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Selection of chilli-pepper seedling's growth promoting rhizobacteria from an Amazonian savanna

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ABSTRACT

The genus *Capsicum* includes economically important species important species, widely used as food and condiment. A critical stage of its production is obtaining quality seedlings, better adapted to transplanting, vegetative and productive development. The use of plant growth-promoting rhizobacteria (PGPR) in the production of high-quality vegetable seedlings can contribute to a more efficient agriculture, less dependent on crop protection products and mineral fertilizers. Therefore, the aim of this study was to select PGPR from pepper plants in the Brazilian Amazon savanna, using the quality index (QI) as a selection criterion and to evaluate their ability to produce promoters of plant growth, such as indole-3-acetic acid (IAA), phosphate solubilization (SCa-P), and biological nitrogen fixation (BNF). The experiment was conducted in Randomized Block Design (RBD) with four replications and 139 treatments, including a positive control (PC) and a negative control (NC). The ten best isolates were identified molecularly and subjected to three biochemical tests. The ten best bacteria demonstrated high growth promotion of pepper seedlings in the *in vivo* tests. Although not all isolates in this group demonstrated SCa-P and BNF capabilities, all of the isolates produced IAA. The bacteria isolated from the rhizosphere promoted the production of seedlings with higher QI, but the selection of bacteria based solely on the production of IAA, SCa-P, and BNF *in vitro* may not be enough to find the best isolates for promoting growth in pepper seedlings, as not all exhibited these mechanisms even though they improved the quality index (QI) of the seedlings.

KEYWORDS: Bioinoculant, *Capsicum annuum* L., horticulture, Solanaceae.

Seleção de rizobactérias promotoras de crescimento em mudas de pimenta na Savana Amazônica

RESUMO

O gênero *Capsicum* inclui espécies de interesse econômico, amplamente utilizada na alimentação e como condimento. Uma fase crítica de sua produção é obter mudas de qualidade que estejam melhor adaptadas ao transplante, desenvolvimento vegetativo e produtivo. O uso de rizobactérias promotoras de crescimento de plantas (RPCP) na produção de mudas de hortaliças de alta qualidade pode contribuir para uma agricultura mais eficiente, menos dependente de produtos de proteção de culturas e fertilizantes minerais. Portanto, o objetivo deste estudo foi selecionar PGPR de plantas de pimentão na savana amazônica brasileira, utilizando o índice de qualidade (IQ) na produção de mudas de pimentão como critério de seleção e avaliar sua capacidade de produzir promotores de crescimento vegetal, como ácido indolacético (AIA), solubilização de fosfato (SP-Ca) e fixação biológica de nitrogênio (FBN). O experimento foi conduzido em delineamento de blocos ao acaso (DBA) com quatro repetições e 139 tratamentos, incluindo um controle positivo (CP) e um controle negativo (CN). Os dez melhores isolados foram identificados molecularmente e submetidos a três testes bioquímicos para avaliar a produção de AIA, a solubilização de SP-Ca e FBN. Os dez melhores isolados demonstraram alta promoção de crescimento de mudas de pimentão nos testes *in vivo.* Embora nem todos os isolados deste grupo tenham demonstrado capacidades de SP-Ca e FBN, todos os isolados produziram AIA. As bactérias isoladas da rizosfera promoveram a produção de mudas com maior IQ, mas a seleção de bactérias baseada exclusivamente na produção de AIA, SP-Ca e FBN *in vitro* pode não ser suficiente para encontrar os melhores isolados para promover o crescimento de mudas de pimentão, pois nem todos exibiram esses mecanismos, embora tenham melhorado o índice de qualidade (IQ) das mudas.

PALAVRAS-CHAVE: Bioinoculante, *Capsicum annuum* L., horticultura, Solanaceae**.**

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INTRODUCTION

Plant food production is an activity of great economic importance around the world and essential for the human diet. Standing out among the vegetables of greatest economic interest is genus *Capsicum*, belonging to the Solanaceae family. *Capsicum* contains approximately 43 species, including *Capsicum annuum* L., considered one of the most economically important, widely used in food and as condiment (Monteiro-Neto et al. 2016; Chinakwe et al. 2019; Bhatt and Maheshwari 2020; Hyder et al. 2020). One of the critical stages in producing these species is obtaining quality seedlings that are better adapted to transplanting and promoting vegetative and productive development (Monteiro-Neto et al. 2016).

Plant growth-promoting rhizobacteria (PGPR) have been used for many years in the search for more-sustainable solutions for agriculture. PGPRs are an ecological alternative to chemical fertilizers due to their ability to solubilize nutrients, fix biological nitrogen, and produce phytohormones, thereby enhancing nutrient absorption and improving plant quality (Liang et al. 2022). Studies with these microorganisms have been carried out around the world on crops such as soybeans, maize, sugarcane, wheat, tomatoes and peppers (Angulo-Castro et al. 2021; Shen et al. 2021; Adedayo et al. 2022). However, beneficial isolates for vegetables are still rare, with most research focused on larger crops (Basu et al. 2021), or on the prevention or control of disease (Messele et al. 2017; Mekonnen and Kibret 2021).

The methods for selecting PGPR include *in vitro* biochemical tests carried out in the laboratory, using criteria such as the capacity for biological nitrogen fixation (BNF), solubilisation of calcium phosphates (SCa-P), production of indoleacetic acid (IAA), and production of siderophores, which are the principal mechanisms directly involved in promoting plant growth (Noh-Medina et al. 2014; Peña-Yam et al. 2016; Basu et al. 2021; Mekonnen and Kibret 2021). Despite the importance of the *in vitro* studies, bacteria selected from the rhizosphere may have a harmful effect or show little or no efficiency when tested in the greenhouse or under field conditions, which is attributed to the absence of interactions with the host plant species (Peña-Yam et al. 2016).

The IAA concentration necessary to stimulate plant growth varies between species. While for some a certain concentration promotes growth, for others the same concentration may have no effect or even cause damage (Noh-Medina et al. 2014; Peña-Yam et al. 2016). Considering this limiting factor when selecting PGPR, *in vivo* testing of all the isolates obtained from the rhizosphere of the target crop is important to prevent ruling out efficient bacteria not selected in the biochemical tests performed in the laboratory.

A rigorous way to evaluate the performance of plants inoculated with PGPR is through the seedling quality index (QI). The QI is a comprehensive metric that assesses seedling quality by considering various factors, such as stem diameter, stem caliper, shoot dry weight, root dry weight, and total dry weight. This index is particularly effective because it offers a holistic evaluation of the seedlings' growth potential, unlike other metrics that may focus on a single aspect, such as height or weight (Currey et al. 2013). This avoids the classification of low-quality seedlings. Additionally, *in vitro* biochemical tests can be used to corroborate the relationship between growth promotion and the target crop, providing additional validation and ensuring that the selected seedlings possess the desired characteristics for optimal growth.

The aim of this study, therefore, was to select PGPR from the rhizosphere of pepper plants grown in a Brazilian Amazonian savanna (Roraima state), using as selection criteria the QI obtained in the production of pepper seedlings, and to evaluate their ability to produce IAA, SCa-P and BNF.

MATERIAL AND METHODS

Study location and sample collection

The research was carried out from September 2018 to June 2019 in a greenhouse and under laboratory conditions, on the Cauamé Campus of the Federal University of Roraima, Boa Vista (2º52'20.83" N, 60º42'44.99" W). An Amazonian savanna in Boa Vista, Roraima state, Brazil, with predominantly flat to gently undulating terrain, featuring parkland savannah and grassy-woody savannah vegetation (Hermógenes et al. 2022).

Pepper plants used for the initial extraction of inoculum were collected from 11 different agricultural properties across the state of Roraima (Brazil) (Figure 1). Three to five healthy plants were selected and extracted together with rhizosphere soil, packed in plastic bags, identified and moistened to keep them alive. After separating the aerial part, the roots were vigorously shaken to remove excess adhered soil without washing, and cut into fragments approximately 1 cm in length, including strongly adhered soil particles. Ten grams of these fragments were placed in Erlenmeyer flasks and 90 mL of sterile saline solution (0.85% NaCl) were added. The flasks were then placed in an orbital shaker where the samples remained for 2 h at 180 rpm at room temperature. Each Erlenmeyer flask represented the rhizosphere of one plant, for a total of 49 samples.

Isolation and purification of bacterial colonies

Serial dilution was carried out on each of the samples to produce a 1 in 10^{-5} dilution in 13 mL glass test tubes, with the aim of reducing the bacterial load. In order to obtain higher bacterial diversity, three culture media were used purely for isolation purposes (Noh-Medina et al. 2014): King B (KB) (King et al. 1954), 523 (Kado and Heskett 1970) and Luria Bertani (LB) (Bertani 1951). Using a micropipette and sterile

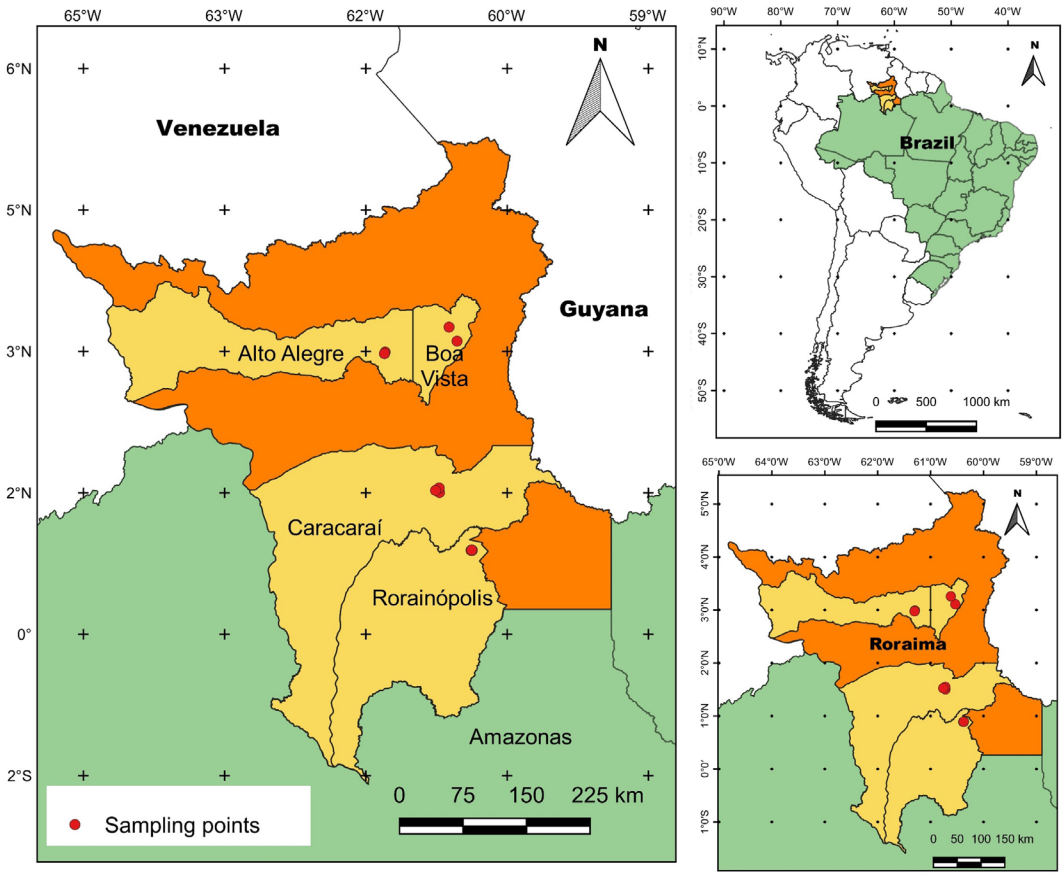

Figure 1. Geographical location of the eleven sites where the plants of *Capsicum* spp. were collected in the state of Roraima, Brazil.

tips, 100 µL of the 1 in 10^{-5} dilution was placed in the centre of the dishes for the KB and 523 media, and 100 µL of a 1 in 10^{-3} dilution for the LB medium. The 10^{-3} dilution was placed in a water bath at 80 °C for 20 minutes, and the solutions were then spread using a Drigalski loop.

The dishes were incubated in a BOD chamber for 24 h at 28°C for the KB and 523 media, and for 72 h for the LB medium, under a photoperiod of 12 h. Bacterial colonies that presented a distinct coloration, shape and border were sub-cultured and purified in the same culture medium in which they were isolated, totaling 137 bacterial isolates. The bacteria were preserved in 2 mL microtubes containing 1 mL of 30% glycerol, and stored in a freezer at -20°C (Noh-Medina et al. 2014).

In vivo tests for the selection of growth-promoting bacteria in green-pepper seedlings

An experiment was set up under greenhouse conditions to select the isolates. Preparation of the inocula and microbiolisation was carried out as per Pelzer et al. (2011). Before microbiolisation, seeds of the Casca Dura Ikeda cultivar of the sweet pepper from Feltrin Sementes®, were disinfected as recommended by Noh-Medina et al. (2014). Seeds soaked in sterile saline solution were used as the control.

The experimental design was of randomised blocks (RBD), with 139 treatments and four replications. Of the treatments, 137 corresponded to the inoculation of the obtained bacterial isolates, with two control treatments: a positive control (PC) with the application of a nutrient solution and no bacterial inoculation, and a negative control (NC) with neither nutrient solution nor bacterial inoculation. One experimental unit corresponded to four seedlings.

The seeds were planted in trays containing four cells (17 cm^3), filled with Orgânica Amazônia® sterile substrate (pH 6.23). The substrate was sterilised by tyndallisation in an autoclave (three periods of 30 minutes at 121°C every 24 h). Two seeds were planted per cell, and thinned after 15 days to leave only one seedling per cell.

The mean temperature inside the greenhouse was 28°C, with a maximum of 48°C and a minimum of 21°C. The mean relative humidity was 75%, with a maximum of 89% and a minimum of 36%. The cells were irrigated daily with 0.0064 mm m^{-2}. Except for the negative control, each cell received 1.7 g of 10-10-10 (N-P-K) + 1 g of ammonium sulphate per

litre of water via the nutrient solution, applying 3 mL per cell each week for 40 days.

The following variables were then determined in the seedlings after 40 days: stem length (SL), measured with a graduated rule (cm); stem diameter (SC) at the base of the seedling, evaluated using a 0.01 precision digital calliper (mm); shoot dry weight (S) and root dry weight (R) - previously washed seedlings were separated into shoots and roots, placed separately in paper bags, kept in a forced air circulation oven at 65°C for 72 h, and the weight determined on an analytical balance with a precision of 0.001 (g); and total dry mass (TDM), obtained from the sum of S and R. From these variables, the quality index (QI) was determined, where QI = TDM × (R:S + SQ), where SQ = SL x SC^{-1} (Currey et al. 2013). Although there are some limitations in this approach the results found in this study demonstrated good results in the selection of PGPR in *C annum* L. seedlings.

The response variables were QI, BNF, Sca-P, and IAA, while the predictor variable included the different bacterial strains and controls. It is important to note that the culture medium was not used as a predictor variable, but rather to enhance the chances of isolating a greater diversity of bacteria. The data were subjected to the Shapiro-Wilk normality test, and the variances were analyzed using Bartlett's homogeneity test at a 5% significance level. Data that did not exhibit a normal distribution required transformation. After verifying the data's normal distribution and the homogeneity of variances, a one-way analysis of variance (ANOVA) was conducted. The Scott-Knott test was used at a 5% probability level to group the treatment means, with calculations performed using R Studio software (R CORE TEAM, 2020).

In vitro biochemical tests of growth-promoting ability

Of the 137 bacteria tested *in vivo* under greenhouse conditions, the ten with the highest QI were selected for performing the following *in vitro* tests: biological nitrogen fixation (BNF), using the methodology suggested by Estrada-de los Santos et al. (2001), the presence of a film in the medium is indicative of a positive test; calcium phosphate solubilisation (SCa-P), using the methodology suggested by Nautiyal (1999); and indole acetic acid production (IAA), using the methodology suggested by Peña-Yam et al. (2016). The bacterium *Azospirillum brasilense* (BR 11001) served as positive control for all the *in vitro* tests. Four replications were used for each isolate.

Molecular identification

For the molecular identification, only the 10 bacterial isolates with the best performance were selected and cultivated in LB liquid medium and incubated at 36°C for 24 h in an orbital shaker at 150 rpm to produce biomass. The biomass was filtered, macerated in liquid nitrogen and the DNA extracted (Wilson 2001). The extracted DNA was analysed on 1% agarose gel and the molecular weight determined by comparison with a 1Kb ladder marker (Invitrogen); the concentration was determined using the NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, USA). Partial sequences of the 16S rRNA gene were amplified for each of the bacterial isolates using the P027F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') primers (Weisburg et al. 1991).

The PCR reactions were carried out using a final volume of 25 µL, containing a 1X reaction buffer [(100 mM Tris-HCl pH 8.8; 500 mM KCl, 0.8% (v/v)], 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.05 U Taq DNA polymerase, 0.2 mM of each primer, 2 ng diluted DNA, and milli-Q water (q.s.p.). The amplifications were performed using the Veriti 96-well Thermal Cycler (Applied Biosystems). The PCR conditions included an initial denaturation at 94°C for 3 min, 40 denaturation cycles at 94°C for 30 s, annealing at 65°C for 45 s, extension at 72°C for 1:30 min, and final extension at 72°C for 10 min.

The PCR products from the bacterial isolates were sequenced using a DNA automated sequencer 3500 Genetic Analyser (Applied Biosystems). The generated electropherograms were analysed visually using the SeqAssem software (Hepperle 2004). The sequences were compared with sequences available from the GenBank database, and aligned using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/BLAST). Sequences from reference isolates of family Enterobacteriaceae were obtained from GenBank and included in the phylogenetic analyses. The sequences were aligned using the ClustalW program (Thompson et al. 1994) as implemented in the MEGA v7 software (Kumar et al. 2016).

Bayesian inference analysis was performed on all sequences using the Monte Carlo chain method. The MrModeltest v2.3 software (Posada and Buckley 2004) was used to determine the model of nucleotide evolution that best fit the data. The model used was GTR+I+G. Phylogenetic analysis was carried out on the CIPRES web site (Miller et al. 2010) using the MrBayes v3.2 software (Ronquist et al. 2012). Markov chains were run simultaneously from random trees for 10,000,000 generations. Trees were sampled every 1000 generations for a total of 10,000 trees. For each analysis, the first 2,500 trees were discarded as burn-in, and the remaining 7,500 trees were used to calculate the posterior probabilities of the branches, determined as the consensus of the majority of the sampled trees. The trees were visualised in Figtree (Rambaut 2009) and exported to graphics software. The *Bacillus thuringiensis* isolate was used as the outgroup. The sequences obtained in this study were deposited in GenBank (accession nos. PP812400 – PP812409).

RESULTS

Of the 49 *Capsicum* spp. plants collected in the 11 locations under study, 41 belong to the species *Capsicum chinense* L. Jacq., divided into four varieties, known locally as Pimenta-de-Cheiro, Olho-de-Peixe, Murupi and Canaime. The eight remaining plants were identified as belonging to the species *Capsicum frutescens* L. (Malagueta). In total, 137 isolates were obtained from the 49 original plant collections and cultivation of rhizospheres in three culture media (KB, 523 and LB).

For the *in vivo* test for PGPR selection using the quality index (QI), the pepper seedlings were separated into three groups: A, B and C (Table 1). At this stage, bacteria with QI<0.03 were discarded. Bacteria inoculated into seedlings with QI ≥ 0.85 formed Group A (ten), those with 0.85 < QI ≥ 0.52 formed Group B (11), and those with QI < 0.52 formed Group C, considered to have no influence on growth promotion. Based on this classification, 77 bacteria were discarded in the evaluation. The mean QI of the pepper seedlings in Group A (QI ≥ 0.85) was superior to the mean value of the positive and negative controls by more than 1400%, and to that of Group B (0.85 < QI ≥ 0.52) by more than 914%.

Based on the phylogenetic analysis, the ten best bacterial isolates from Group A were identified as belonging to family Enterobacteriaceae (Figure 2). The sequence of the A25 isolate was grouped together with isolates of genus *Pantoea* in a highly supported clade (Bayesian posterior probability, Bpp = 1). The sequences of the A04, A16, A26, A33, A45 and A63 isolates were grouped with isolates of genus *Enterobacter* in strongly supported clades (Bpp = 1 and 0.90), and the sequences of the A15, A31 and A88 isolates formed a highly supported clade (Bpp = 0.87) with isolates of genus *Klebsiella*.

Of the ten bacteria evaluated for BNF from group A, only three *Enterobacter* sp. (A45), *Enterobacter* sp. (A16), and *Enterobacter* sp. (A63) were positive for pellicle formation in the culture medium. SCa-P was observed in only two of the bacteria from Group A: in *Enterobacter* sp. (A04) with a halo of 1.82 mm, and in *Pantoea* sp. (A25) with a halo of 9.5 mm; while the control *Azospirillum brasilense* (BR 11001) had a halo of 2.25 mm. The ten bacteria under study showed the ability to produce IAA (Table 2), particularly the *Enterobacter* sp. group, which had a greater variation, 20.9 to 118.5 μg mL^{-1}, and the bacteria identified as *Klebsiella* sp., which produced between 102.2 and 106.9 μg mL^{-1} IAA.

High QI values of pepper seedlings were associated with different combinations of the indicators IAA, BNF, and SCa-P (Table 2). The bacterium *Enterobacter* sp. (A33) had the lowest IAA (20.9 μg mL^{-1}) and a high QI. The *Pantoea* sp. (A25) and *Enterobacter* sp. (A04) isolates showed potential for producing IAA and SCa-P, but with a lower QI than that of *Enterobacter* sp. (A33) and *Enterobacter* sp. (A26). The *Enterobacter* sp. isolates (A45, A16 and A63) were positive for FBN, however this did not result in a higher QI in the pepper seedlings.

Although the QI among group A seedlings ranged from 0.85 to 1.25, statistically there was no difference between them. The isolates most affecting these responses exhibit various growth-promoting mechanisms and different amounts of IAA, with the presence or absence of these mechanisms not affecting the QI index.

Table 1. Effect of PGPR inoculation on the quality index (QI) of *Capsicum annuum* L. seedlings (Casca Dura Ikeda cultivar, Feltrin®) at the selection stage with 137 bacteria, a negative control (NC), and a positive control (PC).

Treatment	Quality Index (QI)	Treatment	Quality Index (QI)	Treatment	Quality Index (QI)
A33 (Group A)	1.25 [a]	C164 (Group C)	0.52 [c]	C100	0.21 [c]
A26	1.11 [a]	C27	0.52 [c]	C130	0.20 [c]
A25	1.10 [a]	C87	0.48 [c]	C108	0.19 [c]
A31	0.96 [a]	C70	0.45 [c]	C76	0.14 [c]
A45	0.93 [a]	C122	0.45 [c]	C128	0.14 [c]
A16	0.92 [a]	C09	0.43 [c]	C119	0.13 [c]
A63	0.91 [a]	C56	0.41 [c]	C103	0.11 [c]
A15	0.89 [a]	C11	0.41 [c]	C107	0.11 [c]
A88	0.85 [a]	C02	0.39 [c]	C99	0.09 [c]
A04	0.85 [a]	C95	0.34 [c]	C57	0.08 [c]
B20 (Group B)	0.78 [b]	C115	0.33 [c]	C165	0.08 [c]
B85	0.78 [b]	C60	0.32 [c]	C111	0.08 [c]
B86	0.71 [b]	C41	0.28 [c]	C167	0.08 [c]
B137	0.66 [b]	C142	0.27 [c]	C131	0.08 [c]
B40	0.66 [b]	C24	0.26 [c]	C129	0.07 [c]
B69	0.60 [b]	C148	0.25 [c]	NC	0.07 [c]
B53	0.58 [b]	C114	0.25 [c]	C123	0.07 [c]
B05	0.58 [b]	C71	0.24 [c]	C135	0.07 [c]
B21	0.57 [b]	C109	0.24 [c]	PC	0.06 [c]
B98	0.56 [b]	C133	0.22 [c]	C125	0.03 [c]
B134	0.55 [b]	C19	0.21 [c]		

Mean values followed by the same letter do not differ by Scott-Knott test at 5% probability.

Table 2. Quality index (QI) of the pepper seedlings, production of indole acetic acid (IAA), diameter of the calcium phosphate solubilisation halo (SCa-P), and effect on film formation in the culture medium (BNF) of the ten best rhizobacteria isolates.

Rhizobacterium	QI[1]	IAA[1] (μg mL^{-1})	SCa-P (mm)*	BNF (+ ou -)
Enterobacter sp. (A33)	1.25	20.9 [f]	-	-
Enterobacter sp. (A26)	1.11	89.6 [c]	-	-
Pantoea sp. (A25)	1.10	42.9 [e]	9.5	-
Klebsiella sp. (A31)	0.96	104.8 [b]	-	-
Enterobacter sp. (A45)	0.93	118.5 [a]	-	+
Enterobacter sp. (A16)	0.92	102.2 [b]	-	+
Enterobacter sp. (A63)	0.91	73.7 [d]	-	+
Klebsiella sp. (A15)	0.89	102.7 [b]	-	-
Klebsiella sp. (A88)	0.85	106.9 [b]	-	-
Enterobacter sp. (A04)	0.85	107.1 [b]	1.82	-

(-) negative for BNF (+) positive for BNF. For IAA, mean values followed by the same letter do not differ by the Kruskal-Wallis test at 5%.

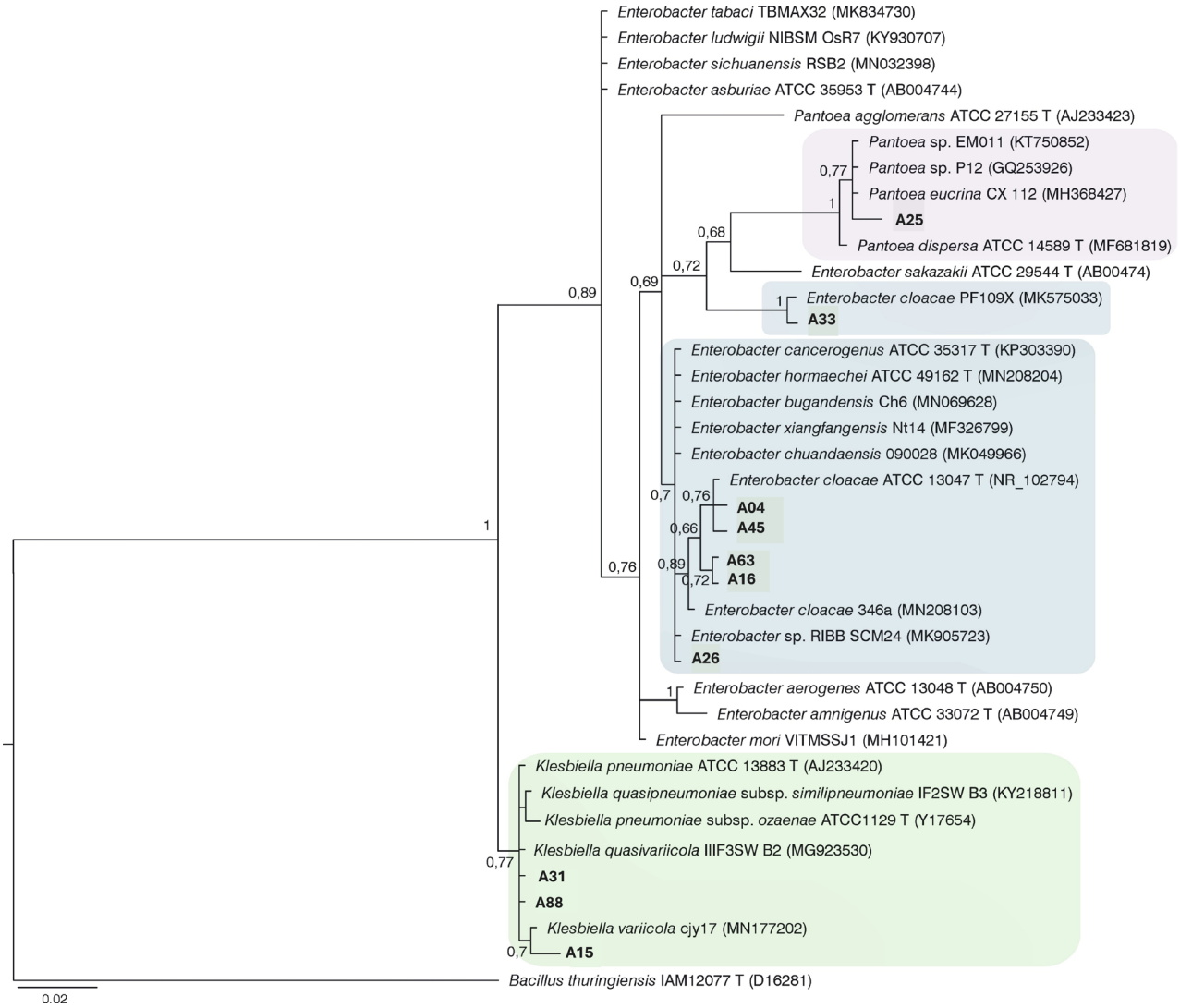

Figure 2. Phylogenetic tree of Bayesian inference based on the partial sequences of the 16S rRNA gene showing the relationships between species of the family Enterobacteriaceae from samples collected from *Capsicum* spp. roots in Roraima, Brazil.

DISCUSSION

Among the *Capsicum* spp. plants collected in the 11 locations under study, the varieties known locally as Pimenta-de-Cheiro, Olho-de-Peixe, Murupi and Canaime, as well as the species *C. frutescens* L. (Malagueta), are species that originated in the Amazon basin (Clement et al. 2010) and are among the pepper species most produced in Brazil. For the production of pepper seedlings, achieving the optimal level of growth and health is crucial to ensure their viability and productivity under field conditions. Since low-quality seedlings do not guarantee good productivity, the use of PGPR is essential for optimizing the process, including the absorption of nutrients and phytohormones, such as indole-3-acetic acid (IAA), gibberellins, and cytokinins (Monteiro Neto et al. 2016; Lobato -Ureche et al. 2023).

The quality index (QI) is considered a good indicator of growth promotion when selecting microorganisms in seedlings, since it includes more than one simple variable, in addition to being commonly used in experiments to evaluate seedling quality (Dickson et al. 1960; Currey et al. 2013; Monteiro-Neto et al. 2016; Silva et al. 2018). During our selection process, all the bacteria with QI < 0.03 were discarded, because they prevented seed germination and caused poor root-development, possibly due to their pathogenicity (Costa et al. 2010; Peña-Yam et al. 2016). However, various bacteria act to promote plant growth, creating a favourable environment for seedling development (Ali et al. 2011; Lim and Kim 2013; Panhwar et al. 2015), as seen in the seedlings inoculated with bacteria from groups A and B, which showed superior responses to those of the controls.

Previous studies of other bacteria on the genus *Bacillus* found that *B. subtilis* increased stem diameter and root volume in the pepper *C. annuum* L. cv Jalapeño, while *B. cereus* increased the number of flower buds; however, two species of *B. flexus* reduced the root volume and total biomass of the seedlings (Peña-Yam et al. 2016). The bacterial genera associated with the rhizosphere of *Capsicum* spp. plants in this study (*Enterobacter*, *Pantoea* and *Klebsiella*), have been reported as growth promoters in several crops, although there are no previous reports of this effect on *C. annuum* L.

Other research found that *Pantoea ananatis* increases the quality of tomato and melon seedlings and the production of pepper (*C. annuum* L.) fruits (Kim et al. 2012). Another study found that pepper (*C. annuum* L.) seeds inoculated with *Bacillus lincheniformis* generated plants of higher quality under water stress, increasing root length and dry matter weight, and raising percentage survival by 80% (Lim and Kim 2013). *Burkholderia cepacia* increased the height, the number of leaves, and the fresh and dry root weight of pepper (*C. annuum* L.) plants, showing the ability to solubilise phosphate and fix nitrogen (Miladiars et al. 2017). The genera *Enterobacter* and *Klebsiella* have been reported as promoting development in rice and wheat crops, respectively, in relation to salt stress (Sapre et al. 2018; Sarkar et al. 2018). Some bacteria may show different behaviour in different crops, such as *E. cloacae*, which promotes growth in maize (Verma et al. 2018).

The *Enterobacter* spp. (A45), (A16) and (A63), evaluated for BNF, were positive for pellicle formation in the culture medium. This film creates an anaerobic environment in the medium, necessary for the hydrogenase enzyme which is responsible for reducing the triple bond of atmospheric N, and quickly transforming it into ammonium (NH_4^+), the form assimilated by plants (Döbereiner et al. 1995; Estrada-de los Santos et al. 2001; Mayz-Figueroa 2004). In studies by Spolaor et al. (2016), bacteria were isolated from *C. annuum* L. and *Solanum lycopersicum* L. that were capable of producing between 0.1 and 1.7 mg L^{-1} of NH_4^+. However, there are bacteria that are able to form pellicles in N-free media, but without producing NH_4^+. As such, when forming a film in a semi-solid medium, or one free of N sources, BNF capacity may be at a level not detected by the methodology. Bacteria of genus *Enterobacter* have the ability to fix N and reduce acetylene to ammonia in *in vitro* tests, but may not be efficient in *in vivo* tests. In certain situations, the growth promoting effect may be induced by other mechanisms, such as IAA production, and not only by BNF (Spaepen et al. 2009).

The genera *Enterobacter* and *Pantoea* are reported in the literature as plant growth promoters due to their ability to solubilize calcium phosphates (Kim et al. 2012; Verma et al. 2018; Chen and Liu 2019). Strains of the genus *Pantoea* demonstrated high efficiency for both organic and inorganic SCa-P (Chen and Liu 2019), while the species *Enterobacter cloacae* can solubilize approximately 84.33 µg mL^{-1} of KH_2PO_4 (Verma et al. 2018). Similarly, rhizobacteria of the genus *Bacillus* isolated from *C. annuum* L. showed a solubilization index (SI) of 1.3 to 1.6 (Peña-Yam et al. 2016). In our study, the rhizobacterium *Pantoea* sp. (A25) showed greater efficiency for SCa-P with a halo of 9.5 mm, while *Enterobacter* sp. (A04) generated a smaller halo, of 1.8 mm.

In studies conducted on the crop of *C. annuum* L., up to 74% of the present rhizobacteria solubilized phosphates between 10 and 881 µg mL^{-1} (González-Mancilla et al. 2017). In rhizobacteria isolated from species of *C. chinense* Jacq., 40% were capable of solubilizing inorganic phosphorus (Noh-Medina et al. 2014). In our study, two out of the ten evaluated rhizobacteria demonstrated potential for phosphate solubilization, which indicates that 20% of the tested rhizobacteria possess this capability.

The species *Enterobacter* cloacae promoted growth in maize by its ability to solubilize phosphate, increasing the P content in dry matter, root length, and total biomass (Verma 2018). However, in tomato plants inoculated with isolates with this potential, seven showed results similar to the control, not promoting seedling growth (Noh-Medina et al. 2014). Our isolate *Enterobacter* sp. (A04) produced a halo of 1.8 mm, which was smaller than the halo produced by *Pantoea* sp. (A25). Nonetheless, the other rhizobacteria in Group A, which did not show phosphate solubilization mechanisms, promoted pepper seedling growth in a similar manner or with a higher QI.

SCa-P can increase the absorption of P and Ca in plants. Rhizobacteria produce organic acids, such as citric, malic, and succinic, which break the bonds between P and Ca, making these nutrients available in alkaline soils. These acids also help solubilize P-Al and P-Fe in acidic soils, where these elements are in insoluble forms. SCa-P, as a method for selecting PGPR, is inconsistent due to specific interactions between organic acids and soil minerals (Bashan et al. 2013).

Different carbon sources in the culture medium can also affect the efficiency of *in vitro* tests, so choosing an inappropriate culture medium may limit the detection of potential isolates (Nautiyal 1999). This method may underestimate efficient species, requiring specific media and molecular techniques to ensure efficient selection (Baldani et al. 2014). Therefore, the SCa-P mechanism, by itself cannot be considered an effective *in vitro* test to evaluate rhizobacteria as promoters of seedlings with higher QI.

Among the principal mechanisms used by bacteria for promoting plant growth is the synthesis of phytohormones, where the production of IAA has been shown to be a widespread property among soil-dwelling bacteria (Noh-Medina et al. 2014). IAA production in bacteria associated with a crop can promote plant growth and is correlated with the ACC deaminase enzyme, protecting plants in situations of

stress (Glick 2014). However, these mechanisms become less important under optimal conditions for crop development. The production of IAA by bacteria can be affected by the pH, temperature, culture medium, and the amount or lack of tryptophan (Costa et al. 2013).

Previous studies show that the production of IAA by bacteria isolated from different species of *Capsicum* ranges from 0.5 to 30 μg mL^{-1} (Mandyal et al. 2012; Noh-Medina et al. 2014; González-Mancilla et al. 2017; Peña-Yam et al. 2016; Bhatt and Maheshwari 2020). Considering that bacteria that produce more than 30 μg mL^{-1} IAA have not yet been reported for genus *Capsicum*, the present study found higher values (Table 2) than those found in the literature for native isolates of the genus. However, in bacteria isolated from *Curcuma longa* (Sundaram and Muralli 2018) and *Bertholletia excelsa* (Chalita 2016) the IAA production was higher.

We observed that inoculation of *Enterobacter* sp. (A33) resulted in the highest seedling QI, producing 20.9 μg mL^{-1} IAA, followed by inoculation of *Enterobacter* sp. (A26) with 89.6 μg mL^{-1} (Table 2). However, the absence of Biological Nitrogen Fixation or Calcium and Phosphate solubilization (evaluated *in vitro* for these two isolates), proved not to be the factor responsible for a high seedling QI. IAA production was a common mechanism among the 10 best selected bacteria. As such, the results found in this research contradict the practice of selecting PGPR based on production of this phytohormone.

CONCLUSIONS

We demonstrated that bacteria isolated from the rhizosphere of pepper plants stimulated the growth of pepper seedlings in *in vivo* tests, and all showed the ability to produce IAA, although not all showed the ability for SCa-P and BNF in *in vitro* tests. Therefore, selection based only on IAA, SCa-P and BNF *in vitro* may not be sufficient to guarantee effective growth of the target crop under *in vivo* conditions. We conclude that a selection based only on *in vitro* biochemical tests using criteria such as IAA production, SCa-P, and BNF needs to be reinforced with other more rigorous variables, such as QI, to ensure that the selected bacteria enhance growth promotion in *in vivo* tests.

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