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Mineral phosphate solubilization activity of gluconacetobacter diazotrophicus under P-limitation and plant root environment

J. M. Crespo[1], J. L. Boiardi[1], M. F. Luna[1,2]*

[1]CINDEFI (UNLP-CONICET, CCT La Plata), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina;
[2]CIC PBA, Comision de Investigaciones Cientificas, Provincia de Buenos Aires, Argentina;
*Corresponding Author: mafla@quimica.unlp.edu.ar

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ABSTRACT

The ability to solubilize insoluble inorganic phosphate compounds by *Gluconacetobacter diazotrophicus* was studied using different culture approaches. Qualitative plate assays using tricalcium phosphate as the sole P-source showed that *G. diazotrophicus* produced solubilization only when aldoses were used as the C-source. Extracellular aldose oxidation via a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) is the main pathway for glucose metabolism in *G. diazotrophicus.* In batch cultures with 5 g l^{-1} of hydroxyapatite as the P-source and glucose as the C-source, more than 98% of insoluble P was solubilized. No solubilization was observed neither using glycerol nor culturing a PQQ-GDH mutant of *G. diazotrophicus.* Solubilizaton was not affected by adding 100 mmol l^{-1} of MES buffer. Continuous cultures of *G. diazotrophicus* showed significant activities of PQQ-GDH either under C or P limitation. An intense acidification in the root environment of tomato and wheat seedlings inoculated with a *G. diazotrophicus* PAL5 was observed. Seedlings inoculated with a PQQ-GDH mutant strain of *G. diazotrophicus* showed no acidification. Our results suggest that *G. diazotrophicus* is an excellent candidate to be used as biofertilizer because in addition to the already described plant growth-promoting abilities of this organism, it shows a significant mineral phosphate solubilization capacity.

Keywords: Gluconacetobacter Diazotrophicus; Phosphate Solubilization; Glucose Dehydrogenase; Pqq; Biofertilizer

1. INTRODUCTION

Phosphorus (P) is after Nitrogen, the most important nutrient limiting agricultural production. Soils are often abundant in insoluble P, either in organic or inorganic forms, but deficient in soluble phosphates essential for growth of most plants and microorganisms. Soluble forms of phosphate fertilizers are widely applied to agricultural soils in order to circumvent P-deficiency but 75 to 90% of added P is rapidly precipitated as insoluble forms and becomes unavailable to plants [1]. Converting soil insoluble phosphates (both organic and inorganic) to a form available for plants is a necessary goal to achieve sustainable agricultural production. Several reports show the ability of different bacteria to solubilize inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate [2]. Among the bacterial genera reported to express a mineral phosphate solubilization (MPS) phenotype are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium* and *Erwinia* [3]. A significant body of evidence has been developed to show that in gram-negative bacteria the expression of a direct extracellular oxidative pathway allows superior MPS capabilities [2]. Through this pathway (also called nonphosphorylating oxidation) glucose is oxidized to gluconic acid and 2-ketogluconic acid directly in the periplasmic space. These strong organic acids can dissolve poorly soluble calcium phosphates present in soils. On the other hand it has been reported that the buffering capacity of soils could limit P solubilization by microorganisms [4,5].

Gluconacetobacter diazotrophicus is a nitrogen-fixing endophytic bacterium able to colonize several plant species [6,7]. *G. diazotrophicus* promotes, besides N_2-fixation, other beneficial effects to plants such as phytormones production and biocontrol towards plant pathogens [6]. Moreover, in some *G. diazotrophicus* strains the ability to promote P solubilization has been demon-

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strated in vitro [8,5]. Considering all these characteristics *G. diazotrophicus* has been described as a plant growth-promoting bacterium.

G. diazotrophicus possess a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) responsible for the periplasmic conversion of aldoses into the corresponding aldonic acid [9]. This is considered the principal pathway for glucose metabolism in this bacterium [10]. It has been reported that in the presence of aldoses and under N_2-fixing conditions, *G. diazotrophicus* is able to express an enhanced production of energy linked to a fully active PQQ-GDH [11]. However, there is no information about PQQ-GDH expression under P-limitation, a condition likely found in soils and it is not known whether buffering could affect MPS by *G. diazotrophicus*. Moreover, it is not known whether this organism expresses an active PQQ-GDH in the rizosphere of plants, where the MPS activity needs to be expressed in order to provide soluble phosphate to plants. The objective of this study was to address the above mentioned issues and assess the potential of *G. diazotrophicus* as a biofertilizer due to its MPS activity coupled to its well-known plant growth promoting abilities.

2. MATERIALS AND METHODS

2.1. Organism and Maintenance

G. diazotrophicus strain PAL 5 (ATCC 49037), kindly provided by Dr. Caballero-Mellado, was maintained on agar slants on a potato medium [12]. Strain MF105, an already described PQQ-GDH negative mutant strain of *G. diazotrophicus* [10], was maintained on the same medium supplemented with streptomycin (400 µg ml^{-1}).

2.2. Cultures and Growth Conditions

Plate assays were carried out using the National Botanical Research Institute's phosphate medium (NBRIP) [13] containing l^{-1}: $Ca_3(PO_4)_2$ (TCP), 5 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g and 10 g of different carbon sources (Figure 1). Bacterial cultures of both strains of *G. diazotrophicus* with a concentration of around 1.10^9 CFU ml^{-1} were centrifuged and resuspended in the same volume of saline solution pH 6.0. A volume of 50 µl of these cell suspensions was plated onto NBRIP medium. The plates were observed two days after incubation at 30 °C.

Batch cultures were performed employing the NBRIP liquid medium with 10 g l^{-1} of glucose or glycerol as carbon source, 5 g l^{-1} of hydroxyapatite (HY, $Ca_{10}(OH)_2(PO_4)_6$) as the sole P source and 2.5 g l^{-1} of $(NH_4)_2SO_4$ (under non-BNF conditions). HY was replaced by K_2HPO_4 (2.0 g l^{-1}) in experiments with soluble P. When the organism was grown under BNF conditions, $(NH_4)_2SO_4$ concentration was decreased to 0.132 g l^{-1} [12]. To evaluate the effect of buffering on MPS activity, NBRIP medium was strongly buffered with morpholineethanesulfonic acid (MES) buffer 100 mmol l^{-1}. Initial pH was adjusted at 6.0 by adding KOH 0.1 mol l^{-1} or HCl 0.1 mol l^{-1}. Bacteria (both, wild type and mutant) were grown at 30 °C in 250 ml liquid NBRIP medium (two flasks per treatment) on a rotary shaker stirred at 200 or 100 rpm for non-BNF or BNF, respectively. Negative controls (no bacteria) were carried out to quantify the solublized P in the culture conditions regardless of microbial activity.

Chemostat cultures were carried out using the modified LGIM medium described by Luna et al. (2000) [11] with glucose 10.0 g l^{-1} or 20.0 g l^{-1} and $NaH_2PO_4.H_2O$ 10 mmol l^{-1} or 0.5 mmol l^{-1} for C- or P-limitation respectively. $(NH_4)_2SO_4$ (2.50 g l^{-1}) was added to cultures grown under non-BNF conditions. Cultures under BNF were carried out without $(NH_4)_2SO_4$ in the culture medium. The strategies to attain BNF conditions were described by Luna et al. (2000) [11]. *G. diazotrophicus* Pal 5 was grown at 30 °C in a 2-l LH (Incelltech 210) fermentation unit with a working volume of 1.0 l. The growth rate (dilution rate) was adjusted at 0.05 ± 0.001 h^{-1}. The pH was automatically maintained at 5.5 ± 0.1 by addition of either 0.5 mol l^{-1} NaOH or 0.25 mol l^{-1} H_2SO_4. Foam formation was prevented by automatic addition of an antifoam agent. Cultures were flushed with air (20 to 25 l h^{-1}). The dissolved oxygen concentration was continuously measured using an Ingold (Wilmington, MA) polarographic probe and maintained at the desired level of air saturation by varying the agitation speed of the impeller. Cultures were considered to be under steady-state conditions when biomass concentration and specific rate of oxygen consumption of cultures remained almost constant (varied less than 5 %), as previously described [11]. After modification in growth conditions, 5 to 10 volume changes were usually required to re-obtain steady state.

2.3. Analyses

Samples of batch (at 8-12 h intervals during 5 days) or continuous cultures (daily during 5-7 days) were taken for pH, absorbance, biomass dry weight, glucose and products quantification. Growth was estimated by measurement of the absorbance at 560 nm and biomass dry weight determined as previously reported [11]. Samples of batch cultures grown in medium with HY were diluted 1:1 (v:v) using 0.1 mol l^{-1} HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated [14]. Samples were centrifuged 10 min at 10,000 g and the resulting supernatant was employed to assay P, glucose, gluconic acid, and extracel-

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lular polysaccharides (EPS). Glucose concentrations in media and supernatants were determined with a glucose oxidase enzymatic kit (Wiener, Argentina). Gluconate concentrations were assayed using a test-kit (Boehringer, Mannheim, Germany). EPS dry weight was determined by adding two volumes of ethanol to culture supernatants; after storing overnight at 4 °C precipitated material was collected by centrifugation (20 min at 7,000 g). The pellets were resuspended in distilled water and dried at 60 °C. Soluble P concentration was measured by the method described by Clesscerl *et al.* (1998) [15]. Oxygen and carbon dioxide concentrations in the emitted gases from the fermentor were determined using a paramagnetic oxygen analyzer (Servomex 1100A, Norwood, MA) and an infrared carbon dioxide analyzer (Horiba PIR 2000, Japan). Gas flow rates were measured with a bubble flow meter. Biomass yields, rates of oxygen consumption, and carbon dioxide production were calculated as previously described [11].

2.4. Enzyme Assays

PQQ-GDH and gluconate dehydrogenase (GaDH) activities were measured spectrophotometrically using 2,6-dichlorophenol-indophenol (DCIP) as the electron acceptor and glucose or gluconate respectively [16,17]. Samples (20 ml) from batch cultures were taken at exponential phase, while glucose was still detectable, and centrifuged for 10 minutes at 12,000 g at 4 ºC. In chemostat, once steady-state conditions were attained, an appropriate volume of culture (70 ml) was withdrawn and centrifuged as described above. Cells were washed twice in phosphate buffer 10 mmol l^{-1} (pH 6.0) containing 5 mmol l^{-1} $MgCl_2$ to a final concentration of 4.50 mg ml^{-1}. This washed cells suspension (WCS) was employed to determine the PQQ-GDH activity in whole cells. The final concentration of cells in the reaction mixture was 0.10 mg ml^{-1} dry weight.

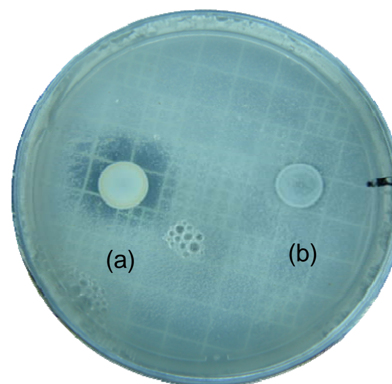
2.5. Acid Production from Root Exudates

Tomato (*Lycopersicum esculentum* cv. "platense italiano") and wheat (*Triticum aestevium* cv. "baguette") seeds were surface sterilized with 2 % sodium hypochlorite for 5 min followed by three washes with sterile water. Seeds were germinated onto water-agar plates (0.5 % agar) at 30 °C during 72 h. The seedlings were inoculated by immersion for 15 min in a *G. diazotrophicus* suspension (either PAL5 or MF105 centrifugated and resuspended as described for plates assays) and placed into tubes containing semi solid agarized Fähraeus medium [18] with 0.1 % methyl red as acid-base indicator. Uninoculated seedlings and a *G. diazotrophicus* suspension were used as negative controls.

3. RESULTS

Plate assays. Qualitative estimation of P solubilization was made in agar plates supplemented with TCP. The MPS phenotype was identified by the production of clearing zones of solubilization around the colony (**Figure 1**). As it can be seen in **Figure 1** appearance of clearing halos was dependent on the nature of the carbon source. Glucose, arabinose, galactose and xylose (all substrates of PQQ-GDH) showed an MPS (+) phenotype. With other carbon sources *G. diazotrophicus* showed growth but was not capable of solubilizing phosphates. When plates were inoculated with strain MF105 (PQQ-GDH (-) mutant) neither the PQQ-GDH substrates nor the other carbon sources showed a MPS (+) phenotype.

Batch cultures. Quantitative estimation of P solubilization was carried out in liquid medium cultures, since this approach is considered more accurate than plate assays [19]. Batch cultures performed with glucose and replacing the soluble P-source by HY, showed a very similar growth behavior to the one reported using soluble P [10]. Soluble P concentration in the media increased together with the gluconic acid. The culture pH dropped during the same period from 6.0 to 2.5 with a concomitant P solubilization that reached around 1,000



Carbon source	Bacterial strain PAL5	MF105
Glucose	+	-
Arabinose	+	-
Galactose	+	-
Xylose	+	-
Lactose	-	-
Maltose	-	-
Gluconate	-	-
Glycerol	-	-
Fructose	-	-

Figure 1. *In vitro* P solubilization by *G. diazotrophicus* (a) PAL5 and (b) MF105. Clearing halo formation (+) and no halo formation (–) by *G. diazotrophicus* growing on NBRIP medium with TCP and different C-sources.

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ppm (**Figure 2**). This amount represents more than 98% of the insoluble P contained in the HY added to the culture medium (**Figure 3**). Once glucose was entirely oxidized (around 80 h of growth) soluble P decreased in the culture supernatants together with gluconate consumption. There was no difference between P solubilization levels under both BNF and non-BNF conditions (**Figure 3**). P concentration of negative controls (non inoculated) remained almost constant, between 2 and 8 mg l^{-1}, along the experiment.

It was observed that *G. diazotrophicus* exhibited a similar P-dissolving capability either in the absence or in the presence of buffer, even at a concentration of 100 mmol l^{-1} of MES (**Figure 3**).

As already described for *G. diazotrophicus* growing with soluble P [10], PQQ-GDH was actively synthesized in glucose-containing batch cultures using HY as the sole P source, either under BNF or non-BNF conditions (**Table 1**).

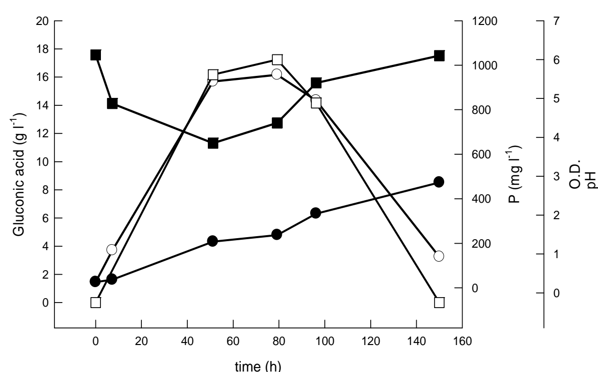

Figure 2. Soluble P, gluconic acid, pH and O.D. measurements of *G. diazotrophicus* PAL5 cultures growing with glucose 20 g l^{-1}, HY 5 g l^{-1} and BNF conditions. (○) soluble P; (□) gluconic acid; (●) O.D. and (■) pH.

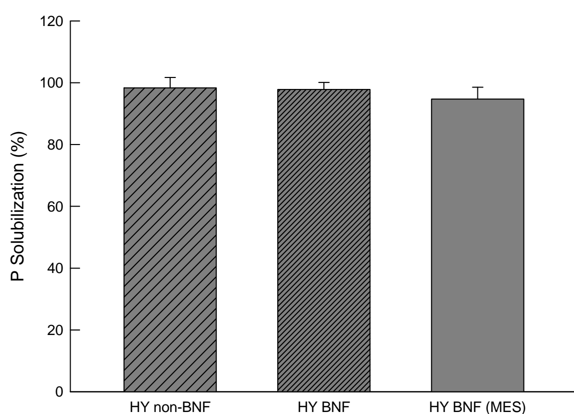

Figure 3. P solubilization by *G. diazotrophicus* PAL5 in cultures with glucose 20 g l^{-1}, HY 5 g l^{-1} under both BNF and non-BNF condition and with MES buffer 100 mmol l^{-1}. P solubilization percentage was determined dividing the maximum soluble P value obtained by the initial P amount in HY.

Cultures of *G. diazotrophicus* PAL5 using glycerol as the sole carbon source and cultures of strain MF105 with glucose, both with HY as the sole P-source, showed pH values over 6.0 all along cultures, indicating no acid production and levels of soluble P below 10 ppm (data not shown).

Continuous cultures. G. diazotrophicus was grown under C- or P-limiting conditions in a chemostat using glucose as C-source. In order to check that growth was indeed C- (or P-limited), additions of the corresponding limiting substrate were made to the culture vessel. An immediate increase in the steady state biomass concentration was observed after the addition of either limiting substrate. Moreover, the residual concentration of the limiting substrate in the supernatants of steady state cultures was below the detection limits of the assays employed (data not shown).

In cultures grown under P-limitation, either with NH_4^+ or N_2 as the N-source, biomass yields were lower than those observed in glucose-limited cultures and showed a significant increase of O_2 consumption. Growth yields of C- or P-limited continuous cultures were not significantly affected by the nature of the N-source (NH_4^+ or N_2), as already observed in a previous work [11] (**Table 2**).

Table 1 *In vitro* PQQ-GDH activities of *G. diazotrophicus* PAL5 in batch cultures.

	Enzymatic activity		
	HY 5 g l^{-1} non-BNF	HY 5 g l^{-1} BNF	HY 5 g l^{-1} BNF (MES 100 mmol l^{-1})
PQQ-GDH	476* ± 31.87	486* ± 30.70	503* ± 13.17

* Enzymatic activities are expressed as nmoles DCIP reduced min^{-1} mg $protein^{-1}$ (assuming 60% protein content in the biomass). Data are mean of at least three repetitions.

Table 2 Continuous cultures of *G. diazotrophicus* PAL5.

	C-limitation		P-limitation	
	non-BNF	BNF	non-BNF	BNF
$Y_{x/s}$	29.2	34.7	13.9	14.9
QO_2	8.44	7.69	11.03	10.37
EPS	nd	nd	3.6	1.41
Gluconic acid	nd	nd	Nd	9.12
C-recovery	98.6	94.5	74.6	102.3
PQQ-GDH	360	406	343	361
GaDH	100.5	95.2	58.0	52.7

$Y_{x/s}$ is expressed as g biomass mol $substrate^{-1}$; QO_2 as mmol O_2 g $biomass^{-1}$ h^{-1}; EPS as g l^{-1}; gluconic acid as g l^{-1}; C-recovery in %. PQQ-GDH and GaDH activities are expressed as nmol DCIP reduced min^{-1} mg $protein^{-1}$ (assuming 60% protein content in the biomass). Data are mean of at least three repetitions. SD was never >10%.

EPS was detected in supernatants of both P-limited

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cultures but only in those growing under BNF conditions gluconic acid could also be detected (**Table 2**). In cultures grown P-limited and with NH_4^+ as the N-source, C-recovery (taking into account biomass, CO_2, EPS and gluconic acid) was not enough to match the C-input (glucose feeded to the culture). On the other hand the supernatant fluids of C-limited cultures contained neither gluconic acid nor any other detectable extracellular product (**Table 2**).

Significant activities of PQQ-GDH were detected independently of the limitation (C or P) or the N-source employed. Similarly GaDH activities were detected under all culture conditions tested but, in this case, with a higher expression under C-limitation in relation to P-limitation.

Acid production from root exudates. **Figure 4** shows red zones of acidification in the root environment of seedlings from both plant species inoculated with a *G. diazotrophicus* PAL5 suspension. On the other hand, no acidification was observed in seedlings inoculated with the mutant strain MF105. Similarly, no acid production was observed in control tubes either inoculated with *G. diazotrophicus* PAL5 without plants or in tubes where non-inoculated seeds were placed.

4. DISCUSSION

PQQ-GDH of *G. diazotrophicus* presented the common behaviour of a broad-substrate aldose dehydrogenase as reported for others PQQ-GDH [20]. This

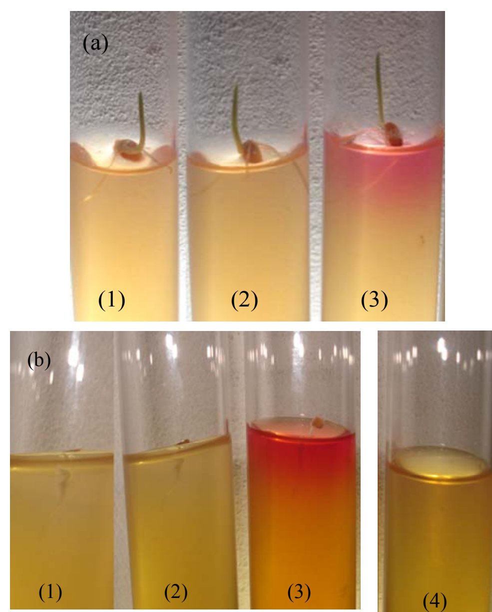

Figure 4. Acidification in the root environment of (a) wheat and (b) tomato seedlings. 1- non-inoculated seeds; 2- MF105 inoculated seeds; 3- PAL5 inoculated seeds; 4- inoculated with PAL5 without seeds.

means the enzyme can oxidize sugars other than glucose but with an aldose function (**Table 1**). Only PQQ-GDH substrates were able to lower the pH of the medium with the consequent solubilization of poorly soluble calcium phosphates. The MF105 mutant, deficient in PQQ-GDH activity, failed to release phosphate from insoluble compounds for every carbon source assayed. These results suggest that, in accordance with Intorne *et al*. (2009) [5], the periplasmic PQQ-GDH of *G. diazotrophicus* PAL 5, which converts aldoses into the corresponding organic acid, was the responsible for the MPS (+) phenotype. Intorne *et al*. (2009) [5] have shown that mutations affecting the production of gluconic acid by *G. diazotrophicus* dramatically alter the MPS phenotype.

It was reported that the exponential growth phase of batch cultures of *G. diazotrophicus* grown in glucose, fixed nitrogen, and soluble P begins when almost 60% of the glucose is converted into gluconic acid [21,9]. During this process an intense aerobic metabolism with a concomitant pH reduction takes place. In our cultures, soluble P increased together with the gluconic acid concentration in the culture medium (**Figure 2**), showing that P solubilization was directly related to acid production. In *Azospirillum brasilense*, reduction in soluble phosphate concentration after a 48 h incubation period can be explained as an auto-consumption of soluble phosphates by the growing bacterial population [14]. However, *G. diazotrophicus* batch cultures performed with soluble phosphates showed that only 10-15 mg l^{-1} of P were used for bacterial growth (data not shown). The reduction of soluble P concentration observed in our cultures could be ascribed to gluconic acid consumption followed by an increase in culture pH, instead of soluble P metabolism by bacterial cells.

PQQ-GDH activity was very similar for all culture conditions tested indicating a constitutive expression of the protein as previously reported [22]. No P solubilization was observed using glycerol as the sole C-source. The same applied to strain MF105 cultures. These results indicate that no P solubilization could take place in the absence of organic acids derived from PQQ-GDH activiy. There was no difference between P solubilization levels under both FBN and non-FBN conditions indicating that P solubilization by *G. diazotrophicus* is not affected by the nature of the N-source. GaDH activity was also observed for all culture conditions indicating the possible presence of 2-ketogluconic acid in supernatants. Nevertheless, if this acid was present, its P solubilization effect would not be as important as the one achieved with gluconic acid because P solubilization patterns were directly related to gluconic acid concentration in the culture supernatants.

It has been reported that the soil buffer capacity af-

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fects MPS by microorganisms [4]. However, in our experiments *G. diazotrophicus* was able to solubilize P in the presence of relatively high MES concentration indicating that buffering would not significantly affect the release of soluble P from HY.

The lower growth yield values observed in chemostat under P-limitation, compared to those under C-limitation, were predictable because in C-limited grown microorganisms, catabolism is tightly coupled to anabolism and high biomass yields are achieved. On the other hand, cultures grown under C-excess conditions exhibit high rates of carbon consumption and low biomass yields and thus, have low energetic growth efficiency as indicated by an increased specific oxygen consumption rate. This behavior is generally coupled to the production of extracellular products (overflow metabolism) [23]. In our case, significant concentrations of EPS (and gluconic acid under BNF) could be detected in culture supernatants when cells were grown under P-limitation and therefore, C-excess. Moreover, in P-limited cultures growing with N-fixed, another unknown extracellular product would have been released since C-recovery could account only for 75% of the consumed glucose.

In spite of the extra energy expenditure for N_2 fixation the biomass yields of cultures grown under BNF conditions were higher (in the case of C-limitation) or, at least, comparable (in the case of P-limitation) to those grown using N-fixed (**Table 2**). It has been reported that cultures of *G. diazotrophicus* grown in glucose (or mixtures of gluconic acid and xylose) under N_2-fixing conditions express an improved growth energetic efficiency because of a higher coupling of the respiratory chain. It was demonstrated that this was linked to the expression of an active aldose oxidation via PQQ-GDH [24]. Once again, it seems that the expression of an active PQQ-GDH and N_2-fixation were the conditions required by *G. diazotrophicus* cultures to direct the electron flow through a more efficient branch of the respiratory chain. In this case, the effect was observed under P-limitation.

Table 2 indicates that PQQ-GDH is not induced by phosphate starvation. Nevertheless, the enzyme was fully active in all cultures. Moreover, under P-limitation and N_2-fixation significant concentrations of gluconic acid could be detected in the culture supernatants.

Tubes containing seedlings developed from seeds that had been inoculated with *G. diazotrophicus* PAL5 showed a significant area of acidification. This acidification can be ascribed to the production of organic acid/s by PQQ-GDH expression. This assumption is made on the basis that no acidification was observed around seedlings from non-inoculated seeds and from those inoculated with the mutant strain MF105, impaired in PQQ-GDH expression. The same was observed in tubes inoculated with *G. diazotrophicus* but without plants. Therefore the simultaneous presence of *G. diazotrophicus* able to express PQQ-GDH and growing seedlings were necessary for acidification. Since root exudates of many plants, including the two used in this study, contain significant amounts of PQQ-GDH substrates [25,26], it is likely that the acidification areas observed in **Figure 4** were caused by some aldonic acid produced by the inoculated *G. diazotrophicus* cells. This result indicates that the presence of *G. diazotrophicus* in the root environment of plants allows the active expression of PQQ-GDH with the concomitant production of organic acids that are able to solubilize P from poorly soluble calcium phosphates, as shown.

Taken together, the results show that *G. diazotrophicus* is able to actively express PQQ-GDH with the concomitant production of organic acids and consequent MPS activity. This activity was not affected by the buffering capacity of the environment. PQQ-GDH activity was expressed under conditions of P-limitation (either with N_2 or NH_4^+ as N-source) and in the root environment of different plant species producing acids from the root exudates. Therefore, in addition to other plant growth promoting activities already described for *G. diazotrophicus* [6], this organism expresses a MPS (+) phenotype which allows its consideration as a promising species for being used as a biofertilizer.

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