PGPR activity of coal solubilizing bacteria

Actividad PGPR de bacterias solubilizadoras de carbón

Atividade PGPR de bactérias solubilizadoras de carvão

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Abstract

Coal solubilizing bacteria (CSB) are microorganisms to able to bio transformed low rank coal, releasing humified organic matter in the process. On the other hand, these bacterial genera have reported previously as plant growth promoting bacteria. The aim of this work was to assess the Plant Growth Promoting Rhizobacteria (PGPR) capacity of five CSB strains: *Bacillus pumilus* (CSB05), *B. mycoides* (CSB25), *Microbacterium* sp. (CSB3), *Acinetobacter* sp. (CSB13) and *B. amyloliquefaciens* (CSB02). For this, the PGPR traits of CSB were evaluated under laboratory conditions: the biological nitrogen fixation capacity, the reduction of acetylene, the synthesis of indole acetic acid (IAA) and the solubilization of phosphates. In a second experiment under plant nursery conditions, PGPR activity of strain CSB05 was evaluated in common bean plants. Under laboratory conditions, it was evidenced that all the evaluated strains produced IAA, solubilized phosphate in a liquid medium, presented atmospheric nitrogen fixation capacity, and only the CSB3 and CSB13 strains reduced acetylene. In the plant nursery experiment, PGPR activity of strain CSB05 was detected in common bean plants, reflected in increases in the height of these plants. These results show that CSB are promising in the PGPR activity, which is interesting to the design of biological products with agricultural and environmental applications, for the management of crops in disturbed soils of the Colombian dry Caribbean.

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Resumen

Las bacterias solubilizadoras de carbón (BSC) son microorganismos capaces de biotransformar carbones de bajo rango, liberando en el proceso materia orgánica humificada y se ha reportado que estas bacterias podrían promover el crecimiento de las plantas. El objetivo de este trabajo fue evaluar la capacidad Plant Growth Promoting Rhizobacteria (PGPR) de cinco cepas de BSC: *Bacillus pumilus* (BSC05)*, B. mycoides* (BSC25)*, Microbacterium* sp. (BSC3)*, Acinetobacter* sp. (BSC13) *y B. amyloliquefaciens* (BSC02). Para esto, fueron evaluados los rasgos PGPR en condiciones de laboratorio: la capacidad de fijación biológica de nitrógeno, la reducción del acetileno, la producción de ácido indol acético (AIA) y la solubilización de fosfatos. En un segundo experimento, en condiciones de casa de malla, se evaluó actividad PGPR de la cepa BSC05 en plantas de frijol común. En condiciones de laboratorio se evidenció que todas las cepas evaluadas produjeron AIA, solubilizaron fosfato en medio líquido, presentaron capacidad de fijación de nitrógeno atmosférico, y solo las cepas de BSC3 y BSC13 redujeron el acetileno. En el experimento en casa malla, se observó actividad PGPR de la cepa BSC05 en plantas de fríjol común, reflejado en incrementos en la altura de estas plantas. Estos resultados muestran que las BSC son promisorias en la actividad PGPR, lo cual resulta de gran interés en el desarrollo de insumos biológicos con aplicaciones agrícolas y ambientales, para el manejo y producción de cultivos en suelos perturbados del Caribe seco colombiano influenciados por la actividad minera.

Palabras clave: rizobacterias promotoras de crecimiento vegetal, fijación de nitrógeno, AIA, solubilización de fosfatos, solubilización bacteriana de carbón.

Resumo

As bactérias solubilizadoras de carvão (BSC) são microrganismos capazes de biotransformar carvões de baixo grau, liberando matéria orgânica humificada no processo, além disso, tem sido relatado que essas bactérias poderiam promover o crescimento das plantas. O objetivo deste trabalho foi avaliar a capacidade Plant Growth Promoting Rhizobacteria (PGPR) de cinco estirpes de BSC: *Bacillus pumilus* (BSC05), *B. mycoides* (BSC25), *Microbacterium* sp. (BSC3), *Acinetobacter* sp. (BSC13) e *B. amyloliquefaciens* (BSC02). Para isso, foram avaliadas as características de PGPR em condições de laboratório: a capacidade de fixação biológica de nitrogênio, a redução de acetileno, a produção de ácido indol acético (IAA) e a solubilização de fosfatos. Em um segundo experimento em casa de malha, avaliou-se a atividade PGPR da cepa BSC05 em plantas de feijão comum. Em condições de laboratório, foi evidenciado que todas as cepas avaliadas produziram IAA, fosfato solubilizado em meio líquido, apresentaram capacidade de fixação de nitrogênio atmosférico, porém, apenas as cepas BSC3 e BSC13 reduziram o acetileno. No experimento em casa malha, a atividade PGPR da cepa BSC05 foi observada nas plantas de feijão comum, refletido no aumento da altura do feijão. Estes resultados mostram que as BSC são promissoras na atividade de PGPR, o que é de grande interesse no desenvolvimento de insumos biológicos com aplicações agrícolas e ambientais, para o manejo e produção de cultivos em solos perturbados do Caribe seco colombiano influenciados pela atividade de mineração de carvão.

Palavras-chave: Rizobactérias promotoras do crescimento vegetal, fixação de nitrogênio, IAA, solubilização de fosfato, solubilização bacteriana do carbono.

Introduction

The application of low rank coal (LRC) lignite-type directly to the soil is an adequate strategy for the conservation of organic matter in dry tropical soils (Cubillos *et al*., 2015), apparently the colonization of these carbonaceous residues by coal bio-transforming microorganisms increases the gradual release of humic substances (HS) (Valero *et al*., 2014). In addition, the porosity and other characteristics of these coals improve the physical properties of the soil and indirectly stimulate microbial activity (Pantoja-Guerra *et al*., 2019; Valero *et al*., 2016).

In previous bioprospecting studies of bacteria in LRC, coal solubilizing bacteria (CSB) were isolated from the rhizosphere of grasses that grow in environments with residual coal particles: *B. mycoides* (CSB25), *B. pumilus* (CSB05), *B. amyloliquefaciens* (CSB02), *Microbacterium* sp. (CSB03) and *Acinetobacter* sp. (CSB13). These bacterial strains are capable to solubilize lignite, releasing HS in the process (Valero *et al.,* 2018; Valero *et al*., 2018). Additionally, some studies have shown that these bacterial genera have PGPR capacity (Idris *et al*., 2007; Tejera *et al*., 2013; Tejera *et al.*, 2011). In the case of *B. pumilus*, in addition to its role as PGPR, its capacity for endophytic colonization of plant tissues has been reported (De-Bashan *et al*., 2010). The PGPR capacity of some coal solubilizing bacteria strains has also been reported (Titilawo *et al*., 2020).

The PGPR are bacterial populations present in the rhizosphere, which have the ability to colonize the root system of plants or their closest environment, generating increases in plant growth (Kloepper *et al*., 1989). These bacteria promote plant growth by direct and indirect mechanisms, especially in soils with nutrient limitations and stress conditions. The most studied direct mechanisms are the nitrogen fixation, phosphate solubilization, production of phytohormones and siderophores, and ACC-deaminase enzymes (Glick, 2012). In addition, there are some indirect mechanisms associated with plant protection against the activity of phytopathogenic microorganisms (Meena *et al*., 2020). Therefore, the aim of this work was to evaluate the PGPR activity of some coal solubilizing bacterial strains, so that their potential in the design of biotechnological products of agricultural and environmental interest can be better known.

Materials and methods

Coal solubilizing bacteria (CSB)

Strains from open-cast coal mines located in the Cesar and La Guajira states, Colombia, identified as *B. mycoides* (CSB25) isolated from the rhizosphere of *Typha dominguensis*; *Microbacterium* sp. (CSB3) isolated from LRC; *Acinetobacter* sp. (CSB13) isolated from coal wash sediments; *B. pumilus* (CSB05) and *B. amyloliquefaciens* (CSB02) isolated from grasses rhizosphere of coal sludge in coal mine wash areas (Valero *et al*., 2012). These bacteria were selected for their ability to bio-transform LRC lignite-type in solid and liquid media, releasing HS (Valero *et al*., 2014).

Evaluation of the PGPR traits of the CSB

Biological nitrogen fixation (BNF) in solid medium: The five CSB in study were massively propagated on nutrient agar, a colony of each strain was taken and inoculated separately in Petri dishes with solid nitrogen-free culture medium (NFB) (Döbereiner *et al*., 1976), then the strains were incubated at 37 °C for five days to observe growth and a change of the medium, from the initial emerald green color to a light blue. These characteristics are characteristic of

nitrogen-fixing bacteria of free-living. The strain *Stenotrophomonas* sp. (BSFG03) was used as a positive control.

Acetylene reduction test: This test was determined by the method established by Hardy *et al.* (1968) whit some modifications. The strains were seeded on trypticase soy agar (TSA) and incubated at 30 °C for 48 h. Subsequently, each one was seeded in triplicate in hermetic vials with 2 mL of Burk liquid medium and were incubated at 30 °C for 48 h. Then, in each vial, was injected with acetylene and it was left to react for 1 h. The production of ethylene generated from the reduction of acetylene by the nitrogenase enzyme was quantified by gas chromatography. For this, a Shimadzu GC 2010 gas chromatograph was used coupled to a mass detector, manual injection in splitless mode (1 mL) and helium carrier gas at a constant flow of 1 $mL \cdot L^{-1}$. The Carbowax/20 M capillary column of 30 m, 0.250 mm diameter and 0.25 µm. The oven temperature was set at 50 °C for 4 min at a flow rate of 36.3 $cm \cdot sec^{-1}$, with a run time of 4 min and retention time of 1.8 min.

Indole-3-acetic acid (IAA) production: 500 µL of each bacterial suspension were inoculated of trypticase soy broth supplemented with 5 mM L-tryptophan. Then, in each vial, was injected with acetylene and it was left to react for 1 h. Then, these were incubated at 28 °C for 24 h in dark conditions. Negative control vials were used as treatment in the same way without inoculation. After, the bacterial cultures were centrifuged of Salkowski's reagent were added to 1.5 mL supernatant. Subsequently, it was incubated for 20 minutes at room temperature in dark conditions, the absorbance was read in a spectrophotometer at 520 nm, and the results obtained were compared with an AIA standard curve at concentrations of 5 $\mu g \cdot mL^{-1}$ to 100 $\mu g \cdot mL^{-1}$.

Solubilization of phosphates: In a preliminary test (data not shown) the ability of the five CSB strains to solubilize phosphate ($Ca_3(PO_4)2$) in solid medium was evaluated. All the strains presented a clear halo around the colonies, which was interpreted as a presumptive result in phosphate solubilization. The phosphate solubilization of the CSBs in liquid medium was then evaluated. One colony of each CSB studied was inoculated in Sundara-Rao and Sinha (SRS) broth, supplemented with tricalcium phosphate ($Ca_3(PO_4)2$) in test tubes. After 24 h, 50 µL of inoculum were seeded in 5 mL of SRS broth, incubated at 30 °C with shaking at 120 rpm for 24 h. Then 250 µL of the inoculum were taken and resuspended in 750 µL of sterile deionized water and 240 µL of paramolybdate blue reagent. As a control, tubes with 750 µL of sterile deionized water and 240 µL of the colorimetric reagent without inoculum and the strain BSFG03 as positive control were taken. A spectrophotometer with a wavelength of 520 nm was used for reading. The results were compared with a phosphorus standard curve at concentrations from 0.1 to 5 $\mu g \cdot mL^{-1}$ (Kumari *et al.,* 2018).

Evaluation of the effect of *Bacillus pumillus* on the growth of *Phaseolus vulgaris* plants

Previous screening works (data not shown) allowed to establish the PGPR potential of CSB strain *B. pumilus* (CSB05). These antecedents allowed selecting the CSB05 strain for in planta trials, under controlled conditions in a phytotron with common bean plants. The CSB05 strain was seeded with nutrient agar for 48 h. Subsequently, a typical and pure colony of *B. pumilus* was inoculated in an erlenmeyer flask with 200 mL of nutrient broth, incubated with shaking at 120 rpm for 72 h. Subsequently, the inoculum concentration was optimized to $1x10^8$ $UFC \cdot mL^{-1}$. For this trial, an experimental design was made with two treatments and five repetitions, the first treatment with the bacterial inoculum and the second without inoculum (control treatment). The common bean seeds were disinfected and sown in pots with 200 g of sterile soil. Then, with a sterile syringe, each pot was inoculated with 10 mL of the biopreparation. The control treatment was inoculated with 10 mL of sterile nutrient broth. The seedlings were kept under plant nursery conditions. After 15 days, the height of the plant and the total biomass were determined.

Re-isolation and identification of *Bacillus pumilus* as an endophytic strain

For the recovery of *B. pumilus* (CSB05) as an endophytic organism, the tissues were cut and disinfected and macerated according to the protocol proposed by Araújo *et al*. (2002). Immediately serial dilutions were made, 100 µL of sample was taken from each of the dilutions and inoculated into Petri dishes with nutrient agar, the boxes were incubated at 28 °C for 5 days. After 5 days, the formation of bacterial colonies was observed, from which colonies whose morphology presumptively agreed with *B. pumilus* were isolated and purified. From these isolates, the DNA was obtained and purified for the identification of the strain by direct amplification by PCR of the 16S ribosomal DNA, its partial sequence (with reading in two directions) and analysis of the sequence.

Statistical analysis

Analysis of variance (ANOVA) was performed with a 95 % confidence level in each of the experiments using the R statistical package. Additionally, multiple comparison tests were performed: Dunnett's test ($p<0.05$) for the experiment of the acetylene reduction, Tukey ($p<0.05$) for AIA production tests, solubilization of phosphates in solid and liquid medium.

Results and discussion

Biological nitrogen fixation (BNF) by CSB in solid medium

The growth of all CSB studied was observed on NFB Agar. In addition, a shift in the bromothymol blue indicator towards a faint blue color was observed, due to the alkalinization of the medium. The positive control for biological nitrogen fixation with the strain of *Stenotrophomonas* sp. (BSFG03), showed growth in the medium and a yellow color turn of the medium, which suggests an acidification of the medium. NFB Agar is a selective culture medium lacking nitrogen sources, so it is presumed that the bacteria that grew in it were able of fixing atmospheric nitrogen through the enzyme nitrogenase, capable of incorporating atmospheric nitrogen into bacterial metabolism (Mirza and Rodriguez, 2012)

Acetylene reduction test - nitrogenase enzyme activity

Figure 1 shows that the strains CSB3 (*Microbacterium* sp.) and CSB13 (*Acinetobacter baumanii*) presented a higher acetylene reduction with statistically significant differences ($p < 0.1$ for both) compared to the other evaluated CSB, which behaved similarly to the control. With this test, the capacity to fix nitrogen of the evaluated strains was indirectly determined, measuring the activity of the nitrogenase enzyme taking advantage of its ability to break the triple bond of acetylene and convert it into ethylene. Therefore, it is possible to infer that the nitrogenase activity was positive for the CSB3 and CSB13 strains, and for the other treatments that correspond to the *Bacillus* genera strains it was negative.

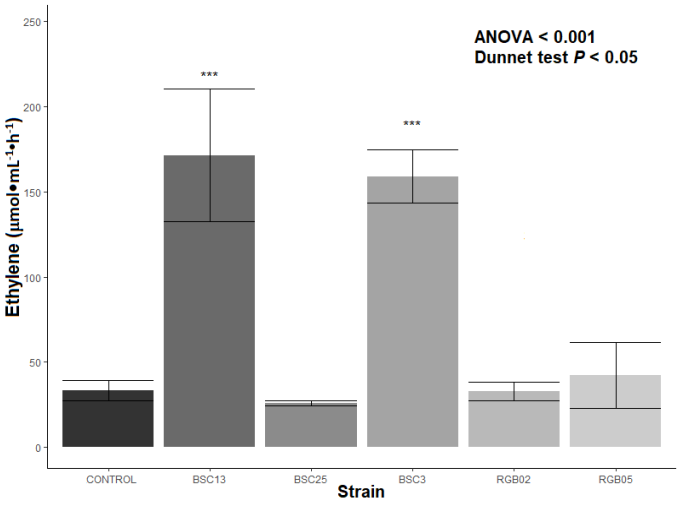

Figure 1. Quantitative biological nitrogen fixation (ethylene generated from the reduction of acetylene) of CSB. The asterisks (*, **, *) indicate significant differences of the treatments with respect to the control. (Dunnett <0.01 *** - <0.05 ** - <0.1 *).**

In other studies, with *B. pumilus* has been reported that is able of reducing acetylene to ethylene (Acuña *et al*., 2010) and other species of the genera *Bacillus* isolated from wheat, corn and rice, have been characterized with high nitrogenase activity (Corrales-Ramirez *et al*., 2016). Although in this test it could be inferred that the CSB25, CSB05 and CSB02 strains do not have the ability to reduce acetylene, it is important to clarify that the Burk`s medium used in this test was devoid of nitrogen, which is a factor that activates sporulation in the genera *Bacillus*, this limits its metabolic activity (Lalloo *et al*., 2009). This result could be the effect of an underestimation.

Production of indole-3-acetic acid (IAA)

All the CSB evaluated were IAA producers, the results showed in figure 2 have the net IAA production, and that is, the estimate in the control treatment (without inoculation) was subtracted to avoid the artifact effect of a possible coloration in the medium. According to figure 2, the production of IAA behaved in a similar way in all the strains, except for CSB3, which was statistically superior to the other treatments, while in the other strains they did not present significant differences between them. The genera *Bacillus*, it is not generally a great producer of IAA, however, some strains capable of producing up to 16 µg·mL^{-1} (Tejera *et al*., 2011). The production of IAA from rhizosphere microorganisms can improve root architecture, increasing the total surface of the root, which consequently can improve the absorption of water and nutrients (Naveed *et al*., 2015)

Quantitative determination of phosphate solubilization of CSB in liquid medium

All strains presented phosphate solubilization values higher than the negative control. The CSB3 strain had a lower solubilization value than the rest of the strains over time, inferring that this strain does not present a significant capacity to solubilize phosphates. The other treatments (CSB25, CSB13, CSB05, CSB02 and BSFG03) behaved similar to each other, showing the ability to solubilize tricalcium phosphate. Solubilization showed lower values at 24 h except for the positive control (BSFG03), which behaved similarly over time. In the figure 3 is showed that the highest solubilization occurred at 48 h, but then at 72 h there was a decrease in soluble inorganic phosphorus values, which is confirmed by Castagno *et al*. (2011), the strains studied by them had a maximum peak at 48 h and a decrease at 72 h. This phenomenon can be explained by alluding to the fact that in an initial stage (up to 48 h of incubation) the phosphate was solubilized to be consumed later (Tejera *et al*., 2013).

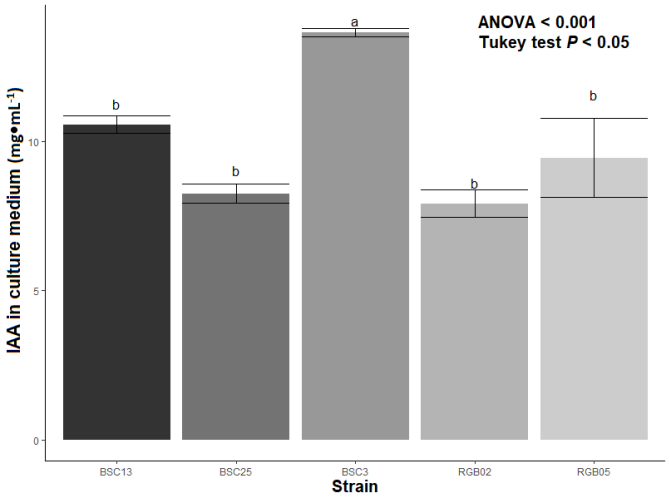

Figure 2. Production of indole-3-acetic acid (IAA) by CSB by colorimetric reaction with Salkowsky's reagent. The letters above the bars represent statistically significant differences (Tukey p<0.05).

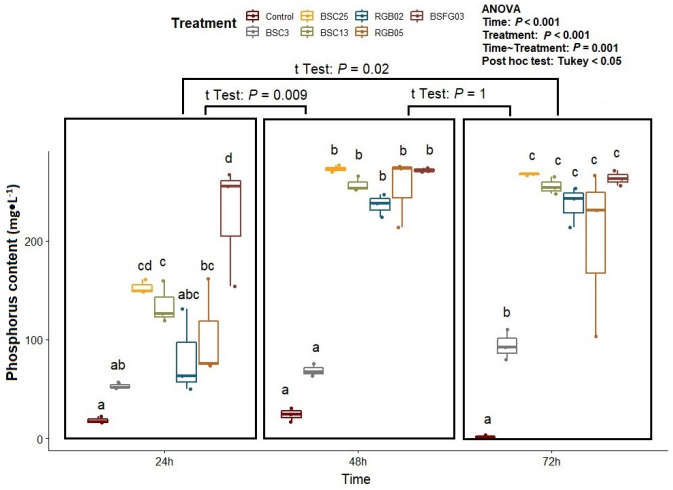

Figure 3. Quantitative determination of phosphate solubilization by CSB in liquid medium over time (24, 48, 72 h). The letters show the significant differences between treatments (Tukey p<0.05).

The genus *Bacillus* is one of the most studied regarding the ability to solubilize phosphates (Tejera *et al*., 2013). The solubilization of phosphates by PGPR allows not only a better assimilation

of insoluble phosphorus from the soil but also the possibility of progressively minimizing the amount of chemical fertilizers used daily in conventional agriculture (Kumari *et al*., 2018).

Evaluation of the effect of *B. pumilus* (CSB05) on the growth of *P. vulgaris* plants

The endophytic CSB05 showed PGPR activity on common bean seedlings under plant nursery conditions, showing significant statistical differences regarding to the control. The variable studied: height was higher than the control treatment as shown in figure 4.

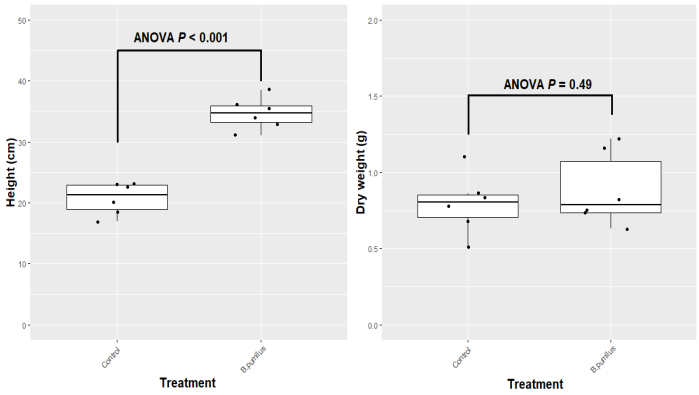

Figure 4. Stem length (left) and total dry weight (right) of *Phaseolus vulgaris*, after 15 days of inoculation with *Bacillus pumilus* (CSB05) compared to the control.

This growth promotion carried out by the endophytic strain CSB05, regarding to the increase in the length of the stem in bean plants could be due to its ability to produce auxin and AIA, which influences both cell division, growth and differentiation and are involved in the growth of stems and lateral roots (Spaepen *et al*., 2014). Although there are few studies on *B. pumilus* as a growth promoter in common bean plants, some studies have shown the ability of *B. pumilus* strains to promote different growth, types of plants, increasing stem and root elongation, and sprout germination, using various mechanisms such as IAA production, phosphate solubilization, production of pathogen biocontroller metabolites (Ansari *et al*., 2019) and even improve the adjacent microbiota after inoculation (De-Bashan *et al*., 2010). Likewise, it is important to note that the CSB05 strain *in vitro* tests presented the ability to solubilize phosphates (figure 3), a key mechanism for plant development in degraded soils, where the microbiota has been negatively affected by the use of fertilizers by current agricultural techniques (Ramírez *et al*., 2014,).

Re-isolation and identification of CSB05 as an endophytic strain

From the processing of plant tissues, it was possible to isolate a bacterial morphotype, with the microscopic and macroscopic morphology characteristic of *B. pumilus* (figure 5), while in plants without bacterial inoculation, no bacterial isolation with these characteristics was recovered. Comparison with the Ribosomal Data Project (RDP) 16S sequence database, using the SeqMatch tool against cultured isolates, indicates that the assembled problem sequence has greater homology with sequences from the species *B. pumilus* (figure 5).

Figure 5. Comparison of the sequence of the 16s rRNA of *B. pumilus* CSB05 against SeqMatch-RD.

Endophytic bacteria establish a close relationship with plants and can positively influence their development, in phytoremediation processes, acting as plant growth regulators, and also as biological controllers, which provides an agronomic benefit that can influence the decline of chemical fertilizers (Pérez and Chamorro, 2013). In similar studies, *B. pumilus* has been reported as endophytic bacteria and also as an effective biological controller producing metabolites that inhibit the activity of phytopathogenic microorganisms (Ren *et al*., 2013).

PGPR bacteria have been projected as a strategy for the phytoremediation of soils with mining residues (De-Bashan *et al*., 2010). Based on the results of the plant nursery evaluation of the endophytic strain *B. pumilus* CSB05, in this study it is concluded that it can increase the growth of the *P. vulgaris* plant. Likewise, in the results of the *in vitro* tests, it was evidenced that the studied strains present between two and three attributes of PGPR, for which it is necessary to implement future greenhouse and field trials to evaluate their PGPR capacity. The results presented in this work agree with those obtained by Titilawo *et al*. (2020), who reported PGPR traits in coal biotransformer bacteria strains.

These PGPR traits allow these bacteria to be screened for further studies and subsequent development of dual-purpose organic amendments, which not only help to release HS in the rehabilitation of soils disturbed by mining, but also allow the effective establishment of species plants in soils disturbed by anthropic activity**.**

Conclusions

The coal solubilizing bacteria strains *Microbacterium* sp. (CSB3), *Acinetobacter* sp. (CSB13), *B. mycoides* (CSB25), *B. pumilus* (CSB05) and *B. amyloliquefaciens* (CSB02) show PGPR traits. The endophytic strain *B. pumilus* (CSB05), stimulates the increase in stem length in *P. vulgaris* plants after 15 days of inoculation and is postulated as a potential biotechnological CSB and PGPR for the design of useful products in the treatment of degraded soils.

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