Phosphate solubilization by *Bacillus* isolates and its influence in a cyanobacterial co-culture

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ABSTRACT

Aims: Phosphorus (P) and nitrogen (N) are essential nutrients required for the growth of crops, with scarce bioavailability in soil. The aim of this work was to study the inorganic P solubilization by three locally isolated bacteria of the genus *Bacillus* (San Luis, Argentina) and its influence in a co-culture with *Tolypothrix tenuis*, a diazotrophic and filamentous cyanobacterium used as a biofertilizer.

Methodology and results: Morphological, biochemical and API50 CH tests clustered strains SL-3, SL-4 and SL-7 as members of the *Bacillus subtilis* group. The solubilization index (SI) on Pikovskaya’s (PVK) agar showed that isolate SL-7 reached the highest solubilization value (SI=2.10), with better results than those obtained with *Bacillus velezensis* FZB42, a recognized plant growth-promoting bacterium (PGPB). In addition, *T. tenuis* was not able to solubilize tricalcium phosphate (TCP) on PVK agar. Individual cultures in Watanabe medium with TCP showed a reduction in the final biomass of 68.61% and their polyphosphate (poly-P) granules decreased considerably in size and quantity in relation to the cultures with soluble P, whose final biomass was 2.74 g/L. Co-cultures with strain SL-7 partially recovered the final biomass, reaching values of 1.37 to 1.42 g/L (*p<0.01*), while co-cultures with strain FZB42 showed no significant differences (*p=0.05*) in relation to the individual cyanobacterial culture with insoluble P.

Conclusion, significance and impact of study: The results of this study indicate that the mixed inoculation of *Bacillus* sp. SL-7 and *T. tenuis* could be a promising biofertilizer for overcoming the limitation of P and N compounds and boosting sustainable agriculture.

Keywords: *Bacillus* isolates, biofertilizer, co-culture, phosphate solubilization, *Tolypothrix tenuis*.

INTRODUCTION

Phosphorus (P) and nitrogen (N) are essential nutrients supplied by the soil and required for the growth of crops. However, soil P exists in various organic and inorganic forms that are insoluble or very poorly soluble, representing a problem for agriculture (Kundu et al., 2009; Prabha, 2016). The addition of inorganic fertilizers has solved P deficiency in the soil, but these fertilizers have long-term harmful impacts on the environment when applied in excess (Kang et al., 2011). On the other hand, although plants can readily take up mineral forms of nitrogen, N availability in the soil is very poor and most of it exists in organic forms (Li and Li, 2014).

Among the different mechanisms by which microorganisms could promote nutrient bioavailability are atmospheric N fixation and transformation of poorly soluble P compounds (Glick, 2012; Rashid et al., 2016). Cyanobacteria are relevant examples of nitrogen-fixing bacteria (NFB) and their polyphosphate bodies constitute a reservoir of P. In addition, cyanobacteria are involved in the biochemical cycles of carbon and oxygen and in the nutritional status and structural stability of soils, making them low-cost and eco-friendly fertilizers (Chittora et al., 2020).

On the other hand, phosphate-solubilizing microorganisms (PSMs) constitute a microbial group used in agriculture to transform complex phosphate...
compounds into simpler forms. This is mainly attributed to the production of organic or mineral acids and the release of extracellular phosphatase (Ivanova et al., 2006; Kundu et al., 2009; Cao et al., 2010). Bacillus, Pseudomonas and Rhizobium are the most efficient bacterial P solubilizers for increasing P bioavailability in soil.

Among the various types of biofertilizers, bacterial inoculants constitute one major group that includes rhizobia, plant growth-promoting bacteria (PGPB), NFB and phosphate-solubilizing bacteria (PSB) as a mixture of microorganisms to achieve enhanced growth, quality and yield of many crops. In this sense, although the use of PSMs to increase P fixed in the soil and thus improve crop production has been abundantly studied, adequate commercial formulations are not yet available (Kalayu, 2019).

Tolyphothrix tenuis, a filamentous N-fixing cyanobacterium, has been studied and proposed as a commercial biofertilizer (Silva and Silva, 2013); however, there are no data evaluating its growth in the presence of tricalcium phosphate (TCP) in co-culturing systems with members of the genus Bacillus. The interactions between different microorganisms play a critical role in a co-culture since the growth of cells of one strain may be enhanced or inhibited by the activities of other microorganisms present in the medium. Moreover, there are many instances in which the use of co-cultures seems to be advantageous over the use of a single microorganism because of the potential for synergistic utilization of the metabolic pathways of all strains in the co-culture (Subashchandrabose et al., 2011).

The aim of this study was to evaluate the P solubilizing capacity of local Bacillus spp. isolates. The best strain was selected for co-culturing with T. tenuis under photoautotrophic and insoluble P conditions. Comparatively, the ability to solubilize phosphate by B. velezensis FZB42, a well-recognized PGPB, was studied in the growth recovery of the cyanobacterium.

MATERIALS AND METHODS

Microorganisms and culture conditions

Isolates of Bacillus spp. were collected from freshwater samples of the Cruz de Piedra Reservoir, San Luis, Argentina (33°15’40.9” S, 66°13’03.4” W) and named SL-3, SL-4 and SL-7. The bacteria were isolated during a previous study concerning the microbiological quality of these recreational waters. Bacillus velezensis FZB42 was purchased from Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Stock cultures were kept frozen in trypticase soy broth supplemented with glycerol 25% (v/v) at −80 °C and activated on trypticase soy agar.

Tolyphothrix tenuis was kindly provided by Rizobacter Argentina S.A. Cyanobacterial stock cultures were maintained at 30 °C in a temperature-controlled room using Watanabe medium (W medium). Continuous illumination was provided by a set of nine fluorescent lamps with a light intensity of 26 W/m² (Silva and Silva, 2007).

Phenotypical characterization of Bacillus spp.

Phenotypic analyses of bacterial isolates were performed on the basis of the methods outlined in Bergey's Manual of Systematic Bacteriology (Logan and De Vos, 2009). The tests included cultural characterization on nutrient agar plate, cell morphology, Gram reaction, endospore formation, motility, aerobic and anaerobic growth, catalase test, casein hydrolysis, Voges-Proskauer test (VP), indole production, tyrosine degradation and growth at different sodium chloride concentrations (3-10% w/v) and temperatures (30-55 °C). In addition, carbohydrate fermentation profiles were determined using the commercial API 50 CH test kit (BioMérieux, Marcy l'Etoile, France). The results were analyzed with the Advanced Bacterial Identification Software (ABIS online) for a preliminary phenotypic characterization (Sorensen and Stoica, 2021).

Phosphate solubilizing capacity

The phosphate solubilization activity of native Bacillus spp. and B. velezensis FZB42 was tested on Pikovskaya’s (PVK) and National Botanical Research Institute’s Phosphate (NBRIP) agar media containing TCP (Nautiyal, 1999). After 4 days of incubation at 30 °C, the halo zone around the streaks was measured. The ability of the isolates to solubilize TCP was estimated by the solubilization index (SI) using the following formula: Total diameter (colony + halo zone)/colony diameter (Edi Premoro et al., 1996). In addition, pH variation was detected at the same incubation time in PVK agar medium supplemented with bromophenol blue ethanol solution (PVK-BP at 0.4% w/v as a stock solution) (Gupta et al., 1994). Tolyphothrix tenuis was also studied in modified PVK and NBRIP media, deprived of carbon and nitrogen sources. Agar plates were streaked on with the cyanobacterium and incubated at 30 °C for 7 days in a wet chamber to avoid evaporation. All the experiments were carried out at least three times.

Polyphosphate granules detection

Two dyes were applied to detect poly-P granules in T. tenuis filaments grown in the presence of soluble and insoluble phosphate (IP). The first one was toluidine blue, which reveals violet inclusions by binding to polyphosphate. Staining was carried out in cells harvested in the stationary phase of both cultures, as described by Brock et al. (2012). They were observed by bright field microscopy (Leitz Wetzlar-Germany), equipped with a 100-fold 1.3 oil immersion objective lens and a micrometer eyepiece. Counts of 200 cells from various samples were performed to estimate the effect of TCP on the content and size of poly-P granules in T. tenuis.
The second dye was 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), which can detect high concentrations of poly-P and other compounds within the cell. A stock solution containing 2.8 mM DAPI was added to a sample of *T. tenuis* culture to a final concentration of 140 μM. After dark incubation, the filaments were examined by means of an Axiovert 40 (Carl Zeiss) fluorescence microscope at 500× total magnification by setting a 436/20 nm excitation filter and 535/30 nm emission filter according to Aschar-Sobbi et al. (2008).

### Co-cultures of *T. Tenuis* and *Bacillus* strains

Individual cultures of *T. tenuis* were developed for 10 days in Petri dishes with 15 mL of W medium and modified W by replacing K₂HPO₄ for TCP (W-iP medium). Each culture was inoculated at approximately 15% w/v with a week-old inoculum and incubated at 30 °C in a controlled temperature room. Illumination was provided continuously by a set of fluorescent lamps fixed on a horizontal board above the cultures (2,300 Lux). Co-cultures of *T. tenuis* with *Bacillus* spp. were developed under similar conditions, using only W-iP medium. Spores of strains SL-7 and FZB42 were prepared by thermal treatment at 80 °C for 15 min from nutrient agar slant cultures and co-inoculated at three different initial concentrations, ranging between 10⁷ and 10⁶ spores/mL.

The final biomass was estimated by dry weight determinations. Cells were washed twice with distilled water and dried at 96 °C until constant weight. In the cultures with TCP, prior iP solubilization was necessary by adding a single drop of HCl 0.1 N. Results were obtained by at least two separate experiments performed in duplicate.

### Statistical analyses

All data were analyzed statistically using GraphPad Prism 8.0 (GraphPad Software Inc., USA). The statistical significance of differences between groups was established using one-way analysis of variance (ANOVA). The acceptance limit was set to 95% significance level. Bonferroni’s test was used to compare the means of the treatments, variability in the data was expressed as error bars, and a *p*<0.05 was considered to be statistically significant.

### RESULTS

Isolates SL-3, SL-4 and SL-7 were strictly aerobic, Gram-positive, rod-shaped, motile and spore-forming bacteria. They all showed casein hydrolysis, catalase and VP-positive reactions, while indole production and tyrosine degradation were negative. In addition, cells were able to grow at 30-55 °C and in the presence of 3-10% NaCl. The characterization of the colonies and the carbohydrate fermentation profiles exhibited differences between strains, with distinctive acid production of lactose, melibiose, raffinose, turanose and xylose (Table 1).

Based on their physiological and biochemical profiles, ABIS online assigned the isolates to the genus *Bacillus* with a similarity of over 95%. Isolates SL-3 and SL-7 showed close similarity with *B. amyloliquefaciens* (97.4%). However, a marked difference in the probability of presumptive identification between these isolates was observed (Table 2). On the other hand, isolate SL-4 presented 95.3% similarity with *B. subtilis/B. vallismortis*, without being able to find differences between these closely related species.

All the *Bacillus* spp. isolates and the FZB42 strain solubilized TCP in PVK medium. SL-7 isolate was the best P solubilizer, reaching a higher SI value than that obtained with strain FZB42, with no statistically significant differences between the remaining isolates (*p*>0.05) (Table 3). However, none of the isolates showed phosphate solubilizing activity when the NBRIP medium was used, and *T. tenuis* could not solubilize iP under these conditions either. Confirmatory qualitative analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SL-3</th>
<th>SL-4</th>
<th>SL-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony form</td>
<td>Round</td>
<td>Flat</td>
<td>Entire</td>
</tr>
<tr>
<td>Shape</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Elevation</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Acid in API system from</td>
<td>D-Lactose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D-Melibiose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D-Raffinose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D-Turanose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D-Xylose</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 1: Distinctive cultural and biochemical characteristics of the *Bacillus* isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Presumptive organism</th>
<th>Similarity (%)</th>
<th>Probability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-3</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>97.4</td>
<td>98.0</td>
</tr>
<tr>
<td>SL-4</td>
<td><em>Bacillus subtilis</em> or <em>B. vallismortis</em></td>
<td>95.3</td>
<td>64.6</td>
</tr>
<tr>
<td>SL-7</td>
<td><em>Bacillus amyloliquefaciens</em></td>
<td>97.4</td>
<td>45.2</td>
</tr>
</tbody>
</table>
Figure 1: Stain of poly-P granules of *T. tenuis* with Toluidine blue (A) or DAPI (B) growing in W and W-iP media.

Table 3: Phosphate solubilizing activity of *Bacillus* spp. and *T. tenuis* in different culture media.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>SI</th>
<th>Organic acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PVK</td>
<td>NBRIP</td>
</tr>
<tr>
<td><em>B. SL-3</em></td>
<td>1.67 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. SL-4</em></td>
<td>1.70 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. SL-7</em></td>
<td>2.10 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. velezensis FZB42</em></td>
<td>1.50 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td><em>Tolyphothrix tenuis</em></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

SI: Solubilization index, PVK: Pikovskaya’s agar, NBRIP: National Botanical Research Institute’s Phosphate agar, PVK-BPB: PVK-bromo phenol blue agar. ND: Not detectable, (+): Yellow halo around the colony. All the experiments were carried out at least three times to calculate mean ± standard deviation. Values with different letters are significantly different at *p*<0.05.

was done on PVK-BPB agar, where similar solubilization halos along with a yellow zone were observed due to the release of organic acids (Table 3). The initial pH showed a decrease to pH 3.4 in the surrounding medium.

Poly-P granules were markedly different in *T. tenuis* grown in W medium with soluble P and TCP (Figure 1). In cells obtained from cultures in W medium, poly-P granules were clearly visible with both toluidine blue and DAPI stains, with all the filaments exhibiting granules ranging from 0.1 to 0.45 µm. In contrast, the granule size was reduced from 0.05 to 0.1 µm in *T. tenuis* filaments from W-iP cultures, with 23.95% of empty cells.

Finally, we tested if iP solubilization by the bacteria could improve the growth of cyanobacterium *T. tenuis* co-cultured with *B. velezensis* FZB42 and the best solubilizing isolate, strain SL-7 (Figure 2). The maximal biomass value of *T. tenuis* was 2.74 g/L in the presence of soluble P; however, it decreased by 68.61% in W-iP medium. Co-cultures with strain SL-7 showed a partial recovery of phototrophic growth, reaching mean values of 1.37 to 1.42 g/L, while biomass values obtained with strain FZB42 ranged between 1.23 and 1.34 g/L. The results showed significant differences (*p*<0.01) for the former of the co-cultures. There were no significant differences between the three concentrations tested in both co-cultures (*p*>0.05), indicating that the growth improvement of *T. tenuis* was independent of the initial spore inoculum of PSB.

DISCUSSION

In recent years, the beneficial effects of *Bacillus* spp. have been the focus of increasing interest as plant growth promoters. Production of enzymes, antibiotics, insecticides, surfactants and several biochemicals, including, among others, organic acids, hormones, siderophores and exopolysaccharides, are responsible for this effective and environmentally friendly approach to improving plant growth. Several members of the *B. subtilis* group have been intensively studied and developed as commercial biofertilizers (Radhakrishnan *et al.*, 2017). In this work, three phosphate-solubilizing
Inclusions are important to restore the overall balance of nutrients and health of the soil, improving the yields of crops and increasing P availability (Krishnaraj and Dahale, 2014; Lestari and Annisa, 2019; Chen et al., 2021). All Bacillus isolates were able to solubilize TCP under laboratory conditions in PVK agar with SI values higher than 1.5, which indicates that they have good solubilizing capabilities, according to Li et al. (2019). Variable results between PVK and NBRIP agar have been previously reported for other microorganisms like Kosakonia cowanii AP01, with higher SI in PVK agar (Panigrahi and Rath, 2019).

When they were grown in PVK-BPB agar, a change of color from blue to yellow indicated acid production by the isolates as a solubilizing mechanism. Regarding organic acids, the production of 2-ketogluconic and gluconic acids has been reported for the bacterial genera Bacillus and Pseudomonas (Muleta et al., 2013; Cisneros Rojas et al., 2017). Vyas and Gulati (2009) have postulated that the structure of the organic acids is more important for phosphate solubilization than their concentration or quantity.

The microscopic analyses of these morphological features of poly-P granules in T. tenius could be useful in estimating P bioavailability. Several studies report that cyanobacteria collected from sampling sites with reduced P bioavailability showed a lower abundance of polyphosphate granules, while polyphosphate granules in cyanobacteria increased due to greater P bioavailability (Whitton and Potts, 2012; Muñoz-Martín et al., 2014).

In Indian agriculture, microalgal and cyanobacterial biomass harvested from wastewater treatment has been used as biofertilizers in an alternative process for the remediation of IP. The release of P from the poly-P granules is a slow and continuous process that depends on the activity of PSMs in the soil, preventing the excess P supply that occurs when inorganic fertilizers are used (Ray and Mukherjee, 2015).

Co-inoculation with the indigenous PSB SL-7 showed a beneficial effect on T. tenius grown in W-IP medium, with better results compared to those obtained with strain FZB42. In this sense, co-inoculation of a PSB as the SL-7 strain, together with this diazotrophic cyanobacterium, increases the potential of beneficial use of these microorganisms as biofertilizers. The mixture of cyanobacteria with arbuscular mycorrhizal fungus and PGPB not belonging to the order Bacillales have demonstrated better growth and grain yield in rice crops (Padmaperuma et al., 2020).

It is known that the co-inoculation of different organisms with or without an organic fertilizer is more beneficial for reinstating soil fertility and organic matter content than a single inoculum (Rashid et al., 2016). Yandigeri et al. (2011) have described Westiellopsis prolifica and Anabaena variabilis as P-solubilizing cyanobacteria and Afkairi et al. (2021) compared the solubilizing ability of organic P sources by an Anabaena sp. strain and a commercial bacterial consortium. On the other hand, different members of the genus Bacillus constitute important PGPB of commercial application due
to their multiple capabilities, but there are no formulations with N-fixing cyanobacteria. For this reason, this proposal could be used in a sustainable agricultural management strategy to restore fertility in soils with low phosphorus availability, reducing the negative impact of artificial fertilizers.

CONCLUSION

Three Bacillus spp. isolates showed iP solubilization capacity on PVK agar and SL-7 strain exhibited the highest SI value. Its use in co-culture with T. tenuis enhanced the cyanobacterial growth, with better results than those obtained with B. velezensis FZB42. Hence, mixed inoculation of a phosphate-solubilizing bacterium and an N-fixing cyanobacterium could be an eco-friendly option for overcoming the limitation of P and N compounds in soils.

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