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PQQ-Dependent Organic Acid Production and Effect on Common Bean Growth by *Rhizobium tropici* CIAT 899

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Abstract *Rhizobium tropici* CIAT 899 is capable of synthesizing inactive apo-glucose dehydrogenase (GDH). To become an active holo enzyme, the GDH requires a cofactor, PQQ. When *R. tropici* CIAT 899 was grown in a broth culture medium containing hydroxyapatite and pyrrolo quinoline quinone (PQQ), pH decreased while the concentration of soluble P increased. The solubilization of hydroxyapatite was associated with the production of gluconic acid and 2-ketogluconic acids. The organic acid production and P solubilization were greatly enhanced when the bacterium was grown with air supply. Effect of *R. tropici* CIAT 899 with (CI+PQQ) and without PQQ (CI) on the common bean growth was examined. Shoot and root weight, and N and P contents in CI+PQQ treatment, were significantly higher than those in control and CI treatment. Nodule weight and acetylene reducing activities were also significantly higher in CI+PQQ treatment than in other treatments.

Key words: Apo-glucose dehydrogenase, PQQ, hydroxyapatite, 2-ketogluconic acid

Many plant growth promoting rhizobacteria (PGPR) which exert a beneficial effect on plant growth are harbored in soil [10, 15]. Species of *Pseudomonas, Azospirillium, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Acinetobacter,* and *Flavobacterium* are known to be active PGPR [14]. Among them, *Rhizobium, Pseudomonas,* and *Bacillus* species are the most powerful mineral phosphate solubilizing bacteria (PSB) for plant growth promotion [7]. PSB can convert insoluble phosphorus into an available form by producing organic acids such as succinic, malic, gluconic, 2-ketogluconic, citric, and acetic acids [22]. Among the organic acids, gluconic acid and 2-ketogluconic acid seem to be the most frequently occurring agents in mineral phosphate solubilization [7].

Pyrrolo quinoline quinone [2,7,9-tricarboxy-1*H*-pyrrolo (2,3-*f*)quinoline-4,5-dione (PQQ)]-dependent glucose dehydrogenase (GDH) can oxidize glucose to gluconate [20]. GDH, which resides in the cytoplasmic membrane, needs PQQ for the holoenzyme [5]. Gluconate is further oxidized to 2-ketogluconic acid by gluconate dehydrogenase (GADH). These two enzymes can be found in bacteria such as *Pseudomonas, Klebsiella,* and acetic acid bacteria [2, 16, 18]. The presence of a PQQ-dependent GDH has been demonstrated in rhizobia. Van Schie *et al*. [24] reported gluconate production from glucose when *Rhizobium leguminosarum* and *Sinorhizobium meliloti* were incubated in the presence of exogenous PQQ. Kim *et al*. [13] reported that *Escherichia coli* DH5α produced gluconic acid from glucose when the *pqq* gene was transformed into the bacterium from *Ranella aquatills*. Therefore, PQQ should be supplemented to bacteria having only apo GDH enzyme for glucose oxidation [1, 25].

This study was carried out to investigate pH, P solubilization, and organic acid production by *Rhizobium tropici* CIAT 899 in a broth culture medium containing 0.4% hydroxyapatite and different concentrations of exogenous PQQ for 10 days. The growth response of common bean by inoculating *R. tropici* CIAT 899 with and without PQQ was studied as well.

MATERIALS AND METHODS

Microorganisms and Culture Medium

Rhizobium tropici CIAT 899 was maintained in a yeast extract mannitol medium (YEM) (mannitol 10 g, K_2HPO_4

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0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, $CaCO_3$ 0.01 g, yeast extract 0.5 g, agar 15 g, pH 7.0, per liter) at 70°C for further experiment [26]. For batch cultures, standard medium (glucose 30 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, NaCl 0.1 g, $CaCl_2 \cdot 2H_2O$ 1.5 g, NH_4NO_3 1.2 g, KCl 0.2 g, yeast extract 0.5 g, hydroxyapatite 4.0 g, pH 7.0, per liter) was used.

Broth Culture

R. tropici CIAT 899 was grown in the standard medium supplemented with 0, 1, 10, or 1,000 nM PQQ. One-hundred ml of the broth was dispended to a 250-ml branched Erlenmeyer flask, and then autoclaved, followed by inoculation of 20 µl cultures of *R. tropici* CIAT 899 (2×10^6 cfu/ml) grown in YEM at 30°C for 1 day. Culture was grown at 150 rpm shaker incubator at 30°C for 10 days with or without air supply. Under air supply condition, air filtered through 0.45-µm Whatman membrane was injected to the culture broth at 30 ml min^{-1}. Samples were withdrawn at different time intervals (0, 1, 3, 5, 7, and 10 days). The experiments were carried out in triplicate. During the incubation period, pH of the medium, and concentrations of phosphate, gluconic acid, and 2-ketogluconic acids were measured.

Analysis of Culture Broth

Medium pH was measured using a pH meter directly by immersing a glass electrode into the culture solution. P concentration (orthophosphate ions) solubilized by *R. tropici* CIAT 899 from hydroxyapatite was measured by the method of Olsen and Sommers [19]. The broth cultures were filtered through a 0.2-µm Whatman membrane filter paper. Fifty µl of filtrates were added into the test tube containing 1.95 ml of distilled water. To this solution, 5 ml of distilled water and 2 ml of ammonium paramolybdate solution were added, followed by 1 ml of dilute $SnCl_2$ solution. After 5 min, P concentration was measured by using a spectrophotometer at 660 nm.

Organic acids in a filtrate were identified by a high performance liquid chromatography (HPLC) with a shodax RSpak KC-811 column (8 mm×30 cm) and UV detector at 210 nm. The mobile phase consisted of 0.1% phosphoric acid solution at a flow rate of 0.5 ml min^{-1}. The organic acids were quantitatively determined by comparing the retention time and peak area of chromatograms with those of the standards.

Plant Growth

Common bean (*Phaseolus vulgaris* L.) plant was grown in an unsterilized mixture (soil:vermiculite:sand=1:2:1, v/v/v) containing 1% hydroxyapatite. The mixture had pH (1:5 H_2O) of 5.3, organic matter of 1.0%, total N of 0.1%, available P of 8.2 mg kg^{-1}, and CEC of 5.3 $cmol^+$ kg^{-1}. Common bean seeds were surface-sterilized by soaking in 90% ethanol for 1 min, followed by a 3% solution of commercial bleach for 5 min. The seeds were then washed in distilled water. Three seeds were sown at 2 cm depth in each pot (700 ml). After emergence, plants were thinned to one plant of the uniform appearance per pot. The treatments were as follows: Control (CON), *R. tropici* CIAT 899 (CI), *R. tropici* CIAT 899 + 10 nM PQQ (CI+PQQ), and Mineral fertilizer (MF). *R. tropici* CIAT 899 was grown in a standard medium with (CI+PQQ) and without (CI) exogenous 10 nM PQQ for 2 days under air supply condition. One ml of inoculum (4×10^9 cfu/ml) per plant was inoculated. Control received the culture medium without both bacteria and PQQ. In MF treatment, fertilizers (N-P-K) were applied 6-8-6 kg per 10 a. Plants were watered with a nutrient solution lacking N and P [26].

Plant Analysis

After 5 weeks, common bean plants were harvested. Plant samples were finely grounded after drying at 65°C for 48 h. A 0.5 g sample was placed in a 100 ml micro-kjeldahl flask with 10 ml of concentrated H_2SO_4. The sample received 0.5 ml H_2O_2 every 10 min for 90 min (total 4.5 ml). After cooling, the solution was filtered through Whatman No. 6 into a 100-ml mess flask. The micro-kjeldahl method was used for N determination, and phosphorus was determined by using a spectrophotometer [9].

Acetylene (C_2H_2)-dependent ethylene (C_2H_4) production by nodulated root was determined to measure the activity of N_2 fixation [6]. After removal of the shoots, nodulated roots were gently collected free of soil. Roots were placed in 100-ml sealed flasks. Ten ml of air was withdrawn and replaced with an equal volume of C_2H_2 (100%). The flask was incubated at 30°C for 60 min. The ethylene was measured by using a gas chromatography (Varian STAR 3400 CX, U.S.A.) equipped with a plot fused silica capillary column (Chrompack CP-A1 203/KCL, 50 m length × 4 µm thin). The temperature of injection port was 250°C, and oven temperature was 100°C. The carrier gas was N_2 at a flow rate of 200 ml min^{-1} at 30 psi. FID detector temperature was 300°C.

RESULTS

Changes in pH and P Solubilization in Culture Broth

Effect of exogenous PQQ on phosphate solubilization by *R. tropici* was measured with and without air supply. The pH of the media rapidly decreased from 6.5 to about 2.8 in 3 days and then remained almost constant for 10 days in 1 and 10 nM PPQ with air supply. In 1,000 nM PPQ, pH decreased to about 4.0 at 3 days, and slowly decreased to 3.8 by 10 days. However, soluble phosphate concentration continuously increased during the incubation period, reaching approximately 550–700 mg/l by 10 days in PQQ treatments (Fig. 1). Control showed a little change in pH and P concentration with air supply (Fig. 1). On the other hand,

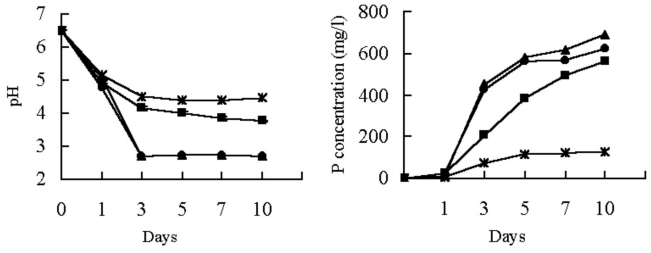

Fig. 1. Effect of different concentrations of PQQ on pH and P solubilization for 10 days in a culture broth containing 0.4% hydroxyapatite by *R. tropici* CIAT 899 with air supply condition.
*, control; ■, 1,000 nM PQQ; ●, 10 nM PQQ; ▲, 1 nM PQQ treatment.

when the culture was not aerated, the pH was maintained above 4, and P concentration was below 120 mg/l in all treatments throughout the incubation period (Fig. 2).

The HPLC chromatogram in Fig. 3 shows gluconic and 2-ketogluconic acids produced by *R. tropici* CIAT 899 in the culture medium. Major peaks were detected at 12 and 13 min elution times. These peaks were identified as 2-ketogluconic acid and gluconic acid, respectively, based on the comparison of elution time of reference compounds. Gluconic acid and 2-ketogluconic acid were drastically released at 3 days in 1 and 10 nM PQQ under air supply condition. The greatest amount of gluconic acid released in the culture medium reached 19.9 g/l in the 10 nM PQQ treatment, while the highest 2-ketogluconic acid was 10.2 g/l in the 1 nM PQQ treatment. Gluconic acid and 2-ketogluconic acid in treatments with air supply were 4–8 times higher than compared to treatments without air supply, where 2-ketogluconic acid in 1,000 nM PQQ was not detected (Fig. 4). In the control without PQQ together with and without air supply, gluconic acid and 2-ketogluconic acid were never detected throughout the experimental period.

Effect on Common Bean Growth by Inoculating *R. tropici* CIAT 899

There were significant differences in shoot, root, and nodule weights of the common bean plant among the

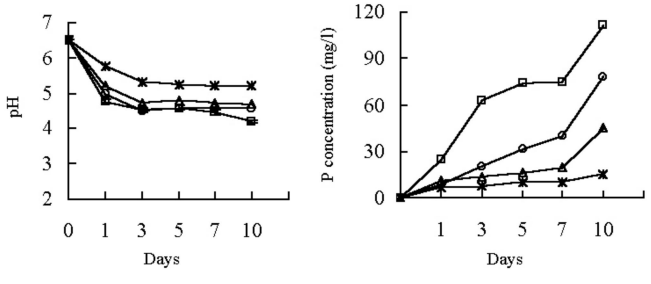

Fig. 2. Effect of different concentrations of PQQ on pH and P solubilization for 10 days in a culture broth containing 0.4% hydroxyapatite by *R. tropici* CIAT 899 without air supply condition.
*, control; □, 1,000 nM PQQ; ○, 10 nM PQQ; △, 1 nM PQQ treatment.

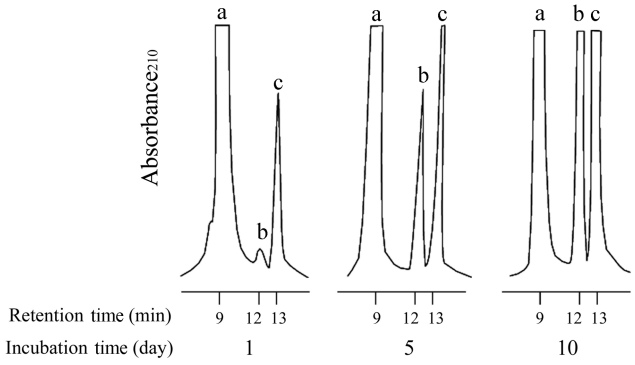

Fig. 3. Identification of organic acids produced by *Rhizobium tropici* CIAT 899 in broth medium supplemented with 10 nM PQQ.
a: Front peak; b: 2-ketogluconic acid; c: gluconic acid.

treatments (Table 1). Shoot, root, and nodule weights in CI+PQQ treatment were significantly higher than those in CON and CI treatment (Table 1). Total N and P taken up by a common bean plant were also significantly greater in CI+PQQ treatment compared to CON and CI treatments. However, the highest growth response was observed in mineral fertilizer treatment (MF) (Table 2): Nodule weight was significantly higher in CI+PQQ treatment compared to other treatments. These results are in accordance with a significant increase in acetylene reducing activities (Table 1).

DISCUSSION

PQQ is known as a cofactor in glucose dehydrogenase (GDH) for glucose oxidation. When nanomolar concentration of PQQ was added to the broth culture of *R. tropici* CIAT 899 with air supply, the soluble P concentration was inversely correlated with pH in the culture medium, which was concomitant with increased organic acid concentrations

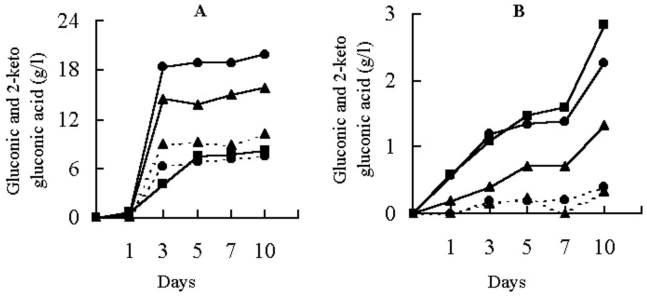

Fig. 4. Effect of different concentrations of PQQ on organic acid production for 10 days in a culture medium containing 0.4% hydroxyapatite by *R. tropici* CIAT 899.
The solid lines represent gluconic acid, while dashed lines indicate 2-keto gluconic acid with (A) or without air supply (B). ■, 1,000 nM PQQ; ●, 10 nM PQQ; ▲, 1 nM PQQ treatment.

Table 1. Growth response and acetylene reducing activity (ARA) in common bean as influenced by *Rhizobium tropici* CIAT 899 (CI), *Rhizobium tropici* CIAT 899+PQQ (CI+PQQ), and mineral fertilizer (MF).

Treatment	Shoot fresh weight (g)	Root fresh weight (g)	Nodule weight (g)	Nodule dry weight (g)	ARA (nmol min^{-1} g fresh $nodule^{-1}$)
Control	3.01	1.50	0.53	0.07	5411
CI	3.35	2.12	0.60	0.07	6618
CI+PQQ	3.69	2.18	1.37	0.18	9292
MF	5.93	3.15	0.72	0.09	6969
LSD(0.05)	0.92	0.52	0.48	0.05	2883

(Figs. 1, 2, 3, and 4). These results indicate that PQQ-dependent holo-GDH activity requires oxygen to overproduce organic acids and depends on the amount of exogenous PQQ. When growing aerobically, *Klebsiella aerogenes* is able to produce gluconate and 2-ketogluconate by means of a direct oxidative pathway [17]. However, gluconate or 2-ketogluconate has never been detected in anaerobic cultures of *K. aerogenes* [23]. Under anaerobic conditions, the synthesis of both the apoenzyme and PQQ is repressed, and glucose dehydrogenase activity decreases. Hommes *et al*. [8] reported that anaerobically grown cells had low GDH activity, and the synthesis of apoenzyme and PQQ was repressed.

Production of gluconic acid by the PQQ-dependent glucose oxidation is due to the presence of apo-GDH in *R. tropici* CIAT 899. When PQQ is supplemented in the medium containing glucose, *R. tropici* CIAT 899 oxidizes glucose to gluconate by reconstituting the holo-GDH. Gluconate as an inducer may activate GADH, which further oxidizes to 2-ketogluconate (Figs. 3 and 4). Boiardi *et al*. [1] found that *R. meliloti* 102F34 possesses a membrane-bound FAD-linked gluconate dehydrogenase which actively oxidized gluconic acid to 2-ketogluconic acid, an intermediate of the ketogluconate pathway. Bernardelli *et al*. [3] reported *S. meliloti* RCR2011 with active GDH, and *R. tropici* CIAT 899 after reconstitution of holo-GDH by the addition of exogenous PQQ extracellulary converted glucose to gluconate, and the latter was further metabolized. Keele *et al*. [11] and Stowers and Elkan [21] reported that gluconate was incorporated and metabolized through Entner-Doudoroff or further oxidized extracellulary, at least partly by the ketogluconate pathway in cowpea rhizobia and *Bradyrhizobium japonicum*.

Rhizobia are well known for their beneficial effect resulting from the symbiotic N_2-fixation with legumes. In particular, phosphate-solubilizing rhizobia can enhance plant growth by increasing P and N uptake [12]. Inoculation with two strains of *Rhizobium leguminosarum* selected for their P-solubilization ability has been shown to improve root colonization and growth promotion and to significantly increase the P and N concentration in lettuce and maize [4]. As shown in our data (Tables 1 and 2), significantly higher plant growth by CI+PQQ treatment was probably due to increased P content solubilized by *R. tropici* CIAT 899. The PQQ added in the culture broth increased organic acid production by reconstituting holoenzyme, which may enhance phosphate solubilization in common bean rhizosphere. The increased phosphate may promote *R. tropici* population as well as nodule formation, which increases nitrogen fixation, as shown by increased acetylene reducing activity (Table 1) and subsequently growth of common bean (Tables 1 and 2). Currently, we are investigating solubilization of rock phosphate by using this bacterium and trying to transfer the *pqq* gene(s) to *Rhizobium* spp.

Table 2. Dry weight, N and P content in common bean as influenced by *Rhizobium tropici* CIAT 899 (CI), *Rhizobium tropici* CIAT 899+PQQ (CI+PQQ), and mineral fertilizer (MF).

Treatment	Dry weight (g $plant^{-1}$)	N in dry tissue (%)	Total N (mg $plant^{-1}$)	P in dry tissue (%)	Total P (mg $plant^{-1}$)
Shoot					
Control	0.56	1.02	5.72	0.27	1.51
CI	0.58	1.05	6.14	0.33	1.96
CI+PQQ	0.73	1.21	8.76	0.74	5.43
MF	1.03	1.36	14.05	1.47	15.09
LSD (0.05)	0.13	0.14	2.12	0.21	1.49
Root					
Control	0.20	1.29	2.63	0.18	0.36
CI	0.22	1.57	3.51	0.25	0.56
CI+PQQ	0.30	1.65	4.81	0.42	1.28
MF	0.38	1.71	6.55	1.51	1.90
LSD (0.05)	0.13	0.26	2.21	0.13	0.72

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