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Isolation and screening of plant growth promoting *Bacillus* spp. and *Pseudomonas* spp. and their effect on growth, rhizospheric population and phosphorous concentration of *Aloe vera*

Deepshikha Thakur, Mohinder Kaur and Atul Mishra

Abstract
An attempt was made to isolate and characterize indigenous phosphate solubilizing strains of *Bacillus* and *Pseudomonas* spp. from the rhizosphere of *Aloe vera* from different agro climatic locations of Himachal Pradesh and evaluate their growth promoting effect on *Aloe vera*. On the basis of *in vitro* plant growth promoting activities, total six isolates, three belonging to *Bacillus* spp. (AvNB-1, AvSB-2 and AvSB-5) and three belonging to *Pseudomonas* spp. (AvHP-1, AvSP-1 and AvSP-7) were selected and used in the pot experiment to study their effect on growth of *Aloe vera*, rhizosphere bacterial population and phosphorus concentration of soil. On the basis of the pot experiment, *Pseudomonas* spp. (AvHP-1) and *Bacillus* spp. (AvSB-1) were found to be the best among all the strains.

Keywords: RAPD, *Bacillus, Pseudomonas, Aloe vera*

Introduction
Demand for medicinal plants are ever increasing as more and more people are fascinated towards plant based medicines. Therefore for meeting the continuously increasing demand of these medicinal plants species by the pharmaceutical industries, it is necessary to multiply these species on large scale with better field establishment and high productivity through the use of bio-based technologies like phosphate solubilizing plant growth promoting rhizoacteria (PGPR).

Aloe vera belongs to the family Xanthorrhoeaceae commonly known as Ghrit Kumari, is the oldest medicinal plant ever known and the most applied medicinal plant worldwide (Pandey and Singh, 2016) [1]. The aloe plant (*A. vera* Tourn. ex Linn. Syn. *A. barbadensis* Mill) is an important and historical medicinal plant, often called a "Doctor in pot" (Gjerstad and Riner 1968) [2]. Aloe species have been used for centuries for the laxative, anti-inflammatory, immunostimulant, antiseptic effects (Shivakumari *et al*. 2007; Mahor and Ali, 2016) [3, 4]. Effect of Plant growth promoting rhizobacteria on endangered medicinal plants *Holostemma ada-kodien* Schultes (Asclepiadaceae), commonly known as Jivanti has also been studied by Thanuja and Ambika, (2010) [5].

Plant Growth Promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield by a wide variety of mechanisms and also offers an attractive way to replace chemical fertilizers, pesticides, and supplements (Wu *et al*., 2005; Ashrafuzzaman *et al*., 2009 and Afzal and Bano, 2008) [6-8]. These bio-inoculants may be highly useful in increasing the growth and biomass yield of plant species (Cakmakci, 2005) [9]. Their application increases the availability of nutrients to plants, control the soil borne pathogens and maintain and sustain the soil fertility and will ultimately help in conservation of medicinal plants and their productivity. Keeping in view the above information the present study was conducted with objectives to isolation indigenous plant growth promoting bacteria from medicinal plants and tests their influence on the growth, rhizosphere bacterial population and phosphorus concentration of soil under *Aloe vera*.

To date, PGPRs have been shown to promote the growth of cereals, ornamentals, vegetables, and food crops (Vessey 2003; Lugtenberg and Kamilova 2009; Mishra *et al*. 2010) [10-12]. However, a limited number of studies have been undertaken regarding the effect of PGPRs on medicinal plants.

Materials and Methods

Isolation and Screening of the bacterial isolates for multifarious plant growth promoting activities

Rhizospheric soil samples of Aloe vera were collected from various locations of Himachal Pradesh i.e. University Forest Product medicinal plant field, University Research Center Neri (Hamirpur), Regional Horticultural Research station Jachh (Nurpur), Krishi Vigyan Kendra Saru (Chamba) and Baidynath medicinal field (Subathu). In total, eighteen isolates of *Bacillus* spp. and six isolates of *Pseudomonas* spp. were isolated on nutrient agar and Kings media respectively. The isolates were then screened for multifarious plant growth promoting activities such as phosphate solubilizing activity **(**Olsen *et al*., 1954) [13], IAA (Gorden and Paleg, 1957) [14] and biocontrol properties such as Siderophore production (Schwyn and Neilands, 1987) [15], Proteolytic activity, hydrogen cyanide (HCN) production (Bakker and Schippers, 1987) [16], antifungal activity of bacterial isolates (Fleming *et al*., 1975) [17] by their respective standard methods.

Evaluation of selected strains of *Bacillus* and *Pseudomonas* species for plant growth promoting activity *in vivo* (pot experiment)

On the basis of screening, total 6 bacterial strains, 3 isolates of *Bacillus* spp. (AvNB-1, AvSB-2 and AvSB-5) and 3 of *Pseudomonas* spp. (AvHP-1, AvSP-1 and AvSP-7), were selected to study their influence on the growth of *A. vera*, rhizosphere total bacterial population, and phosphorus concentration in soil of *Aloe vera* in pot experiment. 15 days old rooted cuttings of *A. vera* were obtained from Deptt. of Forest Product medicinal plant nurseries, UHF, Nauni.

The 6 selected strains were used in *vivo* (pot) for treatment of rooted cuttings of *Aloe vera* (obtained from Deptt. of Forest Product medicinal plant nurseries, UHF, Nauni). 15 days old rooted cuttings of *A. vera* were used for the application of the strains with the inoculum density of $8x10^8$ CFU/ml. The cuttings of *Aloe vera* were dipped in bacterial cultures for 15 minutes and were then planted in the pots. After 15 days of planting, the bacterial inoculum was again applied to the roots of plants. After two months of the plantation, plants were uprooted and growth of plant in terms of height (cm) was measured. Rhizospheric bacterial population and phosphorus content (Olsen *et al*., 1954) [18] was estimated before and after the treatment and the results were compared with the un-inoculated control.

Results

Screening of the bacterial isolates for multifarious plant growth promoting activities

The data pertaining to multifarious plant growth promoting activities of total 25 strains (18 *Bacillus* and 6 *Pseudomonas* isolates) isolated from rhizosphere of *Aloe vera* are depicted in Table 1 and 2. On the basis of screening, 6 strains *viz*. AvNB -1, AvSB -2, AvSB -5, AvHP -1, AvSP -1 and AvSP -7 were selected for the pot trial on *Aloe vera*.

Table 1: Multifarious plant growth promoting properties of *Bacillus spp.* and *Pseudomonas* spp. isolated from rhizosphere of *Aloe vera* L.

Bacteria	Phosphate solubilization		Siderophore production		Auxins[4] Conc. (ųg/ml)	Proteolytic activity[5] (Clear zone)
	Plate assay[1]	Quantitative [2] assay (ppm)	Plate assay[1]	% SU [3]		
***Bacillus* spp.**						
AvCB-1	18.3	7.50	-	-	7	21.8
AvCB-2	15.3	4.60	-	-	2	19.3
AvCB-3	09.3	2.80	-	-	11	17.6
AvCB-4	05.3	2.80	-	23.43	1	15.4
AvCB-5	05.6	3.60	-	21.54	1	16.6
AvCB-7	18.3	9.60	-	-	3	17.3
AvHB-3	10.6	6.50	-	18.34	1	W+
AvHB-4	13.0	4.40	-	19.23	1	16.0
AvHB-6	12.6	6.10	-	-	1	11.3
AvHB-7	18.6	6.90	-	23.56	1	21.8
AvJB-1	27.3	9.40	-	21.12	2	18.6
AvJB-2	11.6	5.90	-	-	8	19.3
AvNB-1	15.3	6.70	-	32.13	15	14.0
AvNB-6	20.3	5.90	-	-	1	14.6
AvSB-2	25.3	8.80	-	-	8	18.1
AvSB-3	20.6	7.80	-	-	12	15.0
AvSB-4	15.3	2.90	-	30.42	15	-
AvSB-5	15.6	4.90	-	35.12	11	16.6
***Pseudomonas* spp.**						
AvHP-1	16.3	5.30	14.0	57.81	2	15.3
AvJP-3	07.3	1.10	14.5	59.24	5	16.6
AvJP-4	28.3	9.70	16.0	63.03	5	-
AvJP-5	17.3	3.00	18.1	67.29	3	-
AvSP-1	24.6	6.90	16.3	63.98	11	12.6
AvSP-7	27.3	7.90	17.6	66.35	9	17.6

1 Phosphate solubilization /siderophore activity expressed in terms of mm diameter of yellow zone / pinkish orange zone produced around the bit on their respective media

2 Phosphate solubilizing activity expressed in terms of ppm of orthophosphate solubilization as calibrated from standard curve of KH_2PO_4.

3 The Siderophore Units (SU) are defined as percent reduction in blue colour as compared to reference

4 Auxins concentration expressed in terms of ųg/ml of indole acetic acid (IAA) produced in supernatant as caliberated from standard curve of IAA

5 Proteolytic activity expressed in terms of clear zone (mm diameter) produced around the well on skin milk agar plates at 37 ^{0}C for 24h.

Table 2: Antifungal activity, HCN and Ammonia production of *Bacillus* spp. and *Pseudomonas* spp. isolated from rhizosphere of *Aloe vera* L.

Bacteria	Antifungal activity (mm. dia)[1] Indicator test fungi					HCN Production[2]	Ammonia Production[3]
	Alternaria spp.	*Fusarium* spp.	*Pencillium* spp.	*Sclerotium* spp.	*Pythium* spp.		
Bacillus sp.							
AvCB-1	W +	-	-	W +	-	-	++++
AvCB-2	17.0	-	-	-	13.6	-	+
AvCB-3	16.6	-	-	W +	-	-	++++
AvCB-4	-	-	-	-	14.3	-	++++
AvCB-5	-	11.3	-	-	-	-	+++
AvCB-7	-	-	-	-	-	-	+
AvHB-3	-	16.2	-	W +	12.6	-	++++
AvHB-4	-	-	-	-	-	-	+++
AvHB-6	15.6	-	-	-	W +	-	-
AvHB-7	-	14.4	-	-	-	-	+++
AvJB-1	W +	W +	-	-	-	+++	+++
AvJB-2	-	-	-	-	-	-	-
AvNB-1	18.6	15.6	-	15.0	-	-	+++
AvNB-6	-	-	-	-	-	-	++++
AvSB-2	19.0	15.2	-	W +	16.3	-	++++
AvSB-3	16.6	-	-	-	12.6	-	-
AvSB-4	-	-	-	-	-	-	-
AvSB-5	15.2	14.0	-	-	15.6	+++	+
Pseudomonas sp.							
AvHP-1	14.3	-	-	-	-	-	+++
AvJP-3	-	W +	-	-	-	-	-
AvJP-4	-	14.4	-	-	-	-	-
AvJP-5	-	-	-	-	10.3	-	+
AvSP-1	-	-	-	-	12.6	-	++++
AvSP-7	-	-	-	-	16.6	++	+++

- Indicates no activity; + Indicate activity; W+ Indicates weak activity

1 Antifungal activity expressed in terms (mm diameter) of clear zone produced around the bit at 28 ± 2 ^{0}C after 72h ;

2 HCN production on king's media expressed in terms of change of colour of paper strips already dipped in Picric acid from deep yellow to orange brown

3 Ammonia production expressed in terms of change of colour of culture broth from faint yellow to deep brown at 30^{0}C for 4days

Effect of Best Selected Isolates on Various Parameters of Aloe Vera after Two Months of Plantation

Effect on growth (height) of *Aloe vera*

The mean growth performance of *Aloe vera* with respect to the treatments i.e. control and inoculum of 6 strains *viz.* AvNB -1, AvSB -2, AvSB -5, AvHP -1, AvSP -1 and AvSP -7 is presented in Table 3. From the data, it is evident that AvHP -1 resulted in maximum mean growth (20.57cm) which was significantly superior from all other strains.

Table 3: Effect and Comparison of *Bacillus* strains (AvNB-1, AvSB-2 and AvSB-5) and *Pseudomonas* strains (AvHP-1, AvSP-1 and AvSP -7) on the growth (height) of *Aloe vera* L. seedlings after 2 months of Plantation

Treatment/ Inoculum density (CFU/ml)	Plant height (cm)						Mean
	Bacillus strains			*Pseudomonas* strains			
	AvNB-1 (T_1)	AvSB-2 (T_2)	AvSB -5 (T_3)	AvHP -1 (T_4)	AvSP-1 (T_5)	AvSP -7 (T_6)	
Control (0)	12.33	12.33	12.33	12.33	12.33	12.33	12.33
$8x10^8$	15.76	19.33	14.53	20.57	19.07	18.80	18.01
Mean	14.05	15.83	13.43	16.45	15.70	15.57	

$CD_{0.05}$

Treatment = 0.44

Bacteria = 0.34

Treatment x Bacteria = 0.6

Fig 1: Effect of *Bacillus* Strains AvNB-1,AvSB-2 and AvSB -5 and *Pseudomonas* Strains AvHP -1, AvSP -1 and AvSP -7 on the growth (height) of *Aloe vera* L. after 2 months of plantations

Effect on rhizospheric bacterial population

The effect of all 6 selected strains, on rhizospheric population of *Aloe vera* is presented in table 4. Recorded data presents that among *Bacillus* strains, AvSB -2 showed maximum increase in rhizosphere soil bacterial population (240.0-278.3 CFU/g soil) with mean difference of 38.3 CFU/g soil, whereas, among *Pseudomonas* strains, AvSP -1 resulted in maximum increase in rhizosphere soil bacterial population (224.7-268.0 CFU/g) with mean difference of 43.3 CFU/g.

Table 4: Effect and comparison of *Bacillus* Strains (AvNB-1, AvSB-2 and AvSB-5) and *Pseudomonas* strains on rhizosphere bacterial population of *Aloe vera* L. seedlings in pot after 2 months of plantation**.**

***Bacillus* strains Inoculum density CFU/ml**	**Rhizosphere bacterial population (10^8 ×CFU/g soil)**		**Mean**	***Pseudomonas* Inoculum density (conc.) CFU/ml**	**Rhizosphere bacterial population (10^8 ×CFU/g soil))**		**Mean**
	Before	**After**			**Before**	**After**	
Control (0)	106.3	144.0	125.2	Control (0)	106.3	142.3	124.3
AvNB-1 (T_1)	210.3	244.3	227.3	AvHP -1 (T4)	238.0	281.3	259.7
AvSB -2 (T_2)	240.0	278.3	259.2	AvSP -1 T(5)	224.7	268.0	246.3
AvSB -5 (T_3)	209.7	235.3	222.5	AvSP -7 (T6)	221.0	261.3	241.2
Mean	191.6	225.5		Mean	197.5	238.2	

$CD_{0.05}$ $CD_{0.05}$

Treatment	=	5.16 Treatment	=	5.31
Bacteria	=	3.65 Bacteria	=	3.76
Treatment x Bacteria	=	7.29 Treatment x Bacteria	=	7.52

Effect on phosphorus concentration of soil

The influence of 3 *Bacillus* strains (AvNB -1, AvSB -2 and AvSB -5) and *Pseudomonas* strains (AvHP-1, AvSP-1 and AvSP-7) on *Aloe vera* is presented in table 5. Recorded data showed that AvNB -1 showed maximum increase in phosphorus concentration (432.9-490.8 kg/ha) after 2 months of plantation, whereas, *Pseudomonas* isolate AvHP-1 resulted in maximum increase in phosphorus concentration (436.6-490.5 kg/ha) with mean difference of 53.9 kg/ha which is at par with treatment AvSP-1. Minimum phosphorus concentration was registered in control (normal saline treatment) in both *Bacillus* and *Pseudomonas* treatments. All the treatments differed statistically from one another.

Table 5: Effect and comparison of *Bacillus* Strains (AvNB-1, AvSB-2 and AvSB-5) on phosphorus concentration of *Aloe vera* L. seedlings in pot after 2 months of plantation.

Treatment inoculum density (conc.) CFU/ml	**Phosphorus concentration (kg/ha)**		**Mean**	**Treatment inoculum density (conc.) CFU/ml**	**Phosphorus concentration (kg/ha)**		**Mean**
	Before	**After**			**Before**	**After**	
Control (0)	430.2	451.0	Control (0)	Control (0)	432.1	452.3	442.2
AvNB-1 (T_1)	432.9	490.8	AvHP -1 (T4)	AvHP -1 (T4)	436.6	490.5	463.6
AvSB-2 (T_2)	437.3	484.2	AvSP -1 T(5)	AvSP -1 T(5)	433.7	483.9	458.8
AvSB-5 (T_3)	430.3	477.2	AvSP -7 (T6)	AvSP -7 (T6)	433.8	473.1	453.4
Mean	432.7	475.8	Mean	Mean	434.0	475.0	

$CD_{0.05}$ $CD_{0.05}$

Treatment	=	8.49 Treatment	=	6.37
Bacteria	=	6.00 Bacteria	=	4.50
Treatment x Bacteria	=	12.01 Treatment x Bacteria	=	9.01

Discussion

The strains isolated from various soil samples from the rhizosphere were assumed to be *Bacillus* and *Pseudomonas* strains respectively, on the basis of morphological and biochemical characterization. The isolates were screened for the plant growth promoting activities and positive results for phosphate solubilisation, auxin production, siderophore production, proteolytic activity and HCN production were obtained by majority of isolates. HCN and antibiotics from different *Pseudomonas* and *Bacillus* spp. have found to suppress the growth of several other microorganism and posses the antibacterial and antifungal properties (Ramette *et al.* 2003) [19]. In general the capability of PGPRs to produce plant growth regulators have been reported by Satter and Gaur (1987) [20] and Malleswari and Bhagyanarayana (2013) [21].

Out of 25 isolates, 19 isolates were also noted to have antifungal activity against various pathogens such viz. *Alternaria, Fusarium, Penicillium, pythium* etc. Rhizosphere microorganisms provide an initial barrier against pathogen attack to the root (Weller, 1988) [22]. Ben, (2005) [23] and Sain *et al*., (2005) [24], have also reported the suppression of fungal pathogens by *Pseudomonas fluorescens* and *Bacillus* sp.

The efficacy of screened and selected six best isolates was then tested under pot trial *in vivo*, in which the isolates showed significant increase in the growth in terms of plant height (cm), rhizobial bacterial population and phosphorous concentration. The improvement in growth promotion of the rooted cuttings of *Aloe vera* may be due to the combination of mechanisms. It seems reasonable to suggest this effect on plant growth parameter (height) is resulted from inoculation with phosphate solubilizing *Pseudomonas* and *Bacillus* species also producing other growth promoting activites such as production of phytohormones i.e. auxins, inhibition of deleterious pathogens or nutrient mobilization and / or ammonification (Azcon *et al.,* 1975, Sattar and Gaur 1987 and Barea *et al*., 1976) [25-27]. Cakmakci, (2005) [28] has also reported the plant growth benefits due to the addition of PGPR include increases in germination rate, root growth, yield, leaf area, chlorophyll content, tolerance to drought, shoot and root weight.

The beneficial effects of inoculation of the PGPRs i.e. *B. pumilus* and *B. licheniformis* on growth of forest plants (alder

and pine) and herbaceous plants such as soybean due to production of auxins and gibberellins have also been reported by Gutierrez Manero *et al.*, (1996) [29]. Growth promotion of *Lupinus albus* seedlings by the inoculation of *Aureobacterium* has also been dedicated to the capacity to auxin and siderophore producing capacity of the strain (Vasudevan *et al.*, 2003) [30].

Recorded data showed that AvNB -1 showed maximum increase in phosphorus concentration (432.9-490.8 kg/ha) after 2 months of plantation, whereas, *Pseudomonas* isolate AvHP-1 resulted in maximum increase in phosphorus concentration (436.6- 490.5 kg/ha). The *Pseudomonas* and *Bacillus* spp. have also been reported as the two most efficient phosphate solubilizing genera by Malleswari and Bhagyanarayana (2013) [31] and Saxena and Sharma (2003) [32].

On the basis of present experimental study, *Pseudomonas* spp. AvHP-1 and *Bacillus* spp. AvSB-1 were found to be best for plant treatments to influence the growth of *Aloe vera*. In a similar experiment by Thanuja and Ambika, (2010) [33], bacterial isolates from rhizoplane of *Holostemma ada-kodien*-an endangered medicinal plant, were screened for their direct growth promoting activities (production of NH_3, IAA and phosphate solubilization) and their efficiency was tested on the growth and dry matter accumulation. The results obtained showed overall improvement in the growth terms of shoot length over control.

Conclusion

The present study concludes that the plant growth promoting bacteria i.e. *Bacillus* and *Pseudomonas* are active solubilizers of insoluble phosphate and produce several growth promoting substances which are not readily available to the plant, thereby incurring beneficial effects on the *Aloe vera*. Thus the use of plant growth promoting rhizobacteria should be increased in agriculture because it offers an attractive way to replace chemical fertilizers, pesticides, and supplements.

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