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Analysis of ecological attributes of bacterial phosphorus solubilizers, native to pine forests of Lower Himalaya

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ABSTRACT

Phosphorus solubilizing bacteria (PSB) isolated from rhizospheric soils of pine forests of lower Himalaya were characterized for phosphorus solubilization and ecological characteristics. Isolated bacterial strains were cultured in broth for determination of their capability to solubilize rock phosphate $Ca_3(PO_4)_2$. Interestingly, eight PSB strains were observed as psychrotolerant i.e. growing and solubilizing P at lower temperature (4 °C) as well as at 28 °C. The P-solubilization by these PSB strains was potentially carried out by acidification which was justified by significant lowering of pH in tested cultures overtime compared to control broth. For ecological characterization, the isolated PSB were evaluated for their interactions against each other and other soil microorganisms i.e. nematodes and fungi isolated from the same Himalayan origin. No inhibition of PSB with P solubilizing fungi, showed their potential for their mutual application to increase P-availability to plants. On the other hand, PSB antagonism to non P-solubilizing fungi and particularly to nematodes would indicate their resistance against plant pathogens postulating the possibility of their application as biocontrol.

1. Introduction

Microbial diversity ranges in billions, belonging to thousands of different species can exist even in one gram of soil (Rossello-Mora and Amann, 2001). Some discrete microhabitats called "hotspots" with high biological activity have been detected (Nannipieri et al., 2003) e.g. aggregates, rhizosphere and preferential flow paths (for non-uniform movements of water and solutes through soil matrix) (Bundt et al., 2001). Biodiversity of an ecosystem is very important and its loss significantly reduces the plant productivity (Liang et al., 2015). Along with microbes like archea, bacteria and fungi, soil also contains organisms like insects, protozoans, nematodes etc. which all together form food web for cycling of energy and organic materials (Nazir et al., 2010). In composition, bacteria are reported to be the most abundant while fungi are placed at second in abundance in any soil (Standing and Kilham, 2007). However, bacteria and fungi often have a shared habitat known as bacterial-fungal interface (Johansson et al., 2004). Bacteria usually exist on fungal hyphae and/or spores, in association with fruiting bodies and even on mycorrhizal roots (Offre et al., 2007). These microbes work in soil primarily in three different ways i.e. saprotrophy, pathogenicity and symbiosis (Pivato et al., 2009).

In any ecosystem, phosphorus (P) is claimed second frequently limiting macro nutrient for plants (Gyaneshwar et al., 2002). P in soil is present in inorganic form as rock phosphate and organic form derived from plants and animals' decay (Behera et al., 2014). The utilization of beneficial microbes ensures P-availability (Richardson et al., 2009) by secretion of various organic acids and enzymes which dissolve P minerals and chelates, directly releasing available P into solution (He et al., 2002). In a symbiotic relationship between Phosphorus solubilizing microbes (PSM) and plants; microbes provide soluble P (Becquer et al., 2014) and plants supply root borne carbon compounds (mainly sugars) for microbial metabolism and growth. In rhizosphere, many interactions of microbes with each other and also with host plant have also been observed e.g. co-inoculation of phosphorus solubilizing bacteria

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(PSB) with N_2 fixers (e.g. *Azospirillum* and *Azotobacter*) or with vesicular arbuscular mycorrhizae (AM) (Zaidi and Khan, 2005).

PSM not only help in growth promotion and P uptake but also contribute in biological control against soil borne phytopathogens (Irshad et al., 2011) and abiotic stresses (Yousefi et al., 2011). The basic mechanism of inhibitory interactions could be due to the production of siderophores, antibiotics, secondary metabolites and/or hydrolytic enzymes (Hwangbo et al., 2003). Particularly the PSB live in association with multitude of other organisms in soil hotspots and metabolically function to access soil phosphate through excretion of phosphatases and organic acids (Artursson et al., 2006). In such associations, bacteria are benefited majorly in carbon based nutrition. For example, soil bacteria acquire nutrition from fungi as (1) extracellular necrotrophy: nutrients release by killing of fungal cells, (2) extracellular biotrophy: nutrients release by actively growing fungal hyphae and (3) endocellular biotrophy: existence of bacteria inside the fungal hyphae (Leveau and Preston, 2008). In rhizosphere, bacteria and fungi can change the chemical composition of root exudates influencing the resident microbial associates (Artursson et al., 2006). Moreover, the secretion of organic acids by fungi and bacteria may also change the dynamics of associated partners (de Boer et al., 2005). Bacteria in soil move towards or along fungal hyphae either by motility or chemotaxis (Nazir et al., 2013, 2014). Furthermore, bacterial numbers also increase along hyphae by utilization of nutrients from fungi (Leveau and Preston, 2008). Boersma et al. (2010) observed that bacteria utilize potentially fungal released compounds as carbon sources.

Other than fungal pressures in soil habitats, bacteria have also to cope with other organisms as well e.g. nematodes which may use them as a prey for nutrition. For example, bacterial community structure and activity changed by bacterivorous nematode predators (Djigal et al., 2004). Bacteria-feeding nematodes have been recognized as a main bacterivorous predator in soil. Venette and Ferris, (1998) found that an adult bacterial-feeding nematode may consume about 10^6 cells per day. Therefore, the microbial interactions are very important in an ecosystem particularly in soil food web functioning. On the same instance, the information on bacterial interactions is limited particularly of PSB with soil associated fungi and nematode grazers. We, thus, devised this work in the poorly understood domain of microbial interactions via focusing on strains from unexplored Himalaya.

Along with biotic factors, cold climate, as found in Himalayan regions, offers extreme conditions as a great living challenge for microbial interactions. Such particular cold habitats are frequently inhabited by cold-adopted (psychrophile and/or psychrotolerant) bacteria (Christner et al., 2000). Such microbes thrive in cold environments due to their unique features e.g. cold shock proteins, short and unsaturated membranous acids, high-specific enzymes, thermolability and genetic changes to thermal shifts (Margesin et al., 2007).

Our study area i.e. Abbottabad is a phosphate rich region, heavily exploited for rock phosphate extraction at a massive scale. It has a plenty of natural vegetation forming forests, mainly comprised of coniferous species *Pinus wallichiana*. There is not much available knowledge about the rhizospheric inhabitants of the area, and about ecological functions of such microbes, particularly PSM, which consequently contribute in stability of natural ecosystem functioning. Therefore, the objectives of the current study were set as following:

(1) Analyses of isolated Himalayan PSB for quantification of available P concentration from inorganic TCP i.e. tricalcium phosphate (2) Recognition and assessment of psychrotolerant PSB for their ecological attributes (3) Evaluation of PSB interactions with other microbiota present in *Pinus* spp. rhizosphere.

2. Material and methods

2.1. Isolated microbes, growth media and maintenance

The bacteria, fungi and nematodes (used in this study) were isolated from Lower Himalayan region of Abbottabad Pakistan. Soil sampling and consequent microbial isolations were done from rhizosphere of different plants (Supplementary table-1) and 13 of these PSB (26%) were identified by 16S rDNA gene sequencing. Two strains (PWB 4 & PWB 57) were Gram positive while the rest were Gram negative, particularly in *Enterobacteriaceae* (PWB 25, PWB 27, PWB 45, PWB 50, PWB 67a) and *Moraxellaceae* (PWB 24, PWB 28, PWB 34) of *gammaproteobacteria,* Bacteroidetes (PWB 13) and alpha proteobacteria (PWB 2, PWB 12) (Nazir et al., 2016). Standard media, as explained in sections below, for microbial growth were prepared for different purposes and sterilized in laboratory (autoclave at 121 °C for 20 min) before use.

For interaction assays, PSB were inoculated in sterilized test tubes, having 10 ml of LB Broth and incubated under shaking condition for 24 h at 180 rpm and 28 °C. The washed bacterial cells were used as inocula for different tests. Moreover, the grown bacterial cultures were mixed with 50% (v/v) sterilized glycerol in 1.5 ml eppendorf tubes and stored at −80 °C for future use.

2.2. Measurement of available P-concentration

For the assessment of PSB strains' ability to solubilize fixed inorganic P, the bacterial cultures were grown in 10 ml tri calcium phosphate (TCP) broth (PVK) in sterilized test tubes under shaking conditions (orbital shaker at 130 rpm) at 28 °C for 24 h. For the assessment of soluble P in the broth, culture supernatant of each bacterial strain was treated with reagents of malachite green method (Irshad et al., 2012). Briefly, a stock solution of P was prepared by dissolving 0.439 g KH_2PO_4 in 30 ml distilled water. Subsequently, 70 ml distilled water was added to get 100 ml volume with 1 g/L P concentration, which was used to prepare a series of standard solutions (400 mg/L, 200 mg/L, 100 mg/L, 50 mg/L, 25 mg/L, and 5 mg/L). To 2 ml of each solution, 400 μl reagent A was added and incubated for 10 min. The solution was incubated for 30 min in the dark after adding 400 μl of reagent B and the light absorbance was noted by UV–vis spectrophotometer at a wavelength of 630 nm. On plotting P concentration versus absorbance a straight line was obtained passing through the origin with line equation $y = 0.0047x$ and $R^2 = 0.9941$.

2.3. Psychrotolerant bacterial strains

For analysis of psychrotolerant efficacy, PSB were cultured on LBA plates and incubated at 4 °C in refrigerator for growth at lower temperature. The strains showing growth on LBA at lower temperature were cultured on fresh plates containing PVK agar supplemented with TCP as sole P-source. The time dependent growth of PSB colonies and P solubilization index (SI) of cold tolerant PSB were determined at 4 °C and 28 °C by using method described previously (Nazir et al., 2016) i.e.

SI = (Colony Diameter + Hallow zone Diameter)/Colony Diameter

Multiple readings were recorded for different replicates and the data is presented as average of at least three biological replicates.

2.4. pH variation of isolated strains

The isolated PSB strains were inoculated in sterilized test tubes having 10 ml of standard PVK (Pekovskaya's) broth as potential source of phosphorus (tricalcium phosphate, TCP). The pH of the