Effect of Crop Productions on Diversity and Efficiency of Phosphate-Solubilizing Bacteria

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Abstract: In order to assess the effects of different crop productions on the diversity and the efficiency of phosphate-solubilizing bacteria (PSB), this study isolated phosphate-solubilizing bacteria from paddy fields and eggplant fields before and after harvest in Kochi prefecture in Japan. In accordance with previous reports in the literature, the results showed that *Klebsiella pneumonia* in the paddy field with water and in the eggplant fields solubilize $Ca_3(PO_4)_2$ better than $FePO_4$ and $AlPO_4$. Especially, *Klebsiella pneumoniae* strain M-AI-2 and *Gluconacetobacter sp*. isolate code Ek01 in the eggplant fields seem to have the capacity to solubilize insoluble forms of $AlPO_4$ and $FePO_4$ which are the main forms of insoluble phosphates in acid sandy soils.

Keywords: Classification, Phosphate-Solubilizing Bacteria (PSB), Crop productions

1. Introduction

Acid-weathered soils of the tropics and subtropics are particularly prone to P deficiency. Worldwide, phosphorus is considered as the principal yield-limiting nutrient along with nitrogen (Zahran, 1999). Phosphorus deficiency is a primary constraint to plant growth in many terrestrial ecosystems (Bonser *et al*., 1996). Phosphorus deficiency occurs especially in acid sandy soils with high levels of P fixation by Fe and Al oxides. The symbiotic nitrogen fixation with legumes is performed by a group of bacteria, commonly called rhizobia. The rhizobia in symbiosis with legumes are the primary source of biologically fixed nitrogen for agricultural systems (Vance, 1997; Kinzing and Socolow, 1994). P fertilization usually results in an enhanced number of nodules, mass and greater N_2 fixation per plant and per gram of nodules (Serraj and Adu-Gyamfi, 2004). Besides increasing nodulation and N fixation, P fertilizer also increases grain yield (Mugwira *et al*., 1997).

Plants use phosphorus for ATP synthesis. ATP is an essential energy-provider molecule for the metabolism of organic compounds containing P such as sugar phosphates, phospholipids, nucleic acids, nucleotides and coenzymes which are key molecules for biological metabolisms (Schachtman, *et al*., 1998). Plants dependent on symbiotic N_2 fixation have therefore high ATP requirements for nodule development and function (Ribet and Drevon, 1996) and need additional P for signal transduction and membrane biosynthesis. However, strains of rhizobia differ markedly in their tolerance to phosphorus deficiency (Beck and Munns, 1985) and P solubilization capacity (Junrungreang *et al*., 2009). Subsequently, nodulation, N fixation, rhizobial multiplication and survival of rhizobia in the soil are particularly affected under low P and acid soil conditions (Graham and Vance, 2003) and the growth rate of most rhizobia strains is reduced by low levels of P (Al-Niemi *et al*., 1997).

Normally, plants and microorganisms produce phosphatases which are released in the rhizosphere and catalyze the hydrolysis of organic phosphate esters to orthophosphate anions. Radersma and Grierson (2004) concluded that root exudation of acid phosphatases and organic acids increase the P solubility in the rhizosphere. The phosphatases efficiency is related to various factors such as the microbial fauna, the soil temperature and humidity and more particularly the associated bacteria communities (Zahran, 1999). Phosphatases activity can be induced by low inorganic phosphorus concentrations in the soil solution, but other factors can play a significant role as the environmental conditions, the physiological state of the plant, the type of rooting system, the age of the plant and the location of ectomycorrhiza on the root (Antibus *et al.*, 1997).

Within the rhizosphere, the solubility of phosphate can be enhanced by the secretion of organic acids in root exudates and enable phytic acid to be more available to microorganisms responsible for its mineralization. Despite its importance in soils and particularly in different crop productions, the isolation of phosphate-solubilizing bacteria and their P solubilization capacity in the phosphorus cycle remains poorly studied and only few studied have aimed at exploring the microbial diversity and its role in the regulation of the cycle of the phytic acid. Finally, the analysis of the ability of bacterial isolates to hydrolyze phytic acid will enable to create a highly valuable basis for the use of these isolates as bio-inoculums or for the use of enzymes in the food industry. The objectives of this research were to isolate phosphate-solubilizing bacteria (PSB) from different field crop productions, evaluate their P solubilization capacity and identify the PSB from different field crop productions in Kochi, Japan.

2. Materials and Methods

2.1 Soil sampling

The experiment was conducted within Kochi prefecture, Japan. Kochi prefecture is characterized by a semi-arid tropical climate with a distinct rainy season from May to September. Soil samples were collected from paddy fields and eggplant fields from the soil surface until a depth of 10 cm in order to isolate phosphate-solubilizing bacteria. The soil samples were analyzed for pH, total N, total P, available P, exchangeable K and soil water content (Table 1).

Table 1 Soil properties in paddy and eggplant fields in different conditions in Kochi, Japan

Soil properties	Paddy field after harvesting	Paddy field with water	Eggplant field in Kochi University	Eggplant field around Kochi University
pH (1:1 H_2O)	4.86	5.45	5.53	6.80
Total nitrogen (%)	0.071	0.126	0.156	0.179
Total phosphorous (mg/kg)	816.33	1,078.72	1,107.87	10,544.22
Available phosphorous (mg/kg)	138.73	312.93	300.29	4,285.71
Exchangeable potassium (mg/kg)	166.77	107.08	244.01	1,527.25

2.2 Isolation of phosphate solubilizing bacteria by enrichment culture

To extract bacteria from soil, 5 g of soil samples were transferred to the National Botanical Research Institute phosphate growth medium (NBRIP). Per liter, this growth liquid medium contains 10 g glucose with 5 g of different insoluble forms of phosphate ($AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$), 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl and 0.1 g $(NH_4)_2SO_4$. Additionally, modified NBRIP media, containing either $FePO_4$ or $AlPO_4$ as the sole source of P, were also used for the initial screening step. The pH of the agar medium was adjusted to 7.0. Tricalcium phosphate was autoclaved separately and the other sterile ingredients were aseptically mixed after autoclaving. Erlenmeyer flasks containing 50 mL of the medium with inoculants were incubated for 7 days at 30 °C on a IWAKI Incubator shaker at medium speed (150 cycles min^{-1}). For the following week, 5 ml of this incubated medium with inoculants were transferred into 50 mL Erlenmeyer flasks again with new liquid medium for 7 more days at 30 °C on a IWAKI Incubator shaker at medium speed (150 cycles min^{-1}). At the end of each week in NBRIP growth liquid media, aliquots of each dilution were spread on NBRIP medium and incubated at 30 °C for 14 days. Colonies were selected from the plates on the basis of the appearance of a clear halo; the clones were further purified on minimal medium based on each insoluble phosphate forms. Once purified, each isolate was stored as a glycerol stock at −80 ° C.

2.3 Mineral phosphate solubilization assays

The phosphate solubilizing (PS) activity of each of the isolates was determined by molybdenum-blue method (Murphy and Riley, 1962). The isolates were grown in NBRIP liquid medium containing different insoluble forms of phosphate ($AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$) for 3 days at 30 °C on a IWAKI Incubator shaker at medium speed (150 cycles min^{-1}). The solubilization efficiencies were determined by reaction with ammonium molybdate for phosphorus compounds as ammonium phosphomolybdate and reduced with a compound ascorbic acid to molybdenum blue. Then, the isolates were incubated for 30 min at room temperature for color development. And finally, the absorption of light in the wavelength range 595 nm was measured by 680 XR Microplate Reader.

2.4 PCR amplification of 16S rRNA and sequencing

The gene-encoding 16S rRNA was amplified from selected strains by the polymerase chain reaction (PCR) using bacterial universal primers proR2 (5′ -AGAGTTTGATCMTGGCTCAG-3′) and 907R (5′ -CCGTCAATTCCTTTRAGTTT-3′) (Weisburg *et al*., 1992). The PCR mix consisted of 0.25 μM of each primer, 1X

PCR buffer and 0.2 U of Taq DNA polymerase. A suspension of cells on MilliQ water, coming from a fresh colony grown on Nutrient Agar, was used as target DNA. The following cycle conditions were used: 85 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 3 min (Lane, 1991).

The PCR products were purified from agarose gels with the PCR Clean-up Gel Extraction Kit (Macherey-Nagel, Germany) and sequenced. The nucleotide sequences were compared using the BlastN program (Altschul *et al*., 1997), and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups.

3. Results

3.1 Isolation of PSB from soil samples

The screening strategy employed during this research enabled the identification of PSB colonies on NBRIP medium containing different insoluble forms of phosphate ($AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$) as sole P source. No colonies exhibiting a clear halo were observed on agar plates supplemented with either $FePO_4$ or $AlPO_4$. Approximately 9 bacterial isolates showed clear halos of $Ca_3(PO_4)_2$ solubilization. Some obvious differences in the size of the halos of different isolates were observed (not shown). This preliminary observation suggested the existence of bacterial isolates exhibiting different degrees of PS efficiencies in the soil samples collected. To confirm this observation, the 9 purified isolates were tested following the protocol of Murphy and Riley (1962), a method previously shown to be a reliable and qualitative indicator of the PS activity of different bacterial isolates. Table 2 shows the OD595 nm shift of the culture supernatants of each of the 9 PSB isolates after a 3-day cultivation period in NBRIP medium. Indeed, some isolates did not show any significant change in the absorbance of the supernatant while others exhibited OD595 nm changes in the absorbance. Furthermore, we noticed that the most dramatic changes in the color of the supernatant correlated with a total solubilization of $Ca_3(PO_4)_2$ in the medium. Based on these results we selected 9 isolates exhibiting the highest PS activities for further studies. The solubilization efficiencies of these isolates were calculated and are shown in Table 2. After evaluating their P solubilization capacity, we concluded that all of the 9 isolates can solubilize $Ca_3(PO_4)_2$ better than $FePO_4$ and $AlPO_4$ especially the isolates with the codes Rk02, Rk03 and Ek04. Moreover, isolate codes Ek01 and Ek04 look interesting for solubilizing $FePO_4$ and $AlPO_4$, which are the main forms of insoluble phosphates in acid sandy soils.

3.2 Effect of soil properties in different field crop productions on PSB

After identification by enrichment culture, we found bacteria from the paddy field with water and from the eggplant field in Kochi University as well as from the eggplant field around Kochi University that could solubilize insoluble phosphate in solid media. Nine isolates from these 3 fields at Kochi were evaluated for their P solubilization capacity. All 9 isolates can solubilize $Ca_3(PO_4)_2$ better than $FePO_4$ and $AlPO_4$ especially the isolates with the codes Rk02, Rk03 and Ek04 that came from crop production in Kochi University (paddy field with water and eggplant fields). These findings might be linked to the soil pH that was between 5.4-5.5 (Table 1) which is not too low and not higher than 6.5. Poehlman (1991) found that the suitable soil pH for rhizobia activity was 6.5 and that rhizobia exhibit varied responses to acidity. Moreover, Taylor *et al*. (1991) reported that acidity had more severe effects on rhizobial multiplication than did Al stress and low P conditions. However, phosphorus is also particularly important for the activity of the rhizobia species. In addition, the growth rate of most rhizobia strains is reduced by low levels of P (Al-Niemi *et al*., 1997). The strains of rhizobia differ markedly in their tolerance to phosphorus deficiency (Beck and Munns, 1985). This P stress response occurred when the medium P concentration decreased below 1 mM.

Table 2 Phosphate solubilizing effectiveness of tested bacteria, 3 days after inoculation.

Isolate Code	Solubilized Phosphate (mgP/l) from		
	$Ca_3(PO_4)_2$	$FePO_4$	$AlPO_4$
Rk01	1,015	0	0
Rk02	1,534	0	0
Rk03	1,580	0	0
Ek01	805	93	0
Ek02	941	0	0
Ek03	943	0	15
Ek04	1,548	47	161
Ek05	770	0	0
Ek06	912	0	0

3.3 Identification of PSB isolates

Nucleotide sequencing of PCR-amplified 16S rRNA genes and sequence comparison with available data in the GenBank using the BLAST algorithm (Altschul *et al*., 1997) allowed us to identify the majority of the PSB isolates

(Table 3). Based on a sequence identification of 94% or greater (Van Waasbergen, 2004), they were all affiliated to the β- or γ- sub-divisions of the Proteobacteria: three isolates were similar to species of the *Klebsiella* genus, another three were similar to *Gluconacetobacter sp*. and one was closely related to *Sphingobacterium sp*. *Klebsiella pneumonia* in the paddy field with water and in the eggplant fields which solubilize $Ca_3(PO_4)_2$ better than $FePO_4$ and $AlPO_4$. *Klebsiella pneumonia* strain M-AI-2 seems to be more interesting to solubilize $AlPO_4$ and $FePO_4$. Moreover, *Gluconacetobacter sp*. isolate code Ek01 in the eggplant field in Kochi University can solubilize $FePO_4$ as well.

Table 3 Identification of PSB isolates from soil samples of paddy fields and eggplant fields in Kochi by 16S rRNA sequencing after inoculation.

Isolate Code	Length of 16S rRNA gene sequenced	GenBank accession no.	Most closely related organism/ Species (Strain)	Accession no.	% Gene identity
Rk01	503	lcl/23633	*Gluconacetobacter sp*.	EF493039.1	97%
Rk02	814	lcl/45645	*Klebsiella pneumoniae*/ BRp_2A	JN644536.1	100%
Rk03	488	lcl/20369	*Klebsiella pneumoniae*/ BRp_2A	JN644536.1	99%
Ek01	546	lcl/19025	*Gluconacetobacter sp*.	EF493039.1	99%
Ek02	710	lcl/39135	*Gluconacetobacter sp*.	EF493039.1	99%
Ek03	990	lcl/26231	*Gluconacetobacter sp*.	EF493039.1	99%
Ek04	938	lcl/51159	*Klebsiella pneumoniae*/ M-AI-2	FJ828890.2	99%
Ek05	966	lcl/30399	*Uncultured bacterium* clone MS-115	GQ477848.1	99%
Ek06	983	lcl/27533	*Sphingobacterium sp*. 21	CP002584.1	94%

4. Conclusion

Klebsiella pneumoniae showed the highest P solubilization capacity in the paddy field with water and eggplant fields. *Klebsiella pneumoniae* strain M-AI-2 and *Gluconacetobacter sp*. isolate code Ek01 in the eggplant field in Kochi University seem to have the capacity to solubilize insoluble forms of $AlPO_4$ and $FePO_4$. Moreover, the soil pH and available P from different crop productions have an effect on rhizobial multiplication and their activities in the soil.

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