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The culture medium volume and the inoculation method should be considered in semi-quantitative screening of calcium phosphate solubilization by bacteria

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ABSTRACT. Agar media with insoluble phosphates are used for semi-quantitative screening of phosphate-solubilizing bacteria based on the solubilization halo formed around the colonies. We show that the volume of the culture medium (15, 20, and 30 mL) and the inoculation method (toothpick vs microdrop) strongly influence the diameter of the solubilization halo, and this should be considered in advance selection of the isolates most efficient in this process.

Keywords: insoluble phosphate; plant growth-promoting rhizobacteria; solubilization halo; microdrop; toothpick.

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Introduction

Since the first studies showing solubilizing activities of insoluble inorganic phosphates by microorganisms, there has been considerable research for their utilization to improve the efficiency of phosphorus fertilization in crops, as well as to better take advantage of the phosphorus adsorbed in soil particles.

The first study showing the ability of bacteria to solubilize inorganic phosphates in agar medium was carried out at the beginning of the twentieth century (Sackett, Patten, & Brown, 1908). From this study, the method of the agar culture medium with insoluble inorganic phosphates sources was adopted, in which visualization of a translucent zone around the colonies is the indication of the bacteria's solubilizing ability. Given the need for establishing selection criteria, Berraquero, Baya, and Cormenzana (1976) proposed the solubilization index (SI) [halo diameter (mm)/ colony diameter (mm)] and the relationship between the SI and the incubation time in which the solubilizing effect appears in the agar medium. Thus, various studies in the literature select phosphate solubilizing bacteria following this method (Hara & Oliveira, 2005; Marra, Oliveira, Soares, & Moreira, 2011; Marra et al., 2012; Oliveira-Longatti et al., 2014; Martins, Lima, Oliveira-Longatti, & Moreira, 2015). However, the solubilizing halo diameter was compared without indicating the volume of the culture medium present in the Petri dishes, and different inoculation methods were used (Peix et al., 2001; Hara & Oliveira, 2005; Rivas et al., 2006; Liu et al., 2015; Martins et al., 2015). Thus, the methods described by Sackett et al. (1908), Berraquero et al. (1976), and Nautiyal (1999) were used, testing different volumes of the culture medium and different bacteria inoculation methods to check their effect on the SI of the phosphate solubilizing bacteria.

Material and methods

The experiment was conducted with strains UFLA04-155, UFLA04-232, and UFLA04-233, identified as *Burkholderia fungorum* (Ferreira, Bomfeti, Soares, & Moreira, 2012), grown in 79 medium (Fred & Waksman, 1928) with the following ingredients (g L^{-1}): K_2HPO_4 0.1, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, mannitol 10.0, yeast extract 0.4, and agar 15, with pH 6.8. These strains were inoculated through the methods of autoclaved toothpick and microdrops in NBRIP culture medium [(g L^{-1}): glucose 10.0, $Ca_3(PO_4)_2$ 5.0,

$MgCl_2.6H_2O$ 5.0, $MgSO_4.7H_2O$ 0.25, KCl 0.2, $(NH_4)_2SO_4$ 0.1, and agar 15.0 and pH adjusted to 6.7], which frequently has been used due to efficiency in selection of phosphate solubilizing bacteria (Nautiyal, 1999). The Petri dishes (Ø 9 cm) received 15, 20, and 30 mL of this medium, measured with a sterile glass graduated cylinder. Two dishes per volume were used for each strain.

For inoculation with an autoclaved toothpick, the strains were removed from the solid 79 medium with a light touch in the colonies and quickly inoculated as a dot in the dishes (Figure 1A**)**. For inoculation by microdrops, the strains were cultivated in liquid 79 medium and adjusted to a 0.5 optical density with saline solution (0.85%), and an aliquot of 20 μL of this bacterial suspension was inoculated in the NBRIP agar medium (Figure 1B). Three equidistant points were inoculated per Petri dish for each inoculation method and incubated at 28°C for thirty days.

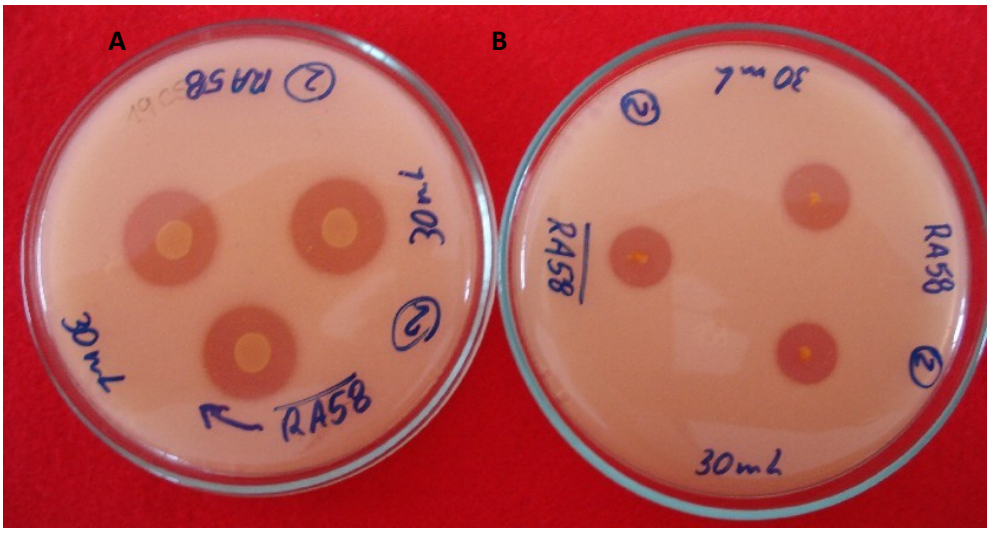

Figure 1. Colonies of phosphate solubilizing bacteria of the strain UFLA 04-155 obtained after inoculation by two methods in 30 mL of NBRIP medium. A - Inoculation by the microdrop method. B - Inoculation by the toothpick method.

The SIs of the strains were calculated according to Berraquero et al. (1976), and evaluations were made at 6, 12, 15, and 30 days after inoculation (DAI) using a digital caliper.

The experimental design was completely randomized in a factorial arrangement with four replications selected at random from six SIs originating from two Petri dishes inoculated for each strain. The SISVAR 5.6 program (Ferreira, 2014) and Scott-Knott test ($p < 0.05$) for comparison of means were used.

Results and discussion

Analysis of variance showed significant interaction between volume of the culture medium and inoculation method for all the strains and in all the evaluations performed ($p > 0.05$) (Table 1), except for UFLA04-233 at 15 DAI, for which there was a significant difference ($F_{2,18} = 285.24$, $P = 0.000$) between the inoculation methods (toothpick SI = 11.71 and microdrops SI = 3.47).

Table 1. Results of analyses of variance showing the interaction between volume of culture medium vs inoculation method for 3 strains of phosphate solubilizing bacteria in all the periods evaluated.

DAI	UFLA04-155	CV%	UFLA04-232	CV%	UFLA04-233	CV%
6	$F_{2,18} = 33.43$, $P = 0.0000$	11.57	$F_{2,18} = 27.79$, $P = 0.0000$	9.47	$F_{2,18} = 27.79$, $P = 0.0000$	9.47
12	$F_{2,18} = 17.80$, $P = 0.0001$	7.00	$F_{2,18} = 41.67$, $P = 0.0000$	7.71	$F_{2,18} = 23.35$, $P = 0.0000$	7.71
15	$F_{2,18} = 10.16$, $P = 0.0011$	13.60	$F_{2,18} = 6.56$, $P = 0.0073$	18.31	$F_{2,18} = 285.24$, $P = 0.6175^{*}$	15.75
30	$F_{2,18} = 16.80$, $P = 0.0001$	7.72	$F_{2,18} = 25.24$, $P = 0.0000$	11.60	$F_{2,18} = 25.24$, $P = 0.0000$	11.60

DAI: days after inoculation. CV: coefficient of variation. *: Not significant at 5% probability.

Regression analysis for slicing the interaction of volume within the toothpick method was significant at the 5% level in all evaluations. However, in the microdrop method, the differences among the SIs throughout the evaluations were not significant.

In general, the SIs of the strains showed a strong relationship to the volume of the culture medium in the Petri dish (Figure 2). This is an indication of dependence on the volume of the culture medium added to the dish, with SIs decreasing as this volume increases (Figure 2).

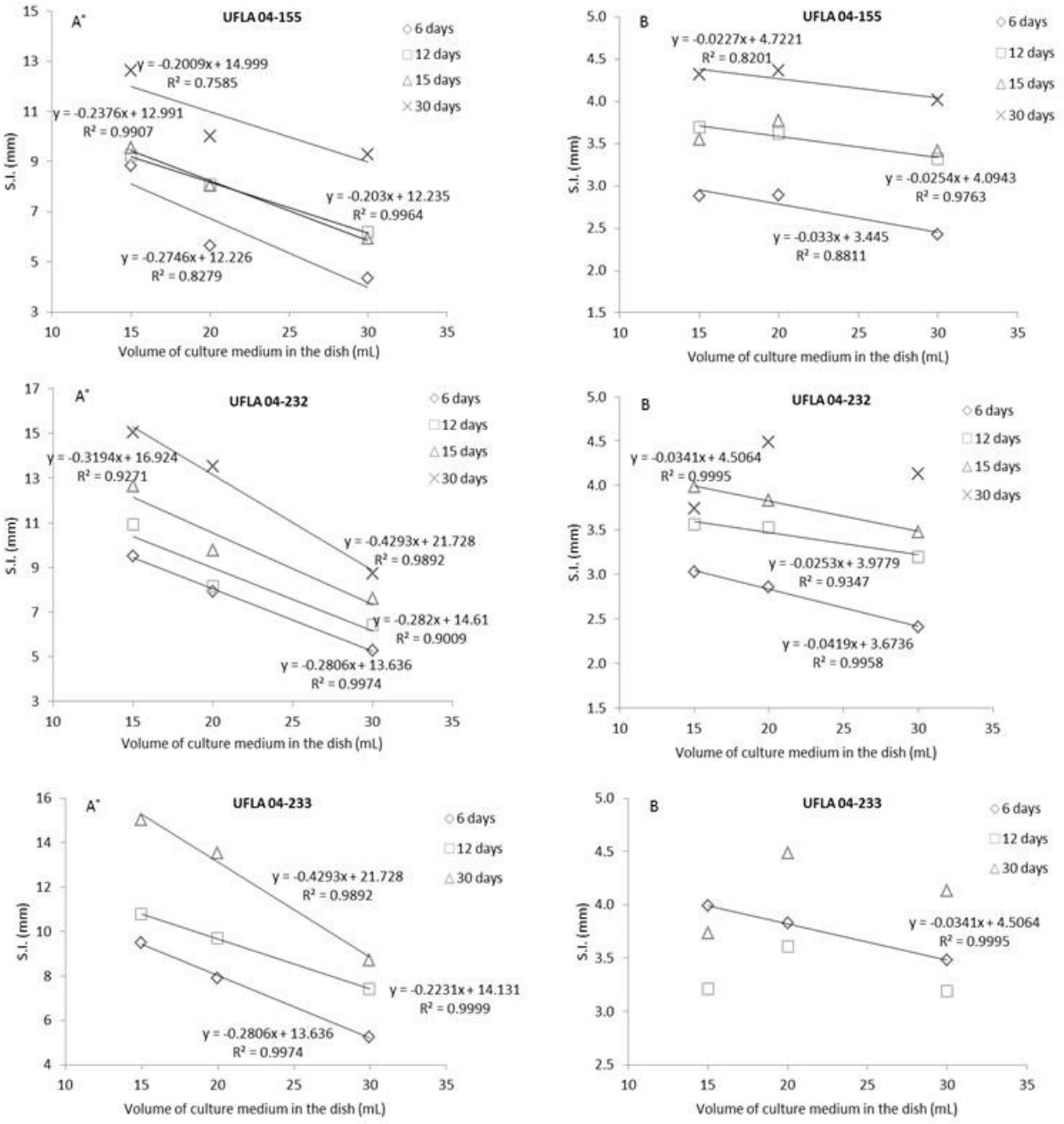

Figure 2. Linear regression analyses for slicing the interaction between volume of culture medium and inoculation method: A) Toothpick, *significant at p = 0.05, and B) Microdrop.

At lower volumes, it is likely that the organic acids released by the strains exploit more the surface area of the culture medium, making solubilization more effective. However, at larger volumes, the volume utilized might also be diluted with the depth of the medium. In the toothpick method, a significant relationship occurred in 100% of the evaluations (Figure 2A). In the microdrop method, in the evaluations that did not exhibit a strong relation, there was a decrease in SIs from 20 to 30 mL of culture medium (Figure 2B). The SIs of the strains inoculated by the toothpick method were significantly greater than when inoculated by the microdrop method (Table 2).

Generally, greater SIs were found in dishes containing 15 and 20 mL of culture medium, with greater frequency in 15 mL. Nevertheless, it is noteworthy that in 15 mL of culture medium in a Petri dish, water loss from the medium is faster than with 20 mL, which may affect bacterial development.

Over time there was an increase in the SIs for all the strains, and the highest indexes were observed on the thirtieth day of evaluation. Considering bacterial diversity, incubation periods can be adopted according to the growth rate of each species. Comparison of different genera of bacteria that have different growth rates should also be taken into consideration.

Table 2. Solubilization index of 3 bacterial strains inoculated by the toothpick and microdrop methods at different volumes of NBRIP culture medium in Petri dishes.

Strain	DAI	Inoculation method	Volume (mL)		
			15	20	30
UFLA04-155	6	Toothpick	8.84aA	5.66bA	4.35cA
		Microdrop	2.89aB	2.88aB	2.42aB
	12	Toothpick	9.26aA	8.07bA	6.18cA
		Microdrop	3.69aB	3.63aB	3.32aB
	15	Toothpick	9.56aA	8.04bA	5.93cA
		Microdrop	3.55aB	3.77aB	3.42aB
	30	Toothpick	12.64aA	10.00bA	9.30cA
		Microdrop	4.32aB	4.36aB	4.01aB
UFLA04-232	6	Toothpick	9.51aA	7.90bA	5.26cA
		Microdrop	3.03aB	2.87aB	2.41aB
	12	Toothpick	10.92aA	8.16bA	6.42cA
		Microdrop	3.56aB	3.52aB	3.21aB
	15	Toothpick	12.65aA	9.76bA	7.59cA
		Microdrop	3.99B	3.83B	3.48B
	30	Toothpick	15.03aA	13.53bA	8.72cA
		Microdrop	3.74aB	4.49aB	4.13aB
UFLA04-233	6	Toothpick	9.51aA	7.90bA	5.26cA
		Microdrop	3.03aB	2.88aB	2.41aB
	12	Toothpick	10.77aA	9.69bA	7.43cA
		Microdrop	3.21aB	3.61aB	3.19aB
	30	Toothpick	15.03aA	13.53bA	8.72cA
		Microdrop	3.74aB	4.49aB	4.13aB

DAI: days after inoculation. Means followed by the same lowercase letters in the same row or by same uppercase letters in the same column do not differ from each other by the Scott-Knott test at 5% probability. UFLA04-233: Non-significant interaction at 5% probability at 15 DAI.

Conclusion

Therefore, although the toothpick inoculation method has greater SIs, the microdrop method is the best option for screening of phosphate solubilizing bacteria because it allows lower variation of the SIs with volume. In relation to volume of the culture medium, 20 mL per Petri dish is ideal because it optimizes good solubilization indexes with saving of resources and reducing water loss, which ensures good development of the strains, especially in evaluation over a longer period.

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