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# Evaluation methods used for phosphate-solubilizing bacteria

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**Phosphorus solubilizers naturally acidify rhizospheric soil and increase phosphorus availability; therefore, their evaluation may help to reduce phosphorus fertilizer use. This work aimed to evaluate the different selection methods and select inorganic phosphorus-solubilizing bacteria as potential plant-growth promoters. Bacterial isolates obtained from sugarcane roots and soil were tested using solid growth media containing bicalcium phosphate and Irecê Apatite ground rock phosphate as phosphorus sources. Seven isolates with high (3), moderate (3) and low solubilization indices (1) and the *Pseudomonas fluorescens* R-243 strain were tested in two liquid growth media, followed by the pH and soluble P in the solution. The same isolates, in the absence of inoculation, were tested in Leonard jars with two high- and low-solubility sources using cowpea as a test species. Forty-four days after planting aboveground dry mass, the phosphorus content and total aboveground phosphorus and substratum phosphorus contents were evaluated. The growth media affected phosphorus solubilization by the bacteria. Evaluation of liquid media was the most reliable method for analyzing bicalcic phosphorus solubilization by the bacteria not linked to pH reduction. Isolates UAGC 17, 19 and 65 should be better studied because they were the best solubilizers in culture media; however, they did not demonstrate the same efficiency when inoculated on cowpea.**

**Key words:** Solubilization, phosphate, P-solubilizing-microorganisms.

## INTRODUCTION

Highly intemperized soils dominate the tropical region and are usually characterized by low nutrient availability, especially phosphorus. This is particularly important due to the complex dynamics of P in soils because Phosphate

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**Table 1.** Solubilization Index (SI), means ± 95% confidence interval, at 3 and 17 days after inoculation of 20 phosphate solubilizing bacteria in solid NBRIP and VERMA growth media.

| Isolate | Rock phosphate | | | | Bicalcium phosphate | | | |
|---|---|---|---|---|---|---|---|---|
| | NBRIP | | VERMA | | Meio NBRIP | | Meio VERMA | |
| | 3 days | 17 days | 3 days | 17 days | 3 days | 17 days | 3 days | 17 days |
| UAGC5 | 0.37±0.37 | 1.00±0.00 | 0.00±0.00 | 0.67±0.33 | 1.11±0.01 | 1.03±0.01 | 1.08±0.04 | 1.10±0.02 |
| UAGC7 | 1.25±0.06 | 1.10±0.10 | 1.24±0.01 | 1.73±0.41 | 1.18±0.08 | 1.36±0.15 | 1.28±0.06 | 1.17±0.04 |
| UAGC8 | 1.22±0.02 | 2.22±0.96 | 0.38±0.38 | 1.06±0.06 | 1.18±0.06 | 1.42±0.07 | 1.16±0.03 | 1.21±0.07 |
| UAGC9 | 1.18±0.02 | 1.00±0.00 | 1.18±0.01 | 1.00±0.00 | 1.21±0.03 | 1.53±0.11 | 1.00±0.00 | 1.00±0.00 |
| UAGC15 | 1.73±0.44 | 1.95±0.18 | 1.76±0.48 | 2.37±0.56 | 1.35±0.07 | 2.07±0.22 | 1.52±0.08 | 2.61±0.11 |
| UAGC16 | 1.15±0.02 | 1.39±0.07 | 0.00±0.00 | 0.00±0.00 | 1.17±0.04 | 1.21±0.13 | 0.00±0.00 | 0.00±0.00 |
| UAGC17 | 1.81±0.42 | 2.96±0.72 | 1.83±0.18 | 2.44±0.44 | 1.49±0.15 | 3.02±0.54 | 1.49±0.14 | 2.06±0.17 |
| UAGC18 | 1.24±0.11 | 1.77±0.08 | 0.70±0.36 | 1.55±0.07 | 1.57±0.07 | 2.66±0.19 | 1.18±0.03 | 2.19±0.10 |
| UAGC19 | 2.34±0.05 | 4.06±0.06 | 1.75±0.14 | 2.76±0.29 | 1.43±0.12 | 3.01±0.28 | 1.51±0.17 | 2.43±0.46 |
| UAGC23 | 1.17±0.04 | 1.25±0.15 | 0.00±0.00 | 1.00±0.00 | 1.15±0.02 | 1.24±0.03 | 1.00±0.00 | 1.08±0.02 |
| UAGC26 | 1.23±0.04 | 1.05±0.05 | 0.00±0.00 | 0.00±0.00 | 1.21±0.04 | 1.14±0.03 | 0.00±0.00 | 1.21±0.10 |
| UAGC29 | 0.00±0.00 | 1.00±0.00 | 0.00±0.00 | 1.00±0.00 | 1.17±0.05 | 1.04±0.02 | 0.00±0.00 | 0.00±0.00 |
| UAGC46 | 1.25±0.06 | 1.53±0.19 | 1.34±0.08 | 1.09±0.05 | 1.36±0.08 | 1.40±0.05 | 1.24±0.02 | 1.28±0.01 |
| UAGC47 | 1.16±0.06 | 1.31±0.05 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| UAGC51 | 1.46±0.10 | 1.77±0.06 | 1.33±0.09 | 1.53±0.02 | 1.81±0.28 | 2.60±0.23 | 1.17±0.02 | 1.40±0.18 |
| UAGC55 | 1.45±0.10 | 2.87±0.10 | 1.11±0.02 | 1.00±0.00 | 1.38±0.11 | 3.25±0.01 | 1.24±0.06 | 1.10±0.01 |
| UAGC57 | 1.36±0.15 | 2.52±0.08 | 1.24±0.08 | 1.08±0.08 | 1.61±0.08 | 3.20±0.22 | 1.19±0.05 | 1.00±0.00 |
| UAGC62 | 1.15±0.03 | 1.21±0.05 | 1.10±0.04 | 0.00±0.00 | 1.48±0.01 | 1.20±0.01 | 1.14±0.02 | 1.13±0.03 |
| UAGC65 | 1.91±0.03 | 3.68±0.01 | 1.47±0.03 | 2.25±0.07 | 1.96±0.28 | 4.51±0.20 | 1.58±0.13 | 2.08±0.05 |
| UAGC70 | 1.23±0.01 | 1.19±0.03 | 0.00±0.00 | 1.41±0.08 | 1.14±0.01 | 1.11±0.02 | 0.00±0.00 | 1.42±0.03 |

readily forms a large number of compounds with Ca, Fe and Al, some of which are highly stable and, thus, essentially unavailable to plants (Yang et al., 2014).

It has long been known that some plants, bacteria and fungi may solubilize some phosphate forms that are usually unavailable to plants and, thus, achieve competitive gains (Krishnaraj and Dahale, 2014). It has been generally accepted that most calcium phosphate solubilization is due to $H^+$ released by the microorganism and the concomitant pH reduction (Dorozhkin, 2002). Other possible mechanisms have been offered, such as organic acid and exopolysaccharide production (Yi et al., 2008; Sindhu et al., 2014), with strong effects being ascribed to media composition (Nautiyal, 1999; Mehta and Nautiyal, 2001; Bashan et al., 2013).

These bacteria may be employed in agriculture through native population management or inoculation of selected strains with higher solubilization potential in a similar manner to that employed for other plant-growth-promoting bacteria (Araújo et al., 2012). One of the major aspects of this selection is the use of *in vitro* testing of the prospective strains to allow for screening of a larger number of strains than would be possible if *in vivo* experiments were the only option to conduct the selection.

Traditionally, this screening is conducted in solid media using a semi-quantitative evaluation of the ratio between a clear halo that is formed due to phosphate solubilization and the colony diameter (Chen et al., 2006). However, many isolates that did not produce any visible halo on solid media could solubilize various types of insoluble inorganic phosphates in liquid medium (Gupta et al., 1994; Mehta and Nautiyal, 2001; Salcedo et al., 2014).

This paper proposes to compare results from solid and liquid media *in vitro* solubilization assays with the strain effects on the available phosphorus under *in vivo* conditions. Thus, it will be possible to select an *in vitro* method that is more suitable as the initial selection steps of a program designed to select strains with the potential for soil inoculation.

## MATERIALS AND METHODS

A collection of 20 endophytic bacterial isolates that were previously identified as inorganic phosphate solubilizers (Lira-Cadete et al., 2012) (some of which were already identified at the genus or species level) were isolated from sugarcane leaves and roots (Table 1) (Silva, 2011). The isolate UAGC 5 is *Pantoea stewartii*; UAGC 7, UAGC 8, UAGC 9. UAGC 16, UAGC 26, UAGC 46 and UAGC 62 are *Pantoea* sp.; UAGC 17 and UAGC 19 are *Klebsiella/Enterobacter*; UAGC 70 is *Enterobacter* sp.; UAGC 15, UAGC 23, UAGC 29, UAGC 47, UAGC 51, UAGC 55, UAGC 57 and UAGC 65 are strains not identified at least at the genus level.

This paper is based on several sequential steps: initial evaluation of solid media composed of rock, tricalcium and bicalcium phosphates; semi-quantitative phosphorus solubilization on solid media; quantitative evaluation of phosphorus solubilization on liquid media; and *in vivo* phosphorus solubilization with cowpea in Leonard jars and with cowpea and sorghum in plastic bags.

**Solid media composition evaluation**

Verma (Verma et al., 2001) and NBRIP (Nautiyal, 1999) solid media were evaluated as the standard preparations and modified to use Irecê Apatite phosphate rock as a phosphorus source based on the 26.05 dag $P_2O_5$/g total P content. The media were inoculated with 72-h-old bacterial broth using the "drop plate" method (Alikhani et al., 2006) and kept at 28°C until bacterial colonies were visible, but no visible halo was formed by any of the isolates.

Due to the lack of visible solubilization, a new experiment was conducted using TSA, VERMA and NBRIP media, all using bicalcium phosphate as the phosphorus source and the same procedure as above. The colony diameter and visible halos were evaluated 3 and 17 days after inoculation to calculate the solubilization index (SI) according to Nautiyal (1999). Descriptive statistical analysis as well as ANOVA and Tukey tests, which considered a factorial 2 x 20 (media x isolates) arrangement, were performed, with three replicates, because no solubilization occurred on the TSA medium. The experiment was repeated once, and the data were transformed according to the SAS Guided Data Analysis Procedure (SAS Institute Inc, 1999).

**Phosphate solubilization in liquid media**

Seven isolates, including the three isolates with the highest SI values (UAGC 17, 19 and 65); three isolates with low solubilization capability and SI values ranging from 1.1 to 1.5 (UAGC 5, 16 and 26); an isolate considered a non-solubilizer (UAGC 47, which only formed a halo on NBRIP media); and the *Pseudomonas fluorescens* (R-243) strain from the Embrapa Agrobiologia collection were selected from the previous experiments. All isolates were grown in TS liquid media at 120 rpm for 72 h.

NBRIP and VERMA liquid media were prepared as per the preceding experiments, and 30 mL were distributed into 50-mL glass flasks for sterilization. Each flask received 300 µL of bacterial broth ($5 \times 10^8$ cfu $mL^{-1}$) and was incubated at 120 rpm at 28 °C. After 2, 4, 6, 8, 12 and 17 days of growth, three flasks with each isolate and media combination were harvested, and 10 mL was collected from each flask. The bacterial cells were separated from the media using a syringe filter membrane with 0.22-µm pores (model 99722 Techno Plast Products AG), and the filtrate was used for pH and soluble P determinations using the water-soluble P method (Embrapa, 1999). Uninoculated treatments were used as blanks for all date and media combinations. Data were analyzed according to an 8 x 2 x 6 (isolates x media x date) factorial arrangement, with three replicates. When the time effects were significant, they were further studied using regression analysis.

***In vivo* solubilization efficiency test in Leonard jars**

The same treatments and an uninoculated control treatment were tested in a greenhouse using Leonard jars and medium texture vermiculite, with cowpea (*Vigna unguiculata*) as the test plant and fertilized with Hoagland solution without P (Hoagland and Arnon, 1950).

Phosphorus was supplied according to the recommended rate for the species (IPA, 2008), and potassium or bicalcium phosphates (500 kg $ha^{-1}$) were used as the soluble or non-soluble P sources, respectively. The P source was weighed and mixed in each Leonard jar.

Cowpea IPA-206 cultivar seeds were disinfected, immersed in distilled, autoclaved water for 24 h and to germinate in autoclaved sand for two days. The bacterial cultures were allowed to multiply in TS media at 120 rpm and achieved a final population estimated as $5 \times 10^8$ cfu $mL^{-1}$. Inoculation was performed by immersing the root into the bacterial broth before transplantation and reinforced by inoculation of 1 mL of the same broth around the seedling.

The plants were harvested 44 days after transplantation for shoot dry mass (SDM), P content (SPC), through nitropercloric digestion followed by vanadium yellow colorimetric determination according to Embrapa (1999) and total P determinations (STP). The substrate P content (SP) was determined using the water-soluble method (Embrapa, 1999).

Statistical analysis was conducted as a completely randomized, 2 x 9 (P source x inoculation treatment) design with three replicates.

***In vivo* solubilization efficiency test in plastic bags**

Two separate experiments were conducted using cowpea and sorghum (*Sorghum bicolor*) with the same inoculation treatments and three phosphorus sources (bicalcium and potassium phosphates and Irecê Apatite ground rock phosphate).

These experiments was conducted using black plastic bags with an autoclaved, medium-texture vermiculite and sand (1:1) mixture and received Hoagland –P solution, as in the previous experiment. Rates of P were again determined using the same criteria, and added per bag. The seeds were directly transferred to the bags. Bacterial broth preparations were performed as in the previous experiment, but inoculation was performed by the addition of 2 mL of the bacterial broth on the seedling crown. The plants were harvested 60 days after seeding, and the same determinations were conducted as in the previous experiment. Statistical analysis was performed separately for each species as a randomized block design with a 3 x 9 (P source x inoculation treatment) design with three replicates.

## RESULTS

### Bicalcium phosphate solubilization in solid culture media

All isolates formed colonies in all culture media, but no apparent phosphate solubilization was found for rock or tricalcium phosphates. Most isolates had similar growth patterns in the same media in both experiments (Table 1), and NBRIP media allowed for larger SIs than VERMA for both experiments and evaluation dates. Isolates UAGC 17, 19 and 65 were among the strongest solubilizers in both experiments and for both media, while UAGC 47 did not present SI, except for NBRIP media in the first experiment. UAGC 5, 16 and 26 demonstrated low solubilization potential, leading to the formation of three solubilization potential groups for the liquid media experiments.

### Liquid media phosphate solubilization

Isolates UAGC 17 and 19 achieved higher solubilization

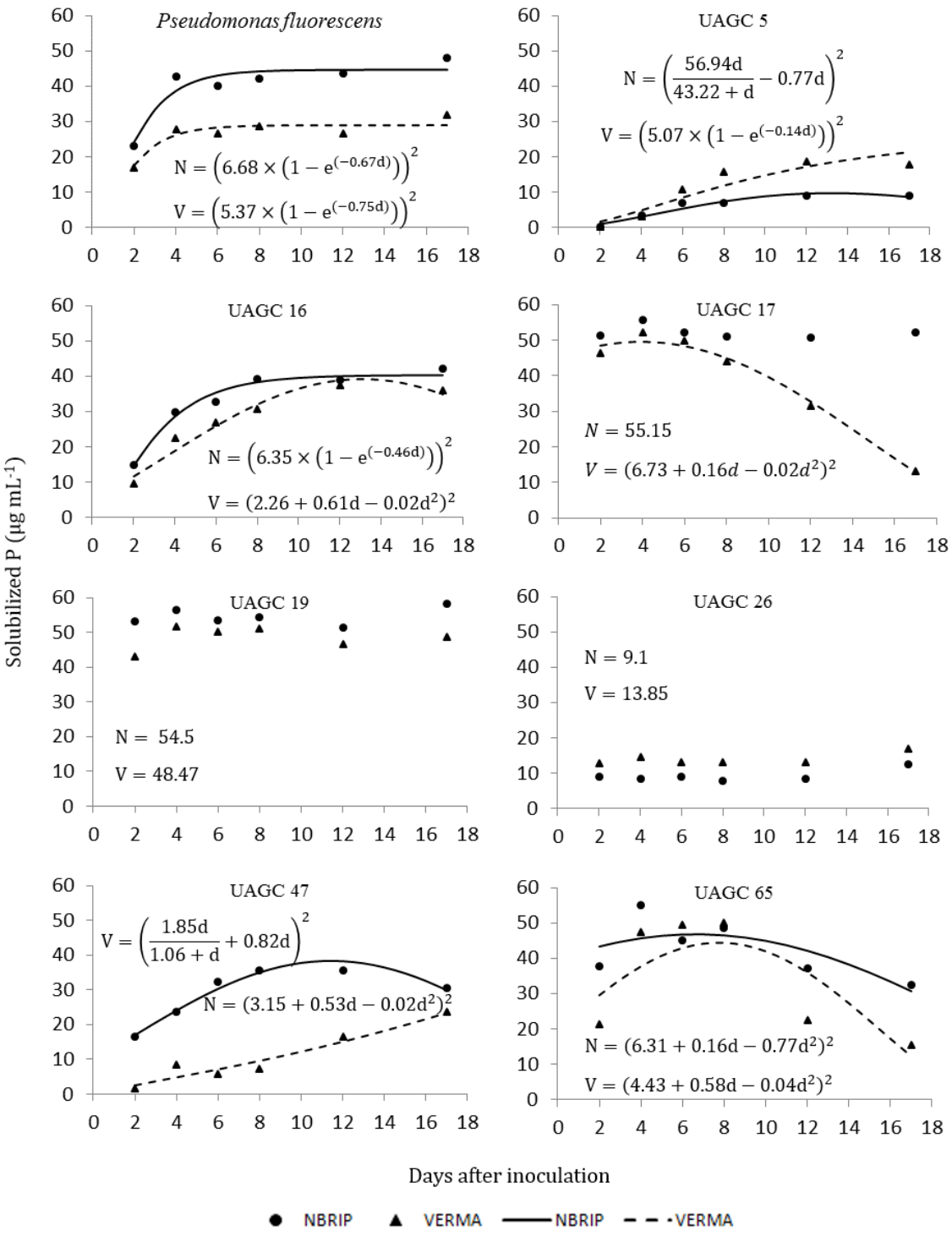


**Figure 1.** Soluble P contents on NBRIP and VERMA liquid media after inoculation with eight phosphate solubilizing bacteria. Each point is the average of three replicates subtracted the average soluble P content of the uninoculated media. d=days after inoculation.

on NBRIP than the standard strain, and both of these and UAGC 26 showed constant solubilization over time, with measurable P levels on the second day and stable P levels up to the 17$^{th}$ day after inoculation (Figure 1). This confirmed their higher-than-average solubilization on solid media. In VERMA media, UAGC 19 maintained

stable P levels, while UAGC 17 showed declining soluble P levels over time.

While isolate UAGC 19 had constantly higher soluble P levels, isolates UAGC 16, 17 and 65 were stronger solubilizers than the recommended strain, but with different solubilization patterns; the highest soluble P levels were achieved at 13, 4 and 8 days after inoculation, respectively.

The remaining isolates solubilized lower amounts of P than the control strain, and showed different curves. While isolates UAGC 5 and 47 were initially poor solubilizers in VERMA media, they increased their solubilization over time and surpassed some isolates with stronger initial solubilization by the end of the experiment. VERMA media only allowed higher soluble P levels for UAGC 5 at the latter half of the experiment, which agreed with the solid media results.

Inoculation strongly reduced the pH two days after inoculation, and each isolate had similar patterns, although different intensities, for both culture media (Figure 2). Isolates UAGC 17, 19 and 65 and the control strain were the strongest acidifiers two days after inoculation, and the pH continued to fall later in the experiment for both media. These findings were in partial agreement with the soluble P levels, which indicated that the acidification of the initial media might be the initial step in P solubilization, and this possibility was strengthened by the coincidence of the lowest pH levels and highest soluble P levels at 11 days after inoculation found for isolates UAGC 16 and 47.

### *In vivo* solubilization efficiency test in Leonard jars

No inoculation effects were observed in the Leonard jars with cowpea because P sources only significantly affected the SDM and STP ($P \leq 0.05$), and no significant ($P > 0.05$) effects were found for SP or SPC (Table 2). The highest SDM and STP were observed for potassium phosphate, most likely due to its higher solubility.

### *In vivo* solubilization efficiency test in plastic bags

The shoot dry mass for cowpea and sorghum was only affected by inoculation when the P source was rock phosphate. This indicates that even the relatively low solubility of bicalcium phosphate supplied enough P to these species (Table 3).

Isolates UAG 19 and 65 and the *Pseudomonas* reference strain were the highest SDM performers for cowpea, but those UAG isolates and UAG 47 did not differ ($P > 0.05$) from the reference strain for sorghum (Table 4). This difference in the response to UAG 47 may be due to some effect of the plant species on solubilization or different P requirements for both species. Importantly, the SDM of plants receiving these strains and rock phosphate did not significantly differ ($P > 0.05$) from those with higher solubility P sources.

As for SDM media, the isolates did not significantly ($P > 0.05$) change the cowpea SPC, except for those receiving rock phosphate, and, again, the highest results were found for strains UAG 19 (although not different from the remaining strains), 47 and 65 and the control strain (Table 3). On the other hand, no significant effects of inoculation on sorghum SPC (Table 4), in which only the P source was significant, were observed, and, again, rock phosphate presented the lowest results (Table 4).

While most isolates enhanced cowpea SP over the corresponding non-inoculated treatments, only UAG 19 showed significant effects ($P \leq 0.05$) for all P sources. When supplied with bicalcium phosphate, none of the UAG isolates, except for UAG 17, showed significant differences ($P > 0.05$) from the standard *Pseudomonas* strain (Table 5). Sorghum STP was significantly affected ($P < 0.05$) by inoculation only when rock phosphate was used as a source, and UAG 47 presented the highest results.

Substrate-soluble P contents did not demonstrate a clear pattern for inoculation or P source, except for the generally lower values for rock phosphate (Table 5).

## DISCUSSION

Nautiyal (1999) ascribed the higher solubilization found for solid and liquid NBRIP media, which was also found in this study, as most likely due to the use of $MgCl_2$ and $MgSO_4$ as Mg sources and/or $(NH_4)_2SO_4$ as the nitrogen source. These effects may also correspond to the strong effects found for different nitrogen sources when the solubilization potentials of defined media were compared, perhaps due to their effect on organic acid production (Dave and Patel, 2003; Sharan et al., 2008; Pallavi and Gupta 2013) or the liberation of $H^+$ when $NH_4^+$ is absorbed by the bacteria (Ahuja et al., 2007). Either of these mechanisms could explain the stronger acidification that was found for NBRIP than for VERMA liquid media (Figure 2), in which the pH difference between the media fell from approximately 0.2 to more than 1 for some of the bacterial strains. However, our data do not indicate which of these is most likely the main factor. Media composition was consistently found to only affect strains on the lower range of the solubilization potential. The differences found for a few isolates between the experiments in the same media are most likely due to the semi-quantitative nature of the method, in which the indication of solubilization depends on the formation of a visible halo that is larger than the bacterial colony, even though it is possible to differentiate a clear zone directly below the bacterial colony through the underside of the Petri dish. These strains were not considered solubilizers using this test, although they presented some solubilization in the liquid media experiments. In addition to the change from

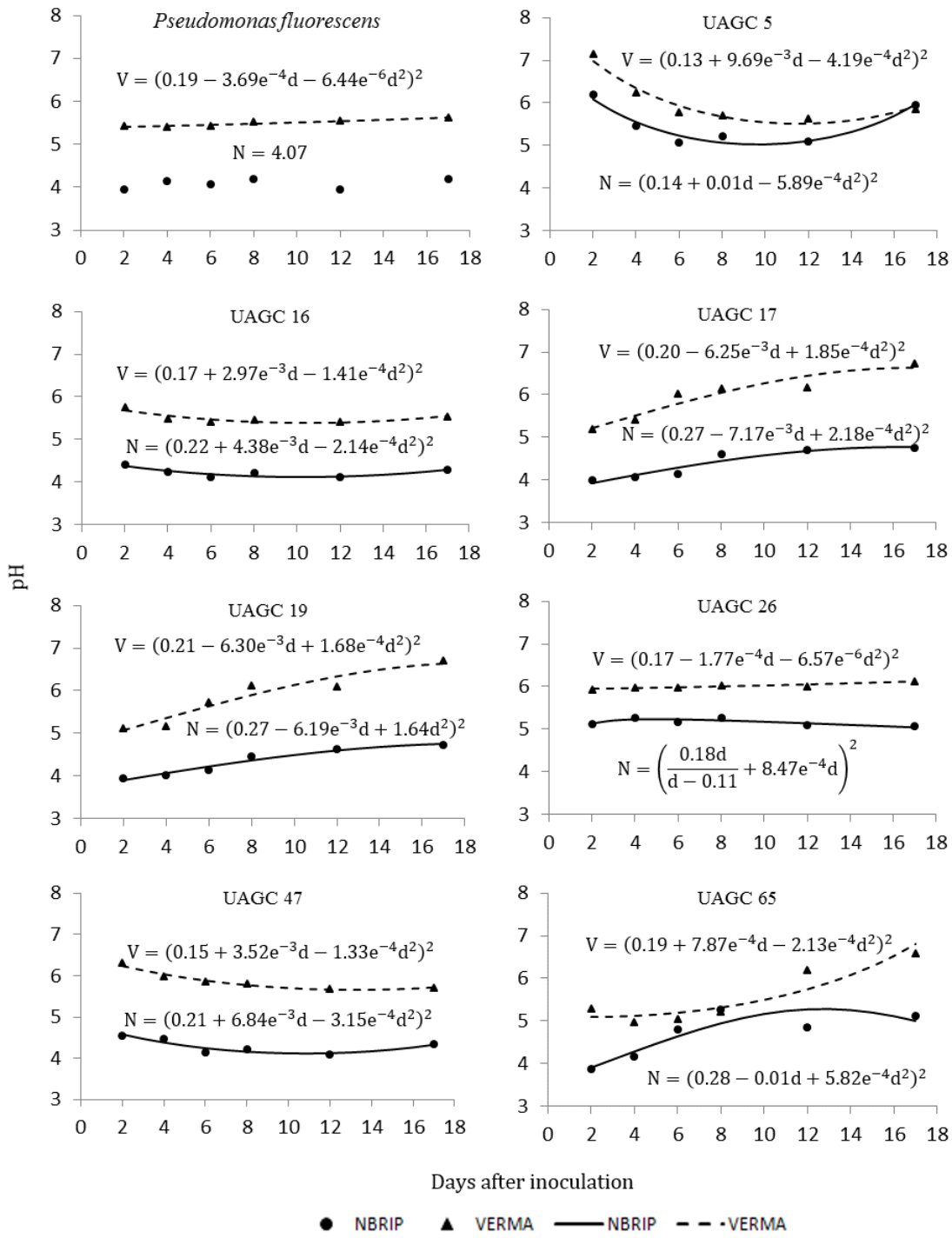


**Figure 2.** pH evolution on NBRIP (N) and Verma (V) media over 17 days of incubation with phosphate solubilizing bacteria. Each point is the average of three replicates subtracted the average pH reduction of the uninoculated media. d=days after inoculation.

non-solubilizer to solubilizer that was found for some strains, others, such as UAGCs 16 and 47 were stronger solubilizers on liquid than on solid media, confirming that liquid media evaluation should be a more sensitive experimental protocol for evaluating phosphate solubilization. The sensitivity difference is most likely due to a combination of the quantitative, rather than semi-quantitative determination, with the stronger diffusion

**Table 2.** Substrate P content (SP); shoot dry mass (SDM), P content (SPC) and shoot total P (STP) of cowpea plants in Leonard jars under greenhouse conditions and two P sources, inoculated or not with phosphate-solubilizing bacteria.

| P source | SP (g kg$^{-1}$) | SDM (g plant$^{-1}$) | SPC (g kg$^{-1}$) | STP (mg plant$^{-1}$) |
|---|---|---|---|---|
| Bicalcium phosphate | 1.10a | 1.743b | 0.121a | 20.9b |
| Potassium phosphate | 1.09a | 2.521a | 0.131a | 33.0a |
| CV (%) | 26 | 23 | 10 | 11 |

Averages followed by the same letter did not differ significantly by the Tukey test. SPC and STP transformed by Log10.

**Table 3.** Cowpea shoot dry mass (SDM) and P content and sorghum SDM after P solubilizer treatments with P sources of different availabilities.

| Inoculation | P source | | |
|---|---|---|---|
| | Bicalcium phosphate | Potassium phosphate | Rock phosphate |
| **SDM Cowpea (g planta$^{-1}$)** | | | |
| Uninoculated | 11.7$^{aA}$ | 13.1$^{aA}$ | 2.4$^{bB}$ |
| UAG 5 | 13.4$^{aA}$ | 12.4$^{aA}$ | 2.8$^{bB}$ |
| UAG 16 | 13.5$^{aA}$ | 14.8$^{aA}$ | 2.9$^{bB}$ |
| UAG 17 | 12.7$^{aA}$ | 12.7$^{aA}$ | 2.9$^{bB}$ |
| UAG 19 | 13.9$^{aA}$ | 14.5$^{aA}$ | 12.4$^{aA}$ |
| UAG 26 | 14.1$^{aA}$ | 12.8$^{aA}$ | 2.9$^{bB}$ |
| UAG 47 | 12.7$^{aA}$ | 13.2$^{aA}$ | 2.0$^{bB}$ |
| UAG 65 | 12.6$^{aA}$ | 12.6$^{aA}$ | 14.6$^{aA}$ |
| *Pseudomonas* | 13.1$^{aA}$ | 13.8$^{aA}$ | 12.2$^{aA}$ |
| CV % | | 12 | |
| **Cowpea Shoot P Content (g.kg$^{-1}$)** | | | |
| Uninoculated | 2.84$^{aA}$ | 2.46$^{abA}$ | 0.91$^{aA}$ |
| UAG 5 | 3.56$^{aA}$ | 5.5$^{aA}$ | 1.11$^{aA}$ |
| UAG 16 | 4.10$^{aA}$ | 3.69$^{abA}$ | 1.83$^{aA}$ |
| UAG 17 | 2.40$^{aA}$ | 1.31$^{bA}$ | 1.18$^{aA}$ |
| UAG 19 | 5.52$^{aA}$ | 2.84$^{abA}$ | 3.18$^{aA}$ |
| UAG 26 | 3.70$^{aA}$ | 2.74$^{abA}$ | 1.30$^{aA}$ |
| UAG 47 | 3.95$^{aA}$ | 4.69$^{abA}$ | 1.53$^{aA}$ |
| UAG 65 | 3.53$^{aA}$ | 3.08$^{abA}$ | 2.10$^{aA}$ |
| *Pseudomonas* | 3.66$^{aA}$ | 3.01$^{abA}$ | 0.92$^{aA}$ |
| CV (%) | | 36 | |
| **SDM Sorghum (g.plant$^{-1}$)** | | | |
| Uninoculated | 28.0$^{aA}$ | 27.2$^{aA}$ | 7.8$^{bB}$ |
| UAG 5 | 25.9$^{aA}$ | 25.9$^{aA}$ | 6.4$^{bB}$ |
| UAG 16 | 28.1$^{aA}$ | 27.2$^{aA}$ | 6.7$^{bB}$ |
| UAG 17 | 26.1$^{aA}$ | 26.8$^{aA}$ | 6.3$^{bB}$ |
| UAG 19 | 28.9$^{aA}$ | 26.7$^{aA}$ | 13.5$^{abB}$ |
| UAG 26 | 30.0$^{aA}$ | 27.1$^{aA}$ | 7.4$^{bB}$ |
| UAG 47 | 30.6$^{aA}$ | 29.0$^{aA}$ | 26.2$^{aA}$ |
| UAG 65 | 26.5$^{aA}$ | 27.1$^{aA}$ | 28.4$^{aA}$ |
| *Pseudomonas* | 25.1$^{aA}$ | 28.4$^{aA}$ | 25.1$^{aA}$ |
| CV % | | 21 | |

Averages followed by the same lower case letter in the column at the 5% significance level according to Tukey´s test.

**Table 4.** Shoot P content in sorghum grown in a greenhouse, inoculated or not with eight phosphate solubilizers, as affected by P source.

| P source | Shoot P content (g kg$^{-1}$) |
|---|---|
| Bicalcium phosphate | 2.80$^{a}$ |
| Potassium phosphate | 2.96$^{a}$ |
| Rock phosphate | 1.64$^{b}$ |
| CV % | 22 |

Averages followed by the same letter do not differ (P>0.05) from each other according to Tukey´s test. Data transformed by √x.

**Table 5.** Cowpea and sorghum total shoot P (STP) and soluble substrate P content (SP) as affected by phosphorus solubilizer inoculation and P sources.

| Inoculation | Phosphorus source | | |
|---|---|---|---|
| | Calcium phosphate | Potassium phosphate | Rock phosphate |
| **Cowpea STP (mg.plant$^{-1}$)** | | | |
| Uninoculated | 3.31$^{bcA}$ | 3.21$^{b}$c$^{dA}$ | 0.21$^{bB}$ |
| UAG 5 | 4.88$^{abA}$ | 6.92$^{aA}$ | 0.32$^{bB}$ |
| UAG 16 | 5.53$^{abA}$ | 5.35$^{abA}$ | 0.53$^{bB}$ |
| UAG 17 | 0.65$^{cA}$ | 0.35$^{dB}$ | 0.34$^{bB}$ |
| UAG 19 | 7.38$^{aA}$ | 4.00$^{abcB}$ | 3.93$^{aB}$ |
| UAG 26 | 5.19$^{abA}$ | 0.76$^{cdB}$ | 0.38$^{bB}$ |
| UAG 47 | 4.87$^{abA}$ | 6.18$^{aA}$ | 0.30$^{bB}$ |
| UAG 65 | 4.23$^{abA}$ | 0.75c$^{dB}$ | 3.07$^{abA}$ |
| *Pseudomonas* | 4.76$^{abA}$ | 4.14$^{abA}$ | 1.13$^{abB}$ |
| CV (%) | | 34 | |
| **Sorghum STP (mg.plant$^{-1}$)** | | | |
| Uninoculated | 7.11$^{aA}$ | 5.80$^{aA}$ | 0.27$^{abA}$ |
| UAG 5 | 9.23$^{aA}$ | 6.56$^{aA}$ | 0.49$^{abA}$ |
| UAG 16 | 9.51$^{aA}$ | 9.20$^{aA}$ | 1.20$^{abA}$ |
| UAG 17 | 5.83$^{aAB}$ | 9.69$^{aA}$ | 0.56$^{abB}$ |
| UAG 19 | 9.30$^{aA}$ | 7.45$^{aA}$ | 3.66$^{abA}$ |
| UAG 26 | 7.22$^{aA}$ | 7.26$^{aA}$ | 0.12$^{bA}$ |
| UAG 47 | 6.42$^{aA}$ | 8.13$^{aA}$ | 9.72$^{aA}$ |
| UAG 65 | 6.96$^{aA}$ | 7.89$^{aA}$ | 7.71$^{abA}$ |
| *Pseudomonas* | 5.59$^{aA}$ | 9.97$^{aA}$ | 6.51$^{abA}$ |
| CV (%) | | 20 | |
| **Cowpea SP (g kg$^{-1}$)** | | | |
| Uninoculated | 0.57$^{abA}$ | 0.66$^{aA}$ | 0.44$^{abA}$ |
| UAG 5 | 0.29$^{bA}$ | 0.33$^{abA}$ | 0.47$^{abA}$ |
| UAG 16 | 1.20$^{aA}$ | 0.15$^{abB}$ | 0.47$^{abAB}$ |
| UAG 17 | 0.12$^{bA}$ | 0.06$^{bA}$ | 0.17$^{bA}$ |
| UAG 19 | 0.33$^{bB}$ | 0.26$^{abB}$ | 1.07$^{aA}$ |
| UAG 26 | 0.18$^{bA}$ | 0.29$^{abA}$ | 0.56$^{abA}$ |
| UAG 47 | 0.15$^{bA}$ | 0.60$^{aA}$ | 0.44$^{abA}$ |
| UAG 65 | 0.51$^{abA}$ | 0.37$^{abA}$ | 0.37$^{bA}$ |
| *Pseudomonas* | 0.51$^{abA}$ | 0.21$^{abA}$ | 0.63$^{abA}$ |
| CV % | | 21 | |

**Table 5.** Contd.

| **Sorghum SP (g kg$^{-1}$)** | | | |
|---|---|---|---|
| Uninoculated | 0.45$^{aA}$ | 0.16$^{bcA}$ | 0.25$^{aA}$ |
| UAG 5 | 0.25$^{aA}$ | 0.73$^{abA}$ | 0.74$^{aA}$ |
| UAG 16 | 0.31$^{aA}$ | 0.84$^{aA}$ | 0.38$^{aA}$ |
| UAG 17 | 0.70$^{aA}$ | 0.16$^{bcA}$ | 0.25$^{aA}$ |
| UAG 19 | 0.40$^{aA}$ | 0.18$^{abcA}$ | 0.28$^{aA}$ |
| UAG 26 | 0.47$^{aA}$ | 0.23$^{abcA}$ | 0.35$^{aA}$ |
| UAG 47 | 0.27$^{aA}$ | 0.11$^{cA}$ | 0.18$^{aA}$ |
| UAG 65 | 0.23$^{aA}$ | 0.38$^{abcA}$ | 0.12$^{aA}$ |
| *Pseudomonas* | 0.25$^{aA}$ | 0.38$^{abcA}$ | 0.36$^{aA}$ |
| CV (%) | | 25 | |

Averages followed by the same lower case letter in the column and uppercase letter in the line did not significantly differ at the 5% significance level according to Tukey´s test. Sorghum STP was transformed by √x.

potential of organic or inorganic acids on liquid than on solid media. This agrees with some previous works using liquid media to evaluate solubilization (Nautiyal, 1999; Alikhani et al., 2006; Traoré et al., 2013).

Another advantage of the liquid media assay is the simpler separation of possible solubilization mechanisms, as observed for the different temporal patterns of acidification and solubilization that was found for isolates UAGC 16 and 47 in which UAGC 16 acidification and solubilization were closely linked, both in time and curve shape. Furthermore, the UAGC 47 curves present a clearer solubilization peak, particularly in NBRIP media, but continue to increase in VERMA media up to the end of the experiment. The later reduction is most likely due to higher P use by the growing bacterial population or to P precipitation, as suggested by Welch et al. (2002); however, our data are insufficient to discern between these possibilities. These different patterns confirm earlier literature (Silva Filho and Vidor, 2000, 2001; Khan et al., 2014) that suggested that, although media acidification most likely plays a major role in phosphate solubilization, it most likely is not the only acting mechanism, at least for these particular strains. The role of other mechanisms in phosphate solubilization is also implied by the similar pH levels that were found for UAGC 5 and the major solubilizers, although its soluble P levels were much lower (Figure 2).

Another point is that while some strains maintained a nearly constant soluble P level, such as UAGC 19, others, such as UAGC 17, presented strong declines in soluble P over time, which may have implications on their efficiency for agricultural use. Some authors (Alikhani et al., 2006; Narsian et al., 2010; Pereira and Castro, 2014) indicate that constant solubilization should be a sought-after characteristic of strains indicated for agriculture use as solubilizers.

A further point that should be emphasized based on the liquid media experiment is the consistent solubilization of bicalcium phosphate, even in the absence of bacterial solubilizer inoculation, which may be of importance under some experimental conditions and should not be disregarded when using this P source for solubilization studies. This is particularly when the test culture has relatively low P requirements, such as cowpea. This inherent solubilization is most likely one of the major reasons for the inability of the Leonard jars experiment to separate the strains and for the absence of the effect of inoculation on the plastic bags that were supplied with bicalcium phosphate, while it was found for rock phosphate (Tables 4 and 5) under the same experimental conditions.

The difference found between the total shoot P and P content responses for sorghum (Table 5) may be due to some other plant-growth effects because the genera of some of these bacteria are known to include plant growth-promoting rhizobacteria (PGPR) strains, such as *Enterobacter* and *Pantoea* (Moreira et al., 2010; Shahid et al., 2012; Chen et al., 2014). This reiterates the need for *in vivo* experiments to evaluate strains before their recommendation as solubilizers (Fernandez et al. 2007; Anzuay et al., 2015)

## Conclusion

*In vitro* liquid media evaluation should be used more often as the default method for phosphate solubilization analysis. However, more studies on the effect of media composition on phosphate solubilization and on the major pathways through which this solubilization occurs are needed.

The phosphate source for *in vitro* and *in vivo* experiments must be strongly considered before strain recommendation for inoculant production because even low solubility sources, such as bicalcium phosphate, may supply enough P for crops with relatively low P demand

to not be affected by strains that substantially increase P solubility in culture media.

## Conflict of Interests

The authors have not declared any conflict of interests.

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