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Buffering capacity affects phosphorous solubilization assays in rhizobacteria

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ABSTRACT

Phosphate solubilizing activity of phosphate solubilizing bacteria (PSB) is affected by buffering capacity of soils. It is well known that the buffering capacity of soils can diminish the efficiency of microbial acid production in phosphorus (P) solubilization process. Hence, in the first step of this study by using solid and liquid Sperber media containing organic or inorganic phosphate, more than 100 isolates were isolated, and finally two capable isolates (C1-1O and C16-2O) with highest potential of phosphate solubilizing activity were candidate for identification, and identified as *Pseudomonas* sp. C16-2O and *Pseudomonas* sp. C1-1O. In the next stage, their efficiency along with six more chosen PSB (including *Pseudomonas putida* P13, *P. fluorescens* Tabriz, *Pantoea agglomerans* P5, *P. agglomerans* P5-n, *Azotobacter* sp. SP5 and *Azotobacter* sp. SP16) in solubilizing phosphorus from two P sources (rock phosphate: RP and tricalcium phosphate: TCP) in the buffered (100 mM Tris–HCl) and un-buffered Pikovskaya's media were evaluated. According to in-vitro experiments, *P. fluorescens* Tabriz, *P. agglomerans* P5-n, *Azotobacter* sp. SP16 and *Pseudomonas* sp. C16-2O showed the highest solubilization overall, ranged from 258 to 252.7 mg L^{-1}. Our results revealed that thoroughly P solubility in buffered condition decreased by 56.2%. Also in the buffered condition, *P. putida* P13 and *Azotobacter* sp. SP5 lost their P-solubilization ability while *Pseudomonas* sp. C16-2O and *P. fluorescens* Tabriz were the best solubilizers and they solubilized 185.3 and 182.7 mg L^{-1} of insoluble P. P solubility in presence of TCP was 2.55 times more than RP source.

1. Introduction

Phosphorus (P) is one of the most essential plant nutrients which profoundly affects the overall growth of plants (Wang et al., 2009). In soils, P exists in many complex chemical forms that differ markedly in their behavior, mobility, and resistance to bioavailability (Jalali and Matin, 2013). Therefore, the fate and transport of soil P vary largely depending on the forms (Reddy et al., 1998). From the various forms of P in the soil, plants only absorb mono and dibasic phosphate which are the soluble forms of phosphate (Jha et al., 2012; Jha and Saraf, 2015). Mineral compounds of P usually contain aluminium (Al), iron (Fe), manganese (Mn) in acidic soils and calcium (Ca) in alkaline soils (Khan et al., 2014) and from the total soil P pool, about 50% of P exists in the organic forms (Goswami et al., 2016), which varies between 4 and 90% in most soils (Yadav and Verma, 2012). In comparison to other nutrients, P concentration in soil solution is much lower and ranges from 0.001 to 1 mg L^{-1} (Brady and Weil, 2002).

To cope with such an extreme P deficiency in soils, most countries import chemical fertilizers, while a large portion of the phosphatic fertilizers applied to soil is rapidly fixed/immobilized and becomes unavailable to plants (Xiao et al., 2011). The insoluble and fixed forms of P, therefore, alter the fertility of soil (Bhattacharyya and Jha, 2011) and limit plant growth (Prejambda et al., 2009; Victoria et al., 2009; Plassard and Dell, 2010). Due to this P fertility problems, growers often apply several fold excess P than required by plants (Goldstein, 1986) which after accumulation/deposition gradually results in soil pollution and pollution of other water resources such as lakes, streams, and groundwater (Reddy et al., 2002).

The insoluble forms of P can be converted to soluble P by Phosphate Solubilizing Bacteria (PSB) inhabiting different soil ecosystems (Khan et al., 2013; Sharma et al., 2013). Although thousands of PSB have been isolated in the past, these belong to only a few bacterial genera, namely: *Pseudomonas*, *Bacillus*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Azospirillum, Aerobacter*, *Rhizobium, Flavobacterium*, *Erwinia* (Rodriguez and Fraga, 1999; Goswami et al., 2016), *Serratia, Enterobacter*, *Paenibacillus*, *Escherichia*, *Pantoea*, and some other members of the *Enterobacteriaceae* family (Malboobi et al., 2009; Kämpfer, 2007).

Several workers have documented their findings in order to have better understanding that how the microbial populations cause the solubilization of insoluble P (Illmer and Schinner, 1995; Khan et al., 2007, 2009; Buch et al., 2008). The P-solubilizing activity is determined by the microbial biochemical ability to produce and release metabolites such as organic acids that through their hydroxyl and carboxyl groups, chelate the cations (mainly calcium) bound to phosphate, the latter

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being converted into soluble forms (Glick, 2012; Goswami et al., 2016). From the various strategies adopted by microbes, the involvement of low molecular mass organic acids secreted by microorganisms has been a well-recognized and widely accepted theory as a principal means of P-solubilization, and various studies have identified and quantified organic acids and defined their role in the solubilization process (Maliha et al., 2004; Khan et al., 2010; Marra et al., 2012). Consequently, the acidification of microbial cells and their surrounding leads to the release of P-ions from the P-mineral by H^+ substitution for Ca^{2+} (Mullen, 2005; Trivedi and Tongmin, 2008). Furthermore, production of phosphatases and phytases by PSB has major role in releasing phosphate from organic phosphate sources (e.g. phytate) (Rodriguez and Fraga, 1999; Goswami et al., 2016).

However, buffering capacity of the medium reduces the effectiveness of the PSB in releasing P from tricalcium phosphate (TCP) (Stephen and Jisha, 2009). In soil, pH buffering capacity is governed mostly by protonation/deprotonation of acidic group on organic matter, oxides and hydroxides, dissolution/precipitation of carbonates and etc. (Hamza, 2008). In many cases, the P-solubilization ability of PSB in natural soils is different from that observed under in-vitro conditions. Indeed, most PSB have been isolated using neutral and un-buffered media, although it is well known that both the acidity and the buffering capacity of soils could limit microbially mediated P solubilization (Gyaneshwar et al., 1998). Irrespective of in-vitro P solubility potential of different isolates, it is reported that due to buffering capacity of soil, stabilization of pH reduces solubilization potential by bacteria (Ae et al., 1990; Hariprasad and Niranjana, 2009). Qureshi et al. (2012) reported that PSB solubilized more available P by the production of organic acids which acts like chelates. Deubel and Merbach (2005) found that a significant amount of P was released from TCP through the chelating activity of carboxylic anions which solubilize more phosphorus and play a greater role than acidification under natural soil (buffered) conditions. The buffering capacity of soils would limit solubilization of soil phosphates by microorganisms as it has been shown, solubilization Ca-P complexes are mediated mainly by lowering the pH of the medium (Maliha, 2004). We should be aware that buffers may affect bacterial growth and make difference between in-vitro finding compared to land experiments, for instance, some reports indicate a negative effect of Tris (Tris(hydroxymethyl)amino methane) buffer, some authors have raised some of its beneficial effects, by enhancing the availability of carbon, vitamins and hormones when promoting the increase of bacteria in the growth media (Provasoli and Pintner, 1980; Woelkerling et al., 1983; Fabregas et al., 1993), but it seems that simulation of soil buffered condition by application of buffers (such as Tris, MES and etc.) in in-vitro assays give reliable results to isolate efficient PSB from soil samples. Hence in the present study we isolated and identified PSB strains and assessed their capacity to solubilize P under buffered and non-buffered conditions with different insoluble P sources, and results showed that ignoring soils buffering conditions in in-vitro experiments can fail to detect the really effective microbial strains.

2. Material and methods

2.1. Collection of soil samples

Surface (0–25 cm) soil samples were collected from sub-humid (Fandoghlou-Ardebil) and semi-arid (Namin-Ardebil) regions in Iran from the lands under cultivation of legumes, cereals and non-cultivated areas such as grassland and virgin lands. Totally, 24 different soil samples were taken in summer 2012. Samples were immediately transferred to the lab and stored at 4 °C till use.

2.2. Isolation and screening of phosphate solubilizing bacteria

Soil samples were dissolved in sterile distilled water and then serially diluted up to 10^{-7}. Then, 100 µL of $10^{-4}-10^{-7}$ serially diluted samples were spread on organic and inorganic Sperber agar and the plates were incubated at 26 °C for 3–5 days. The organic Sperber medium used was inositol hexa phosphoric acid (IHP) (ingredients g L^{-1}: agar 16, glucose 10, yeast extract 0.5, $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.25, IHP 5 mL adjusted to pH 7). The mineral Sperber medium was mineral phosphate (ingredients g L^{-1}: agar 16, glucose 10, yeast extract 0.5, $CaCl_2$ 0.1, $MgSO_4$ 0.25, TCP or RP 5, pH 7). After incubation, the bacterial colonies of PSB were detected by clear zones (Vyas et al., 2007) and blue color around them (Sarikhani et al., 2010) presumably due to solubilization of mineral (TCP) and organic (IHP with chromogenic substrate (BCIP or 5-bromo 4-chloro 3-indolyl phosphate)) phosphorus sources, respectively. Purification of these isolates was done by re-streaking on the fresh Sperber or nutrient agar (NA) media. After that, bacterial isolates were spot inoculated on Sperber's medium supplemented with insoluble organic and inorganic P sources. Diameter of halo zones around colonies and intensity of blue color were measured on first, third and fifth days of the incubation of plates at 28 °C. Isolates with highest halo zone diameter and blue color intensity were selected for further assays and more identification. Ratio of the halo diameter (HD) to the colony diameter (CD) was used as P solubility index (HD/CD) in solid Sperber medium containing TCP (Edi–Premono et al., 1996) while blue intensity in Sperber medium containing IHP + BCIP was checked as organic-P solubility by phosphatases (Malboobi et al., 2009; Sarikhani et al., 2010).

Quantitative estimation of phosphate solubilizing ability by candidate isolates from the last stage was carried out using Erlenmeyer flasks (100 mL) containing 30 mL of liquid mineral (TCP) and organic (IHP) Sperber's media inoculated in triplicate with the bacterial isolates (500 µL inoculums with approximately 10^8 CFU mL^{-1}). Sterile non-inoculated medium served as control. The flasks were incubated for 7 days at 26 °C in shaker incubator at 150 RPM. The concentration of solubilized phosphate in culture supernatant was determined to observe the kinetics of solubilization of each isolate by yellow color method and robust isolates were specified. Concentration of P was determined spectrophotometrically by ammonium-vanadate-molybdate method (Hach DR/2000 spectrophotometer; Hach Company, Cambridge, UK) (Sarikhani et al., 2016).

2.3. Determination of phytase activity of PSB with Wade's reagent

Phytase activity of isolated bacteria was assessed qualitatively by application of Wade's reagent. In this method phytase activity is being scored by eliminating substrate (phytate) in the assay medium. Determination the presence of phytate in medium with colorimetric method is based on the discoloration of the purple Fe^{3+}–sulfosalicylate complex (Wade reagent) by phytate. Two mL of the supernatant aqueous extract of the bacterial cultures, 2 mL of the Wade reagent consisting of 0.03% $FeCl_3.6H_2O$ and a 0.3% sulfosalicylic acid solution was added and the presence of phytate was determined by the discoloration of the medium (from purple to colorless) (Velickovic et al., 1999). Hence, we expect in bacterial isolates with phytase activity after adding Wade's reagent purple color was developed (Deaker et al., 2011).

2.4. Molecular characterization of bacterial isolates

Bacterial ribosomal gene analysis was performed as described by Weisberg et al. (1991). The extraction of chromosomal DNA was done according to Sambrook and Russel (2001). Gene fragments specific for the highly variable region of the bacterial 16 S rRNA gene was amplified by PCR as described by Loffler et al. (2000) using the universal primers 24 F (5-AGAGTTTGATCMTGGCTCAG-3) and 1525 R (5-AAGGAGGTGATCCAGCCGCA-3). PCR was performed in a 20 µL reaction mixture containing 2 µL of 10X PCR buffer, 0.4 µL of 10 mM dNTPs, 0.8 µL of 50 mM MgCl2, 0.2 µL of each primer (10 pmol), 0.2 µL of 10 U $µL^{-1}$ Taq-DNA polymerase, 1 µL genomic DNA and rest of the PCR