated and some strains were not identified. Similar to our work, Abdeljalil *et al*. (2016) [1] isolated 25 efficient PGPR from tomato rhizosphere which were subjected to morpho-biochemical identification. Most of the strains were belonging to genus *Bacillus*. Besides this *Chryseobacterium*, *Enterobacter*, and *Klebsiella* were also isolated. Type of plant species also had profound effects on microbial community dynamics, with the effect of soil type typically exceeding that of plant type (McSpadden Gardener, 2004) [22]. Physiological traits, such as multilayered cell wall, stress resistant endospore formation, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes, contribute bacilli to their survival under different environmental conditions for extended periods of time.

Table 1: Enumeration of culturable rhizospheric bacterial population associated with tomato seedlings from different locations

Location	Rhizosphere soil bacteria population (× 10^5 cfu g^{-1})			Percentage of P-solubilizers (%)
	Nutrient agar (NA)	Pikovskaya agar (PVK)	P-solubilizers	
Fagu	125	116	43	37.07
Habbni pull	118	102	80	78.43
Tickri	100	32	16	50.00
Pandha	200	80	68	85.00
Gaura	195	100	32	32.00
Chail	50	21	21	100.00
Nando	71	18	18	100.00

Table 2: Endophytic bacterial population associated with associated with tomato seedlings from different locations

Location	Endorhizobacterial population (×10^5 cfu g^{-1} roots)			Percentage of P-solubilizers (%)
	Nutrient agar (NA)	Pikovskaya agar (PVK)	P-solubilizers	
Fagu	200	150	140	93.00
Habbni pull	-	-	-	-
Tickri	150	-	-	-
Pandha	100	-	-	-
Gaura	19	12	12	100.00
Chail	20	2	2	100.00
Nando	85	52	46	88.00

Table 3: Quantitative estimation of phosphate solubilization exhibited by bacterial isolates in NBRIP broth at different days of incubation

Isolates	Phosphate solubilization (µg ml^{-1})				
	Days of incubation				
	3	5	7	9	11
FR-II	283.00	405.83	342.17	326.33	325.71
H-I	143.83	219.33	278.42	273.00	235.08
HB-I	298.00	557.17	545.08	471.33	412.17
HB-II	218.00	270.08	253.11	235.08	235.00
HB-III	160.23	218.29	341.56	245.51	181.22
TX-I	170.50	235.08	308.83	294.00	218.00
TX-II	457.17	545.08	667.17	607.58	553.00
TX-III	112.21	190.76	218.0	215.67	147.23
PX	267.58	290.64	370.50	368.83	324.25
PZ-I	311.65	495.08	601.33	515.5	459.23
PZ-III	475.43	512.58	460.22	331.20	215.65
G-II	368.83	432.58	370.08	293.82	161.11
G-IV	162.83	229.33	268.42	239.00	205.08
CH-I	190.50	225.08	278.83	204.00	118.00
CH-III	217.00	357.17	345.08	271.33	212.17
CH-IV	167.58	190.64	270.50	268.83	224.25
ND-II	275.43	312.58	260.22	231.20	175.65

Table 4: Nitrogen fixing and antagonistic activities of bacterial isolates against fungal pathogens

Isolates	Growth on nitrogen free medium	Antifungal activity (% GI)	
		Fusarium oxysporum	*Rhizoctonia solani*
FR-II	+	54.44	42.22
H-I	ND	51.11	ND
HB-I	+	46.66	46.66
HB-II	ND	38.88	ND
HB-III	+	ND	ND
TX-I	ND	ND	44.44
TX-II	+	62.00	42.22
TX-III	+	42.22	42.22
PX	ND	ND	ND
PZ-I	+	ND	54.44
PZ-III	ND	54.44	51.11
G-II	+	60.00	ND
G-IV	ND	ND	ND
CH-I	ND	ND	ND
CH-III	+	42.22	ND
CH-IV	+	42.22	46.66
ND-II	+	ND	51.11

Table 5: Morphological and biochemical characterization of bacterial isolates against fungal pathogens

Isolates	Colony characteristics	Biochemical tests									Probable identification
		Gram reaction	MR	VP	Indole	Citrate	Oxidase	Catalase	Urease	Starch	
FR-II	Small, circular, raised	+	-	+	-	-	+	+	-	+	*Bacillus* sp.
H-I	Large, rhizoidal, raised	-	-	-	-	+	+	+	-	-	*Pseudomonas* sp.
HB-I	Large, flat, smooth	+	-	+	-	+	+	+	+	-	*Bacillus* sp.
HB-II	Small, rhizoidal, raised	+	-	+	-	-	+	+	-	+	*Bacillus* sp.
HB-III	Large, rhizoidal, raised	_	_	+	-	+	-	+	-	-	*Enterobacter* sp.
TX-I	Large, glistening, mucoid	+	_	+	_	+	_	+	-	+	*Bacillus* sp.
TX-II	Small, rough, rhizoidal	+	-	+	-	-	+	+	-	-	Unidentified
TX-III	Small, circular, flat	+	-	+	-	+	+	+	-	+	Unidentified
PX	Small, circular, rough	+	-	-	-	+	-	+	-	-	*Acinetobacter* sp
PZ-I	Small, rhizoidal, lobate	+	-	+	-	+	-	+	-	+	*Bacillus* sp.
PZ-III	Small, rhizoidal, raised	+	-	+	-	+	+	+	-	+	*Bacillus* sp.
G-II	Large, rough, raised	-	-	-	-	+	+	+	-	-	*Pseudomonas* sp
G-IV	Large, irregular, raised	+	-	+	-	-	-	+	+	-	*Bacillus* sp.
CH-I	Large, irregular, lobate	-	-	+	-	-	-	+	-	-	Unidentified
CH-III	Large, irregular, smooth, lobate	-	-	-	-	+	+	+	-	+	*Pseudomonas* sp
CH-IV	Large, circular, flat	+	-	+	-	+	-	+	-	-	*Bacillus* sp.
ND-II	Punctiform, flat, glistening	-	-	+	-	-	-	+	+	-	Unidentified

Conclusion

The results obtained for this study are quite encouraging in the way that many of the rhizobacterial and endorhizobacterial isolates exhibited strong in-vitro potential for P-solubilization and antifungal activity. Thus, these isolates can further be evaluated for more plant favorable characteristics as well as their response under field conditions so that these may be developed as successful soil-inoculants for improvement of plant growth.

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