

Short communication

Conventional farming disrupts cooperation among phosphate solubilising bacteria isolated from *Carica papaya*'s rhizosphere

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ABSTRACT

We tested the legacy of the farming system (conventional or organic) on the interactions among phosphate solubilizing bacteria (PSB) and phosphate (Pi) solubilisation. We determined the *in vitro* Pi solubilisation and production of organic acids by nine PSB (four from conventional and five from organic farming) isolated from the rhizospheres of *Carica papaya*, grown in monoculture or in pairs (66 combinations). When grown in monoculture, PSB isolated from conventional farming solubilised more Pi, but when grown in pairs, these PSB solubilised the least Pi. Furthermore, when the pairs included only PSB isolated from conventional farming, no Pi-solubilising cooperation was observed, that is, Pi solubilisation by pairs was lower than in monoculture. When PSB isolated from organic farming were present in the pairs, ~40% of the combinations resulted in Pi solubilising cooperation. Organic acid production was not related with Pi solubilisation. Our work demonstrated the existence of a farming system legacy influencing the biotic interactions among PSB, and that the output of the interactions (cooperation or antagonism) was not phylogenetically determined. Data highlighted that biotic interactions should be considered when screening for potential plant growth promoting bacteria.

1. Introduction

The productivity of most ecosystems, including agricultural systems, is limited by nutrient availability, namely phosphorus (P). However, in conventional farming, 60–90% of the soluble inorganic phosphate (Pi) applied to soils as fertilizer is rapidly immobilized after application, making it unavailable to plants (Richardson and Simpson, 2011; Estrada et al., 2013). Therefore, understanding the role of biotic interactions in governing bio-availability of P should enhance the economic and environmental sustainability of agriculture (Liu et al., 2007; Oliveira et al., 2009). In contrast, organic farming replaces synthetic fertilizers and pesticides with biological inputs (Liu et al., 2007), so microbial processes are essential for its productivity and sustainability (Liu et al., 2007; Babalola, 2010). Several soil microbes have been identified as promoters of plant growth due to their ability to solubilize

Pi (Richardson et al., 2009; Singh et al., 2014). However, the use of Pi-solubilizing inoculants provides inconsistent results, even when the microbes have potential for high Pi solubilisation (Richardson, 2001). One of the key mechanisms involved in Pi solubilisation by microbes is the secretion of low molecular weight organic acids. In this process, released protons acidify the rhizosphere, directly increasing Pi solubilisation (Sharma et al., 2013). Soil Pi concentration influences the phenotype of Pi-solubilising bacteria (PSB) and soil PSB community composition (An et al., 2014).

Even though cooperation between soil microbes occurs naturally (Mitri and Foster, 2013), the large quantities of chemical inputs currently used in conventional farming have had a great impact on soil productivity and also on its functionality, which has led to increased interest in improving the understanding of soil ecology (and how this can be influenced by management), and consequently in promoting

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cooperation between rhizospheric microbial populations (Barea et al., 2005).

Our objective was to test the legacy of the farming system (conventional or organic) on the interactions among PSB leading to Pi solubilisation. We hypothesized that the high Pi concentrations typical of conventional farming, would not eliminate the Pi solubilisation trait from the bacterial community, but would instead select for more independent Pi-solubilising networks. In contrast, under low Pi concentrations, typical of organic farming, stronger functional interactions in Pi solubilisation between the microbes would be expected. Therefore, we expected to observe more cases of cooperation in Pi solubilisation between PSB isolated from organic farming than from the conventional. We used nine PSB previously isolated from the rhizosphere of *Carica papaya* (four from conventional and five from organic farming – Melo et al., 2016), and determined their *in vitro* Pi solubilisation potential and production of organic acids when growing in monoculture and in pairs (66 combinations).

2. Material and methods

2.1. Experimental design and sampling

The experimental design comprised one factor (farming system) with a completely randomized block design. Each block consisted of 10 × 10 m plot (three per farming system), while each repetition consisted of a rhizospheric soil sample (6 per block).

Soil was sampled in October 2012 from an area in Espírito Santo state, Brazil where *Carica papaya* L. (Golden variety) is produced under two farming systems (organic (19°14'13.40"S 40°4'38.73"W) and conventional (19°12'22.9"S 40°05'52.0"W) – for details please see Melo et al., 2016). The experimental area has a plain topography and a deep soil classified as moderate type A Dystrophic Yellow Podzolic with a sandy-medium texture. Rhizospheric soil samples were collected at 0–20 cm depth. Each sampled soil parcel contained 6 plants spaced at 20 cm. Four soil subsamples were collected from around each plant, at 8 cm from the plant stem. Nine bacterial strains were isolated from the rhizosphere of *Carica papaya*, as described in Melo et al. (2016): four from conventional farming (*Enterobacter* sp. C, *Klebsiella* sp. C1, *Burkholderia vietnamiensis* C and *Klebsiella* sp. C2) and 5 from organic farming (*Burkholderia cepacia* O, *Burkholderia vietnamiensis* O, *Klebsiella* sp. O1, *Klebsiella* sp. O2 and *Leclercia* sp. O). All the isolates were characterized as Gram – (Melo et al., 2016).

2.2. Growth and Pi solubilisation of PSB grown in monoculture

PSB growth was quantified using the method described by Magalhães et al. (2016). *In vitro* Pi solubilisation was quantified by the malachite green method adapted from D'Angelo et al. (2001); a pre-inoculum was made of each strain in 10 mL NB medium. This pre-inoculum was incubated at 28 °C and 160 rpm overnight, then 1 mL of the inoculum was transferred to an Erlenmeyer containing 20 mL of NBRIP medium with tricalcium phosphate (Nautiyal, 1999), which was incubated at 28 °C and 160 rpm for 24 h. Subsequently, an aliquot of 2 mL was taken to determine the soluble Pi. Samples were centrifuged at 12,000 × g, for 10 min to sediment insoluble particles present in the medium, the supernatant was filtered using Whatman filter paper 42. Culture medium without inoculum was used as control. For each isolate, three replicates were made.

2.3. Effect of PSB coculture on Pi solubilisation

Coculture assays were designed to test whether synergistic effects and farming system influenced Pi solubilisation. Three groups of PSB combinations were considered: i) Group 1–10 combinations of the PSB isolated from conventional farming; ii) Group 2–21 combinations of the PSB isolated from organic farming; and iii) Group 3–35 combinations of

all nine PSB strains (Melo et al., 2016). Cocultures were grown in NBRIP liquid medium, and solubilised Pi was quantified as described above. For the coculture assays, the inoculum was reduced from 1 mL to 500 µL of each bacterium strain, to maintain total cell concentration (Malboobi et al., 2009).

The effect of coculture on Pi solubilisation was calculated using a feedback approach, according to Kardol et al., (2007) as follows:

$$\text{Effect of coculture on Pi solubilisation} = \frac{(\text{Pi coculture} - \text{Average Pi monoculture})}{\text{Average Pi monoculture}}$$

Average Pi monoculture corresponds to the average of Pi solubilisation of each PSB isolate when grown in monoculture; while the Pi coculture corresponds to the Pi solubilisation of the PSB grown in coculture. Positive effects of coculture on Pi solubilisation correspond to co-operation, while negative effects of coculture on Pi solubilisation correspond to antagonism.

2.4. ROA-organic acid – high performance liquid chromatography (ROA-HPLC)

The pre-inoculum for the analysis of the organic acids was prepared using the methodology described above. ROA-HPLC separations were performed using a YoungLin Acme 9000 HPLC system (Seoul, Korea) equipped with a YoungLin Acme 9000 UV–vis detector, an isocratic pump and autosampler. The samples were analysed on a Rezex ROA Organic Acid H⁺ organic acid column (5 µm, 7.8 mm × 300 mm; Phenomenex, (Torrance, CA) operated at ambient temperature with a 0.005 N H₂SO₄ mobile phase flowing at 0.6 mL/min. Aliquots of 10 µL were injected and all chromatograms shown in this report were detected at a wavelength (λ) of 210 nm. Sample analysis was completed in 40 min. For each standard organic acid (citric, gluconic, malic, acetic, fumaric, propionic, oxalic and succinic), the linear calibration curve was determined using five concentrations. The peaks were identified by comparing retention times and the UV spectra of standard acids (Table S1 and Figs. S1 and S2).

2.5. Statistics

The effect of the farming system legacy on PSB parameters was tested using a two-way ANOVA, with farming system and PSB isolate as fixed factors. Bonferroni *post hoc* multiple comparisons tested for differences ($p < 0.05$) in PSB parameters between treatments. Effects of PSB coculture on Pi solubilisation were analysed by the *t*-student test ($p < 0.05$), with independent samples compared to zero (no effect). In all cases, preliminary analyses were performed to ensure that there was no violation of statistical assumptions (including the Levene's test to check for homogeneity of variances). SPSS (version 23.0, IBM, Inc.) was used for all these analyses.

3. Results

3.1. Effect of the farming system on PSB grown in monoculture

All the PSB strains showed similar growth (Fig. 1-a) under the tested conditions. In contrast, the amount of Pi detected in the growth media was influenced by the isolate, the farming system, and the interaction between these two factors, ranging between 11 and 177 µg Pi mL⁻¹ (Fig. 1-b), showing there was no relation between Pi solubilisation and growth (Fig. 1). PSB growth and Pi solubilisation were always accompanied by the acidification of the growth media and by the production of organic acids (Table S2).

All bacterial strains showed similar profiles of the organic acids excreted into the growth media (Fig. S1). Gluconic acid was always the most abundant acid produced by the PSB strains (Table S2), but no

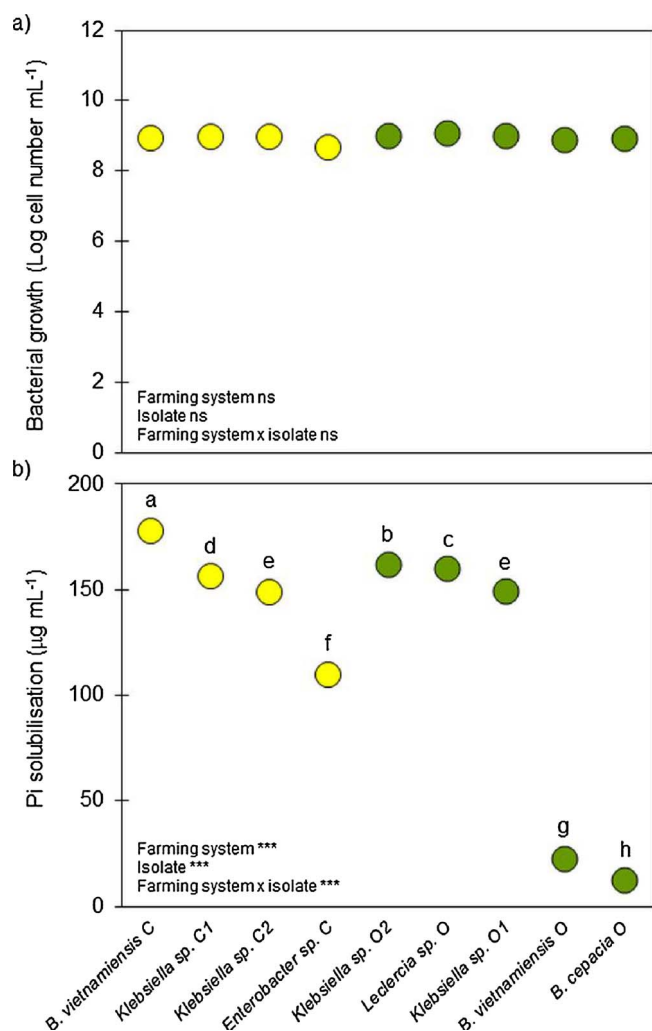


Fig. 1. Legacy of the farming system on bacterial growth (a) and Pi solubilisation (b) of the nine PSB isolated from the rhizospheres of *C. papaya* grown in monoculture. Significant effects are shown: * 5% level, ** 1% level and *** 0% level; ns is non-significant. Different letters show significance at the 5% level. Symbols (yellow for conventional and green for organic) are the mean \pm 1SE (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relation between accumulation of gluconic acid (or any other organic acid) and Pi solubilisation by the distinct strains was observed.

3.2. Effects of farming system on PSB grown in pairs of isolates

There was no positive effect of coculture on Pi solubilisation (*i.e.* no Pi-solubilising cooperation) when the bacterial strains came exclusively from conventional farming (Group 1 – Fig. 2). In contrast, in all cases of Pi-solubilising cooperation, at least one of the bacterial strains had originated from organic farming (Groups 2 and 3). P-solubilising cooperation was observed in ~50% of the combinations in Group 2 and ~30% of the combinations in Group 3. PSB growing in the coculture assays within Group 1 solubilised less Pi (~160 $\mu\text{g Pi mL}^{-1}$) than those within Groups 2 and 3 (~230 $\mu\text{g Pi mL}^{-1}$ – Table S4). There was no relation between Pi solubilisation and growth parameters in the coculture assays (data not shown).

The selected filtrates of the growth media obtained from the coculture assays were analysed for their organic acid profiles. We selected pairs that showed cooperation and antagonism, including strains with relatively high and low Pi solubilisation capacities (Table S3). No oxalic acid was detected. Gluconic acid was always the most abundant acid in

all the growth media (Table S3), but no relation between accumulation of gluconic acid (or any other organic acid) and Pi solubilisation was observed.

4. Discussion

Our study clearly shows that there was a farming system legacy influencing the biotic interactions among PSB, and that the output of the interactions (cooperation or antagonism) was not phylogenetically determined. Furthermore, data highlighted that biotic interactions should be considered when screening for potential plant growth promoting bacteria.

4.1. Legacy of the farming system on PSB strains growing in monoculture

All the PSB used belong to genera already described as including Pi-solubilising strains (Yu et al., 2011; Shahid et al., 2012; Estrada et al., 2013). However, no phylogenetic relation could be established in terms of the *in vitro* Pi solubilisation, since strains from the same species (*Burkholderia vietnamiensis*), but isolated from different farming systems, presented the highest (156 $\mu\text{g mL}^{-1}$) and the second lowest (20 $\mu\text{g mL}^{-1}$) Pi solubilisation (Fig. 1). PSB growth was always accompanied by an acidification of the growth medium (Table S2), which may be a consequence of the secretion of low molecular weight organic acids (Khan et al., 2014). However, the PSB that acidified the growth medium the most did not solubilize the most Pi. This lack of relation between acidification and Pi solubilisation cannot be attributed to differences in growth (Illmer and Schinner, 1995; Hwangbo et al., 2003), since all PSB strains presented similar growth (Fig. 1). The direct oxidation of glucose to gluconic acid is a main route of Pi solubilisation (Vyas and Gulati, 2009; Gulati et al., 2010; Oteino et al., 2015). Indeed, gluconic acid was the most abundant organic acid produced by our PSB (Tables S1 and S2), but the ability of strains to produce organic acids, and gluconic acid in particular, was not proportional to their capacity to solubilize Pi. Again, no phylogenetic relation could be established in terms of gluconic acid production, as evidenced by the *Burkholderia vietnamiensis* strains from the distinct farming systems (Table S2).

4.2. Legacy of the farming system on PSB strains growing in pairs of isolates

As hypothesized, the farming system modified the interactions among PSB (Fig. 2). High Pi concentrations (conventional farming) did not eliminate the Pi solubilisation trait from the bacterial community, but rather it selected for more independent Pi solubilisation networks, resulting in antagonistic interactions. The lack of cooperation between PSB isolated from conventional farming may reflect the ecology of the strains' provenance depicted by the lack of competition for nutrients (Mitri and Foster, 2013; An et al., 2014). In contrast, ~40% of the combinations including PSB isolated from organic farming, even when cocultured with PSB isolated from conventional farming, cooperated in solubilising Pi, showing that the organic farming favoured cooperation, which may maintain optimum conditions of resource availability and pH. As shown by Fonseca et al. (2017), these observations support the hypothesis that, under limited nutrient availability, rhizosphere colonization is more influenced by the plant and promotes stronger functional interactions among the microbiome, while in richer environments, the microbial colonization of the rhizosphere is more stochastic.

There was no consistent phylogenetic trend associated with cooperation or antagonism in Pi solubilisation, which may indicate that the interactions are established at the level of the isolate. For example, only 10% of the coculture assays with only *Klebsiella* strains (from conventional and/or organic farming) resulted in Pi-solubilising cooperation. On the other hand, in ~60% of the cases of Pi-solubilising cooperation, *Klebsiella* was one of the PSB in coculture.

Our data may explain many of the unsuccessful field trials with PSB strains selected on the basis of their performance under laboratory

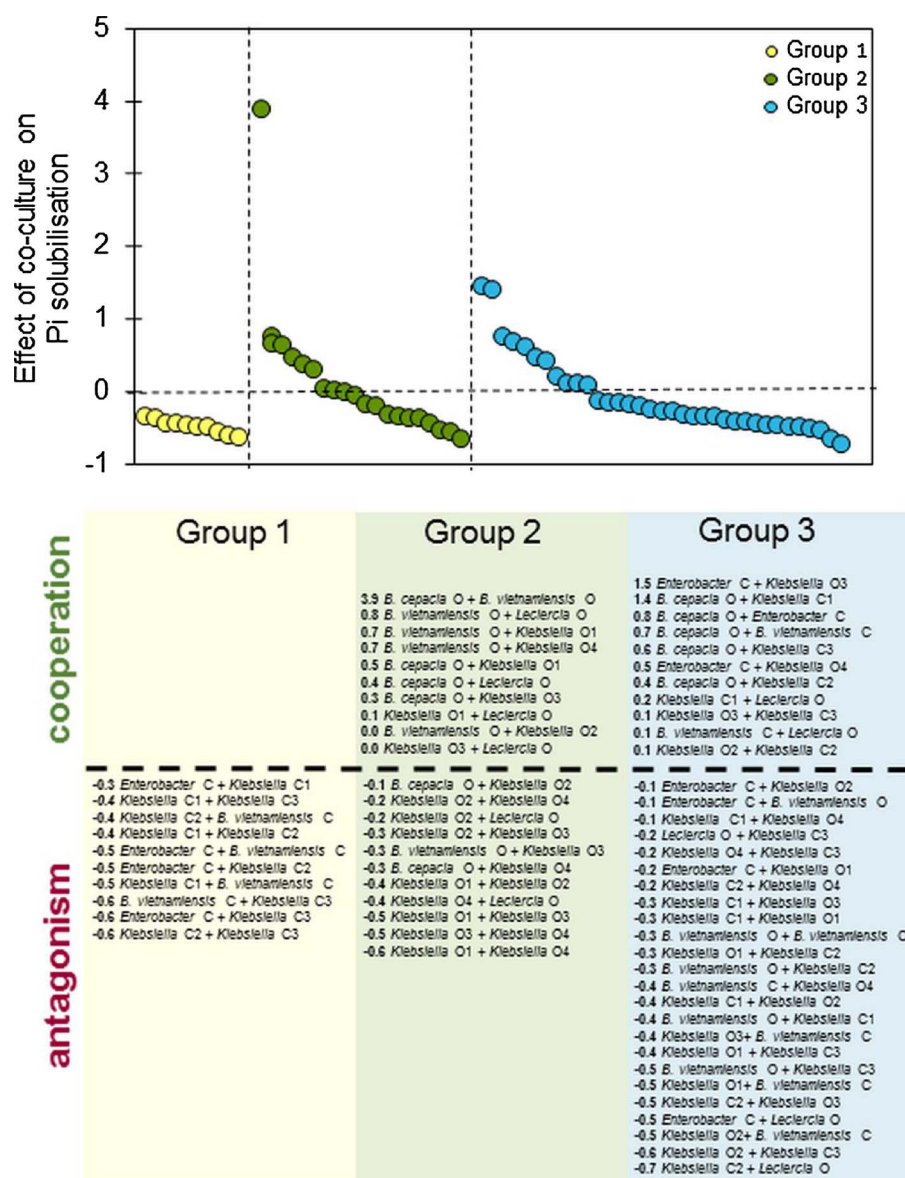


Fig. 2. Legacy of the farming system on the effect of coculture on Pi solubilisation when PSB were grown in pairs of isolates. PSB pairs resulted in significant effects of coculture on Pi solubilisation being positive (i.e. cooperation) or negative (i.e. antagonism). Symbols (yellow for combinations of PSB isolated from conventional, green for combinations of PSB isolated from organic and blue for combinations between the 9 PSB) are the mean \pm 1SE (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions, and also highlight the specificity of the strains involved in consortia formation with the objective of creating biofertilizers that perform well under reduced P fertilization. In terms of applications, these results show the need to consider the ecology of the rhizosphere when isolating and identifying plant growth promoting rhizobacteria, and to develop performance tests using soil. Our established consortia have the potential for a high cooperative performance as PSB in crop soils, and we envisage field tests, to determine their biofertilizer capability. Our dataset refers to *in vitro* experiments that, although highlighting the importance of considering microbial ecology in biofertilizer design, need to be confirmed under field trials.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.apsoil.2017.11.015>

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