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SHORT REPORTS

Isolation and characterization of phosphate solubilizing bacteria from Western Indian Himalayan soils

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Abstract Previous studies confirmed the existence of diversified microbial flora in the rhizosphere of Himalayan Red Kidney Bean (RKB) (*Phaseolus vulgaris* L.). Therefore, fifteen different temperate and subtropical regions of Western Indian Himalaya (WIH) were explored for the isolation of RKB rhizosphere-associated Phosphorus (P) solubilizing bacteria. On the basis of qPCR analysis, three soils, i.e., Munsyari, Kandakhal and Nainital soils were selected for the isolation of P solubilizers. Among 133 isolates, three bacteria viz. *Lysinibaccilus macroides* ST-30, *Pseudomonas palleroniana* N-26 and *Pseudomonas jessenii* MP-1 were selected based on their P solubilization potential. Moreover, in vitro seed germination assay was performed to investigate their effectiveness against four native crops viz. (*Cicer arietrinum* L.), (*Vigna radiata* L.), (*Pisum sativum* L.) and (*Zea mays* L.). Treated seeds showed significant increase in germination efficiency over their respective controls. The results suggest that *Lysinibaccilus macroides* ST-30, strain is a potential plant growth-promoting bacterium for chickpea (*Cicer arietrinum* L.) and, therefore, could be implemented as a low-cost bio-inoculant in hill agriculture system.

Keywords Western Indian Himalaya · Phosphate solubilizing bacteria · Bioinoculant · qPCR

Introduction

Phosphorus being the second most important nutrient for plant is not readily available as it is present in insoluble form in soil and does not have large atmospheric source as nitrogen. It is absorbed by plants in the form of $H_2PO_4^-$ and HPO_4^{2-} ions. These are called labile phosphorus and are readily absorbed through the roots. However, these labile phosphorus ions become unavailable to the plants by interacting with the surrounding environment and thus requiring repeated application of fertilizers. Nevertheless, major part of the unused or unabsorbed fertilizer remains in the soil until it is not eroded by the natural factors. Therefore, efforts are being made by the government, R&D agencies as well as fertilizer companies to increase the efficiency of phosphorus absorption by plants. The best alternative of chemical-based phosphatic fertilizer is to increase the reliance on phosphorus solubilizing bacteria (PSB). It is primarily classified as bio-fertilizers and is one of the most cost-effective and sustainable ways to increase the phosphorus absorption efficiency. These bacteria solubilize the complex phosphate compounds present in soil into the simpler readily absorbable form. Also, there is no requirement of their additional application as inactive phosphorus is readily present in the soil which will be solubilized steadily by these bacteria, thus providing for a sustainable source of phosphorus to the crops.

Agriculture is the major source of sustenance for the people dwelling in high altitude agro-ecosystems of Himalayas. Farmers of WIH rely on pristine natural farming methodologies and are far away from the use of

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hazardous chemical-based farming techniques. In this context, WIH agro-climatic region could prove as a treasure for adaptable potential PSB. In these habitats, microbial P solubilization is of particular interest since the low concentration of bio-available P is one of the key limitations for plant and soil microorganism growth (Duc et al. 2009). Previously, we have confirmed the presence of diversified vast microbial assemblage in rhizospheric soil of Himalayan Red Kidney Bean (RKB) (Suyal et al. 2015a, b). Moreover, seven diazotrophs were isolated from the WIH RKB rhizosphere and their proteome was documented (Suyal et al. 2014a, b; Soni et al. 2015). Furthermore, psychrophilic *Pseudomonas migulae* S10724 (JX173286), which was originally isolated from WIH RKB rhizosphere, was reported to promote the growth of *Vigna radiata* (L.) Wilczek (Suyal et al. 2014a, b). In view of the above, the present study aims to isolate and characterize P solubilizers from RKB rhizospheric soil from WIH. Furthermore, we have also investigated the effectiveness of P solubilizing potential strains on the seed germination efficiency of four native crops which could be explored for improved crop production and sustainability.

Materials and methods

Sampling sites and sample collection

RKB rhizospheric samples were collected from fifteen different regions of WIH as per the method described earlier (Suyal et al. 2015a, b) (Table 1, Fig SM1). Soil samples were collected in triplicates and then mixed to make a single composite sample from each site.

Total soil DNA extraction and qPCR analysis

Total DNA from the soil was extracted as described previously (Suyal et al. 2015a, b). Copy numbers of 16SrDNA and PQQ genes from the collected soil samples were quantified using iCycleriQTM Multicolor (Bio-Rad Lab, Hercules, USA) qPCR machine as per earlier description (Miethling et al. 2000; Kim et al. 2003; Soni and Goel 2010).

Isolation, Screening and quantification of P-solubilization

Isolation of P solubilizers was done on National Botanical Research Institute's phosphate growth medium (NBRIP) agar medium at 30 °C (Rani et al. 2013). Furthermore, all the isolated bacteria were qualitatively screened for P-solubilization potential through solubilization index on Pikovskaya's agar plates at 30 °C for a week (Singh et al. 2013; Rani et al. 2013). The selected isolates were sequenced using 16S rDNA sequencing as described previously (Table SM2) (Suyal et al. 2014a, b).

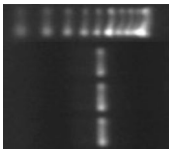
In vitro seed germination assay

In vitro seed germination assay was conducted to assess the efficacy of selected bacterial strains on germination of four local crops varieties viz. chick pea (*Cicer arietrinum* L.

Table 1 Comparative 16S rDNA and PQQ gene abundance in different sampling sites as revealed by qPCR analysis. Each value is the mean of three replicates. Values in parentheses indicate standard error

S. no.	Sampling site	Latitude, longitude	Elevation (m)	Climate	Copy No. (per g of soil)	
					16SrDNA gene	PQQ gene
1	Surkhanda	30°24′41″N, 78°17′17″E	2757	Temperate	1.19×10^{10} ($\pm 1.11 \times 10^{2}$)	1.12×10^{2} ($\pm 1.08 \times 10^{2}$)
2	Munsyari	30.07°N, 80.23°E	2200	Temperate	1.59×10^{12} ($\pm 1.36 \times 10^{3}$)	1.38×10^{3} ($\pm 2.98 \times 10^{2}$)
3	Chakrata	30°41′46″N, 77°52′10″E	2118	Temperate	1.50×10^{10} ($\pm 1.37 \times 10^{3}$)	3.11×10^{2} ($\pm 1.21 \times 10^{2}$)
4	Nainital	29.23°N, 79.30°E	2084	Temperate	2.69×10^{11} ($\pm 2.31 \times 10^{3}$)	1.62×10^{3} ($\pm 1.90 \times 10^{2}$)
5	Mussoorie	30°27′15″N, 78°5′0″E	2005.5	Temperate	1.47×10^{9} ($\pm 2.21 \times 10^{2}$)	2.35×10^{2} ($\pm 1.01 \times 10^{2}$)
6	Pauri	30°8′54″N, 78°46′26″E	1814	Sub-temperate	3.57×10^{8} ($\pm 3.01 \times 10^{2}$)	5.38×10^{2} ($\pm 2.92 \times 10^{2}$)
7	Lansdowne	29°50′35″N, 78°40′44″E	1700	Sub-temperate	1.10×10^{10} ($\pm 1.14 \times 10^{2}$)	4.38×10^{2} ($\pm 1.09 \times 10^{2}$)
8	Mahabgarh	29°52′40″N, 78°27′18″E	1650	Sub-temperate	2.96×10^{10} ($\pm 1.91 \times 10^{2}$)	1.09×10^{3} ($\pm 1.11 \times 10^{2}$)
9	Chamba	30°21′59″N, 78°23′49″E	1524	Sub-temperate	1.27×10^{9} ($\pm 2.01 \times 10^{3}$)	2.89×10^{2} ($\pm 1.78 \times 10^{2}$)
10	Kandakhal	29.52°N, 78.34°E	1427	Subtropical	4.63×10^{10} ($\pm 1.12 \times 10^{2}$)	3.11×10^{3} ($\pm 1.4 \times 10^{2}$)
11	Dugadda	29°48′28″N, 78°36′30″E	932	Subtropical	7.40×10^{9} ($\pm 1.48 \times 10^{2}$)	5.99×10^{2} ($\pm 1.63 \times 10^{2}$)
12	Aamsour	29°47′10″N, 78°35′13″E	745	Subtropical	2.80×10^{10} ($\pm 3.01 \times 10^{2}$)	1.90×10^{2} ($\pm 1.33 \times 10^{2}$)
13	Satpuli	29°55′10″N, 78°42′33″E	657	Subtropical	7.17×10^{9} ($\pm 1.91 \times 10^{2}$)	3.38×10^{2} ($\pm 2.61 \times 10^{2}$)
14	Srinagar	30°13′31″N, 78°47′42″E	560	Subtropical	1.03×10^{10} ($\pm 1.44 \times 10^{3}$)	4.36×10^{2} ($\pm 1.95 \times 10^{2}$)
15	Kotdwar	29°45′24″N, 78°29′17″E	454	Subtropical	1.80×10^{10} ($\pm 2.66 \times 10^{3}$)	1.01×10^{3} ($\pm 1.93 \times 10^{2}$)

Table 2 P solubilization potential of bacterial strains under study. Each value is a mean of three replicates

S. no.	Strain I.D.	P solubilization index[a]	P solubilized (μg/ml)	PQQ gene amplification
1	ST-30	62 mm	713.11	
2	N-26	8 mm	381.29	
3	MP-1	7.2 mm	314.43	

[a] Solubilization Index (SI) $= \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$

var. PG-186), mungbean (*Vigna radiata* L. var. Pant Mung 4), field pea (*Pisum sativum* L var. Arkel), maize (*Zea mays* L var. Sankar Makka 2) as per earlier studies (Kumar et al. 2014).

Results and discussion

Soil samples were collected from different temperate and subtropical climatic regions of western Indian Himalayas. qPCR analysis reveals that the highest copy no. of 16SrDNA and PQQ genes was observed in Munsyari, Kandakhal and Nainital soils and, therefore, these three soils were selected for the isolation of P solubilizers (Table 1).

A total of 133 bacterial isolates were isolated from the above-mentioned soil on NBRIP agar medium and all were point inoculated in Pikovaskya Agar medium to check the zone index formed by them (Fig SM2). Bacterial colonies showing solubilization index $\leq$7 mm are selected for P quantification. Halo zones production on solid media and efficient release of phosphate in NBPIP is due to the release of several organic acids like citric, keto, glyoxalic succinic butyric and malic (Kelel et al. 2014). Several reports are available on the isolation of P solubilizers from Himalayan regions (Singh et al. 2013; Panda et al. 2016). Recently, Elias et al. (2016) have isolated 38 fungal isolates from the rhizosphere of RKB; however, the associated bacteria were not studied.

Three bacterial colonies ST-30, N-26, and MP-1 have shown zone solubilization index of 62, 10 and 7.2 mm, respectively, and therefore selected for further quantification studies (Table 2). Significantly, the highest P solubilization potential of ST-30 was recorded 713.11 μg/ml which corresponds with its largest solubilization index (SI) shown on Pikovskya Agar plate. MP1 has solubilized 398.14 μg/ml P followed by N-26 (381.29 μg/ml) (Table 2; Fig SM3). Further, all these bacterial cultures have shown positive amplification for PQQ gene too which is an ideal marker for identification of P solubilizers (Kim et al. 2003; Anzuay et al. 2013) (Table 2). Pyrroloquinoline quinone (PQQ), a cofactor required for gluconic acid synthesis, is involved in P solubilization and antifungal action (Kaur et al. 2006).

In vitro seed germination assay reveals the significant increase in bacteria treated seeds over their respective controls. In chickpea (*Cicer arietrinum* L. var. PG-186), ST-30 treated seeds have shown the highest germination rate of 98% which is at par of seed germination rate of N-26 (97.5%) followed by MP1 (91.5%) (Table 3). Untreated control has shown 78% germination. In mungbean (*Vigna radiata* L. var. Pant Mung 4), untreated

Table 3 Effect of potential bacterial strains on seed germination efficiency of different crops under in vitro conditions after 72 h of germination

Crops	Treatments	In vitro seed germination assay (% germination of the seeds)
Chick Pea (*Cicer arietrinum* L. var. PG-186)	Control	78.00 ± 1.3^{a}
	ST-30	98.00 ± 0.77^{d}
	N-26	97.50 ± 0.76^{d}
	MP-1	91.50 ± 0.76^{bc}
	SEm	4.179
Mungbean (*Vigna radiata* L. var. Pant Mung 4)	Control	84.33 ± 1.21^{ab}
	ST-30	88.17 ± 0.61^{c}
	N-26	82.67 ± 0.89^{a}
	MP-1	86.67 ± 0.89^{bc}
	SEm	2.869
Field Pea (*Pisum sativum* L. var. Arkel)	Control	75.33 ± 0.88^{a}
	ST-30	85.67 ± 0.88^{c}
	N-26	83.33 ± 3.18^{bc}
	MP-1	87.33 ± 0.88^{cd}
	SEm	7.429
Maize (*Zea mays* L. var. Sankar Makka 2)	Control	65.67 ± 1.20^{a}
	ST-30	88.33 ± 0.88^{d}
	N-26	87.67 ± 0.88^{d}
	MP-1	84.00 ± 0.58^{c}
	SEm	2.226

Data were analyzed through SPSS 16.0. Duncan's Multiple Range Test was applied. Values in parenthesis indicate homogenous subsets at significant difference ($P \leq 0.05$). Each value is the mean of three replicates

Alphabetic values indicate homogenous subsets at significant difference ($P \leq 0.05$). Each value is the mean of three replicates

control seeds were 84.33% germinated, while the highest germination was observed in MP-1 (86.67%) which is at par of ST-30 (88.17%) followed by N-26 (82.67%). In field Pea (*Pisum sativum* L var. Arkel), untreated control has shown 75.33% germination. MP1-treated seeds have shown the highest germination rate of 87.33% which is followed by ST-30 and N-26 with 85.67 and 83.33% germination, respectively. In maize (*Zea mays* L var. Sankar Makka 2), untreated control seeds were 65.67% germinated, while among treated seeds the highest germination was observed in ST-30 (88.33%) followed by N-26 (87.67%) and MP1 (84%). The present study revealed that *Lysinibaccilus macroides* ST-30 strain is an efficient P solubilizer and showed plant growth-promoting properties against chickpea (*Cicer arietrinum* L.) followed by *Pseudomonas palleroniana* N26 and *Pseudomonas jessenii* MP1 as evident from seed germination assay. These findings are in agreement with those of Rani et al. (2013) who have evaluated the effect of phosphate-solubilizing *Commamonas aquatica* 710B and *Pseudomonas putida* 710A in *Vigna radiata* (L.) wilczek. Moreover, several earlier reports reveal successful implementation of the PSB as the PGPBs (Rani et al. 2013; Singh et al. 2013). Selvakumar et al. (2013) revealed the solubilization of rock phosphate using *Pseudomonas* spp. isolated from the rhizoplane of wild grass from Indian Himalayas. Furthermore, Majeed et al. (2015) analyzed the effect of plant growth promontory rhizobacteria isolated from wheat rhizosphere of Himalayan region of Kashmir. Bergottini et al. (2015) have studied the bio-inoculation effect of yerba mate seedlings (*Ilex paraguariensis* St. Hill.) with native plant growth-promoting rhizobacteria.

Conclusion

In conclusion, this preliminary study provides the clue about the effectiveness of P solubilizing strains *Lysinibaccilus macroides* ST-30, *Pseudomonas palleroniana* N-26 and *Pseudomonas jessenii* MP-1 against chickpea. They will facilitate the development of microbial inoculants for the agriculture in fluctuating hill environments. However, successful implementation of these strains needs further investigation.

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Compliance with ethical standards

Conflict of interest The authors hereby declare no conflict of interest.

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