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Article

Synergistic Phosphate Solubilization by *Burkholderia anthina* and *Aspergillus awamori*

Buddhi Charana Walpola, Hyo-Ju Jang, and Min-Ho Yoon*

Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, Daejeon, 305-764, Korea

Single or co-inoculation of phosphate solubilizing bacterial and fungal strains (*Burkholderia anthina* and *Aspergillus awamori* respectively) was performed separately to assess their synergistic and antagonistic interactions and the potential to be used as bio-inoculants. Co-inoculation was found to release the highest content of soluble phosphorus (1253 μg ml^{-1}) into the medium, followed by single inoculation of fungal strain (1214 μg ml^{-1}) and bacterial strain (997 μg ml^{-1}). However, there was no significant difference between single inoculation of fungal strain and co-inoculation of fungal and bacterial strain in terms of the phosphorous release. The highest pH reduction, organic acid production and glucose consumption were observed in the sole A. *awamori* inoculated culture medium. According to the plant growth promotion bioassays, co-inoculation of the microbial strains resulted in 21% and 43% higher shoot and root growth of the mung bean seedlings respectively as compared to the respective controls. Therefore, co-inoculation of B. *anthina* and A. *awamori* showed better performance in stimulating plant growth than that in inoculation of each strain alone. However, assessment period of the present study being short, we recommend in engaging further experimentation under field conditions in order to test the suitability of the strains to be used as bio-inoculants.

Key words: *Burkholderia anthina*, *Aspergillus awamori*, Phosphate solubilization, Co-inoculation

Introduction

Phosphorous shortage is becoming a matter of concern and a major constraint for sustaining the soil fertility in the most tropical and some subtropical areas due to acidic nature of these soils. In these regions, phosphate ions are either adsorbed onto the surface of soil minerals or precipitated by free aluminum and iron leading to the widespread phosphorus deficiency (Frossard et al., 2000). To overcome this deficiency, farmers used to apply several-fold excess phosphorous than actually the plant needs. This excess application of chemical phosphatic fertilizer may cause environmental degradation as well as increase the cost of production.

It has been reported that plant growth promoting rhizobacteria (PGPR) including phosphate-solubilizing microorganisms (PSMs) are able to solubilize the unavailable forms of P in soil by acidification, chelation, and exchange reaction in the soil environment (Maliha et al., 2004; Ponmurugan and Gopi, 2006).

However, soil inhabits several diverse groups of microorganisms and there is a competition in among them owing to synergistic and antagonistic interactions (Sylvia et al., 2005). Metabolic activity, nutrient requirements of microorganisms and environmental factors may involve in determining the dominant species of the microorganisms within the soil. Similarly, PSMs exhibit synergistic and antagonistic interactions with each other and therefore, it is important to understand as to how PSMs compete or cooperate with each other in the soil before using them as bio-fertilizers.

PSMs have widely been used as inoculants to increase phosphorous uptake and crop yield, and there are several previous reports regarding plant growth promotion and increase of phosphorous availability due to co-inoculation of PSMs under green house as well as field conditions (Reyes et al., 2002; Zaidi et al., 2003; Khalid et al., 2004; Hameeda et al., 2006; Chen et al., 2008;). However, adequate laboratory methods are needed for better understanding of the interactions of the inoculated microorganisms within the soil.

This study evaluated the effect of co-inoculation of phosphate solubilizing bacterial and fungal strains (*Burkholderia anthina* and *Aspergillus awamori*) respectively on solubilization of inorganic phosphate in the growth medium.

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*Corresponding author : Phone: +82428217888
E-mail: mhyoon@cnu.ac.kr

Materials and methods

Isolation of bacterial and fungal strains The phosphate solubilizing bacterial strain, *Burkholderia anthina* was isolated from tomato growing rhizosphere soil samples collected from green houses at Chungcheongnam-do , Gongju- gun area in South Korea. Fungal strain, *Aspergillus awamori* was isolated from waste mushroom bed of *Agaricus bisporus* from Chungcheongnam-do , Buyeo-gun area in South Korea. Pure cultures were maintained as a glycerol suspension (30% v/v) at -80°C until use.

Inoculum preparation and inoculation A single colony was transferred into 100 ml flasks containing 25 ml nutrient broth and was grown aerobically in flasks on a rotating shaker (150 pm) for 48 hr at 30°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration 10^8 CFU ml^{-1}, and resulting suspensions were used to inoculate sterilized 500 ml Erlenmeyer flasks (n = 3) containing 200 ml National Botanical Research Institute Phosphorus (NBRIP) liquid medium containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2$. $6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999) as single bacterial inoculation. Another set of sterilized 500 ml Erlenmeyer flasks (n = 3) containing 200 ml NBRIP liquid medium were inoculated by transferring 10 mm diameter mycelia disc from a fully sporulating culture as single fungal inoculation. For co-inoculation, both bacterial and fungal cultures were mixed and then used inoculate NBRIP liquid medium described earlier. Flasks were then incubated for 8 days with continuous shaking at 30°C. Sterilized non-inoculated medium served as a control. A 10 ml sample of each cultured and control were taken into centrifugation tube at 2, 5 and 8 days after inoculation and centrifuged in each day for 10 min at 8,000 rpm. The clear supernatant was used to determine the release of phosphorous into the medium, the medium pH, the residual glucose contents and the organic acid production.

Assay of phosphorous release and medium pH Phosphorous release into the medium was assayed using the phospho-molybdate blue color method (Murphy and Riley, 1962). The pH of the culture medium was recorded with the pH meter equipped with glass electrode.

Assay of residual glucose content The residual glucose content of the culture medium was assayed using DNS (3, 5- dinitrosalicylic acid) method as described by Miller (1959).

Assay of organic acid production To determine the organic acid composition of the different cultures, aliquots from the supernatants were analyzed using high-performance liquid chromatography (HPLC-Model). The used column was Inertsil ODS 3V and a UV detector set to 210 nm at 40°C. Mobile phase consisted of 0.008 M H_2SO_4 run at a flow rate of 0.2 ml min^{-1}. HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of pure organic acids (gluconic acid, oxalic acid and citric acid) injected separately. Peaks were identified by retention times against a set of standards from known three organic acids.

Plant growth promotion bioassay on mung bean (*Vigna radiata*) Plant growth promotion ability with the single and co-inoculation of *Burkholderia anthina* and *Aspergillus awamori* was determined by pot culture assays lasted for 4 weeks. Seeds of mung bean were soaked in bacterial and fungal single or co-culture suspensions separately for about 30 min prior to sowing. After 4 weeks, seedlings were uprooted, washed under running water and root to shoot length ratios were measured.

Results and Discussion

Periodic changes in the pH of NBRIP medium, soluble phosphorus content released from the $Ca_3(PO_4)_2$ containing NBRIP medium and residual glucose contents due to single or co-inoculation of *B. anthina* and *A. awamori* incubated for 8 days are presented in Fig. 1, 2 and 3 respectively.

Co-inoculation was shown to release the higher contents of soluble phosphorus (1253 μg ml^{-1}) into the medium, followed by single inoculation of fungal and bacterial strains with 1214 and 997 μg ml^{-1} of soluble phosphorus respectively (Fig. 1). However, there was no significant difference between single inoculation of fungal strain and co-inoculation of fungal and bacterial strain in terms of phosphorous release. The results clearly depicted that co-inoculation enhanced phosphate solubilization into the liquid culture medium. This is in

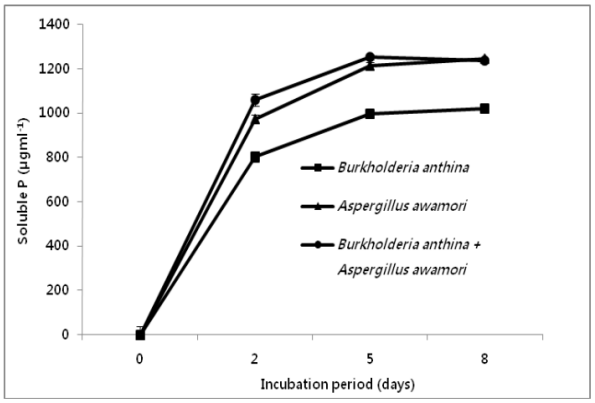

Fig. 1. Effect of single and co-inoculation of *Burkholderia anthina* and *Aspergillus awamori* on phosphate solubilization. Values given here are the means (n = 3) ± standard deviation.

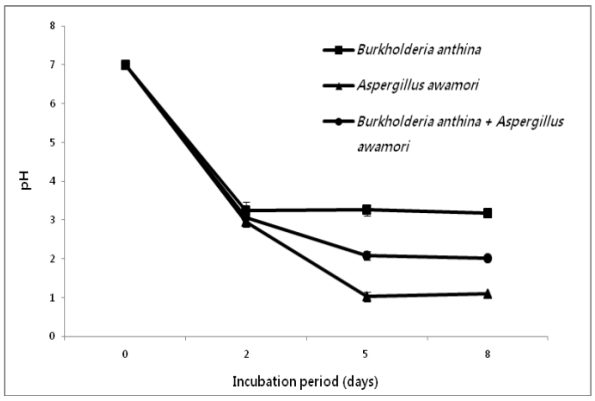

Fig. 2. Effect of single and co-inoculation of *Burkholderia anthina* and *Aspergillus awamori* on pH change. Values given here are the means (n = 3) ± standard deviation.

agreement with Bras and Nahas (2012) who observed similar trends of phosphate solubilization by *Burkholderia cepacea* and *Aspergillus niger* co-inoculated medium. The average phosphorous released in their experiments were recorded as 570 μg ml^{-1} for bacteria, 740 μg ml^{-1} for fungi and 760 μg ml^{-1} for the co-culture at the end of the incubation period. Our results also showed that the contents of soluble phosphorus released into culture medium in all cases increased significantly during the first 5 days of the incubation and then remained at peak for further few days.

Bacterial and fungal inoculation caused reduction in pH of the culture medium (Fig 2). In fungal inoculated medium, it was reduced to 1.02 after 5 days of incubation and in bacteria inoculated medium, the pH was reduced to 3.24 after 2 days of incubation. In co-inoculated medium, it was reduced to 2.09 after 5 days incubation. Both single

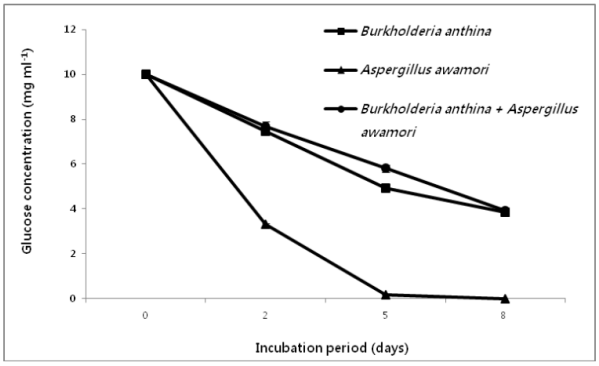

Fig. 3. Effect of single and co-inoculation of *Burkholderia anthina* and *Aspergillus awamori* on residual glucose content. Values given here are the means (n = 3) ± standard deviation.

and co-inoculation showed negative correlation (r = -0.934 ± 0.1, $P \leq 0.05$) between soluble phosphorus content and pH in the culture medium.

A similar strong negative correlation (r = -0.962 ± 0.1, $P \leq 0.05$) was observed between residual glucose content and phosphate solubilization. On the 5^{th} day of incubation, glucose contents were completely consumed by medium inoculated with fungi (Fig. 3). However, 61.6% and 60.9% reduction in glucose levels were observed in media inoculated with bacteria, and the co-culture, respectively after 8 days of incubation.

Table 1 shows the organic acid production by single and co-inoculation of *B. anthina* and *A. awamori.* Gluconic acid was the major organic acid produced by both single and co-inoculated culture medium followed by oxalic acid and citric acid (Table 1). The bacterial strain was not capable of producing citric acid. Similar to phosphate solubilization, no significant difference was observed in organic acid production between single inoculation of fungal strain and the co-inoculation of fungal and bacterial strains. In agreement with this work, gluconic acid was the major organic acid produced by *Burkholderia cepacia* DA23 (Song et al., 2008), *Burkholderia cepacia* CC-A174 (Lin et al., 2006), *Aspergillus flavus* and *Aspergillus niger* (Maliha et al., 2004). Production of organic acids other than gluconic acids such as oxalic acid, citric acid and succinic acid have been reported to be produced by *Aspergillus flavus*, *Aspergillus niger* (Maliha et al., 2004). Singh and Amberger (1991) reported the production of significant levels of glycolic acid, oxaloacetic acid, succinic acid, fumaric acid, malic acid, tartaric acid and citric acid by *Aspergillus niger* during the straw composting with rock

Table 1. HPLC analysis of organic acids production by single and co-inoculation of *Burkholderia anthina* and *Aspergillus awamori*. Values given here are the means of three replicates (n = 3). ND- Not Detectable

	Gluconic acid mg ml^{-1}			Oxalic acid mg ml^{-1}			Citric acid mg ml^{-1}		
	Day 2	Day 5	Day 8	Day 2	Day 5	Day 8	Day 2	Day 5	Day 8
Burkholderia anthina	18.71^{a}	19.63^{a}	17.52^{a}	0.81^{b}	0.95^{b}	0.84^{ab}	ND	ND	ND
Aspergillus awamori	11.35^{b}	17.21^{b}	9.95^{b}	1.21^{a}	1.81^{a}	0.94^{a}	1.91^{a}	2.09^{a}	2.07^{a}
Burkholderia anthina + *Aspergillus awamori*	11.21^{b}	16.59^{b}	9.12^{b}	1.18^{a}	1.76^{a}	0.91^{a}	1.83^{a}	2.01^{a}	1.95^{ab}

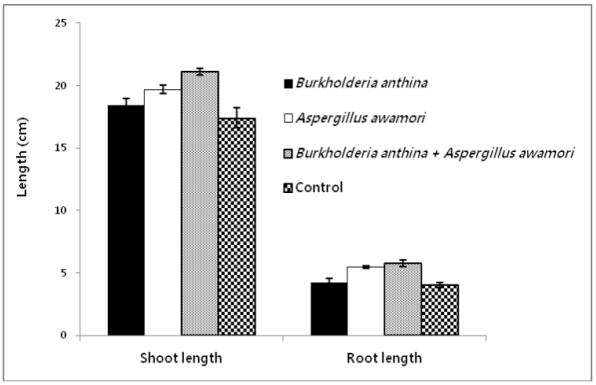

Fig 4. Effect of single and co-inoculation of B*urkholderia anthina* and *Aspergillus awamori* on Shoot length and root length of mung bean seedlings. Values given here are the means of three replicates (n = 3).

phosphate.

Organic acid production increased with the incubation period, reaching to the maximum at day 5. Similarly, decrease in pH and glucose contents, increase in soluble phosphorous contents was observed during the same period. Therefore this inverse relationship between phosphate solubilization and organic acid production with pH suggested that production of organic acids played a significant role in the acidification of the culture media. Single inoculation of *A. awamori* was shown to release the highest amount of organic acids compared to co-inoculation. This was evident from higher pH reduction and complete consumption of glucose contents within 5 days of incubation. Therefore, organic acid production is not the sole factor responsible for phosphate solubilization as reported previously by Chen et al. (2006).

According to the plant growth promotion assay, both single and co-inoculation of bacterial and fungal strains had significantly different effects on shoot and root growth as compared with the non-inoculated seeds. As shown in Fig. 4, shoot and root growth enhanced by 6%, 13%, 21% and 5%, 35%, 43% respectively for the single inoculation of *B. anthina* and *A. awamori* and co-inoculation of strains compared with the un-inoculated seedlings. Co-inoculation resulted in significantly higher shoot and root length compared to the single inoculation. Though no significant difference was observed in phosphate solubilization between fungi and co-culture medium, a significant difference in shoot and root length was observed between fungi and co-inoculated seedlings during plant growth promotion assay. This may be due to enhanced phosphorus nutrition and other plant growth promoting activities due to synergistic action of the co-inoculated medium. Phosphate solubilizing microorganisms can enhance plant growth through several different mechanisms in addition to providing available phosphorus to plants (Mundra et al., 2011). Several researchers have reported that synergistic interaction between bacteria and fungi (Bras and Nahas, 2012; Suri et al., 2011). Suri et al. (2011) observed synergistic interaction between Arbuscular Mycorrhizal Fungi (AMF) and Phosphate-Solubilizing Bacteria when co-inoculated to Maize plant.

In concluding, co-inoculation of the strains *Burkholderia anthina* and *Aspergillus awamori* showed better performance in stimulating plant growth than inoculation of each strain alone. However, being short assessment period of the present study we recommend in engaging further experimentation under field conditions in order to test the suitability of the strains to be used as bio-inoculants.

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