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Evaluation Of The In Vitro Antagonistic Potential Of Rice Plant-Associated Endophytic Bacteria Against Burkholderia Glumae

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ABSTRACT

Burkholderia glumae is the etiological agent of the disease called bacterial panicle blast of rice, which causes great economic losses in the agricultural sector. The objective of this research was to evaluate the in vitro antagonistic activity of endophytic bacteria isolated from rice varieties against Burkholderia glumae. A completely randomized design was applied for the in vitro antagonistic activity of endophytic bacteria against B. glumae and to establish differences between the population density present in rice varieties and the type of colonized tissue. Likewise, the Duncan multiple range test was used to establish significant statistical differences ($p\text{-value} < 0.05$) between colonized tissues and percentage of inhibition of endophytic bacteria. A total of 148 morphotypes of endophytic bacteria were isolated from the varieties F2000, FMocarí and F473. The F2000 variety showed a higher population density ($p\text{-value} < 0.05$). The root was the tissue with the highest population density of endophytic bacteria ($p\text{-value} < 0.05$) when compared to the rest of the tissues. The morphotypes H2M1LIM and P4M2LIM were molecularly identified by 16S rRNA gene as Burkholderia cepacia, R1M2LIM and R3M3LIM as Bacillus subtilis and T5M7 as B. cereus which showed in vitro inhibition against B. glumae. These species are characterized by the production of volatile and non-volatile secondary metabolites that have the ability to control growth in vitro against pathogens. The application of endophytic bacteria with antagonistic potential may become in the future a great alternative to replace the application of agrochemicals and chemical fertilizers.

Keywords: agriculture, antagonism, Oryza sativa, production, yields

INTRODUCTION

Rice is considered one of the main foods in the family food basket and is characterized by its high nutritional content (Martínez et al., 2005). In Colombia, rice cultivation occupies the first place in terms of economic value among short-cycle crops (Maqueira et al., 2009). Rice is ranked as the second largest rice producing country in Latin America and the Caribbean after Brazil and Peru; and globally it ranks 22nd with a share of 0.4% (FAO, 2013; Mazuera, 2010). Rice is the third largest agricultural product after coffee and maize (Espinal et al., 2005). It represents 6% of agricultural production and 11% of the country's agricultural activity, and is considered an important source of employment and income (Rives et al., 2007). The value generated by this product is equivalent to 63% of the value of coffee cultivation (Espinal et al., 2005; Zapata & Vélez, 2011).

To improve the yield of this crop, the application of chemical fertilizers is necessary, as well as the control of diseases, which at certain times of the year are one of the main and most important limiting factors in rice yield (Ham et al., 2011; Kim et al., 2018; Lee et al., 2016; Mizobuchi et al., 2016). Burkholderia glumae Kurita and Tabei 1967 (Burkholderiaceae) is the etiological agent of the disease called bacterial panicle blast of rice (Pedraza et al., 2018). The symptomatology of this disease is evidenced in the panicle of the plant, causing grain abortion which generates large economic losses in rice production (Ham et al., 2011).

Conventional agricultural practices using agrochemicals to control the disease are very effective. So far, the chemical agent capable of controlling the disease is oxolinic acid, but it has caused environmental problems and has led to changes in soil microbiota (Ham et al., 2011; Hikichi et al., 2001; Valdez et al., 2020). Furthermore, this agrochemical is banned in countries because it is considered a risk due to the formation of resistant strains and contamination of water bodies (Maedea et al., 2007; Manulis et al., 2003). For this reason, biological control has been taken as an alternative for disease control and management.

Endophytic bacteria are associated with different plant tissues without causing any disease. They are considered an important biotechnological tool for improving crop yields through their plant growth-promoting capacity via phosphate solubilization, siderophore production, nitrogen fixation and ACC deaminase production (Khan et al., 2020; Krishnamoorthy et al., 2020; Matsumoto et al., 2021; Rho et al., 2018; Wang et al., 2021). In turn, they produce secondary metabolites that inhibit pathogen growth and induce systemic resistance to their host (Álvarez et al., 2020; Ramos et al., 2020). Under this perspective, endophytic bacteria have become a very important tool in replacing the application of agrochemicals that are currently affecting the environment (Gazara et al., 2020). For this reason, the aim of this study was to evaluate in vitro the antagonistic activity of endophytic bacteria isolated from rice varieties against B. glumae etiological agent of rice panicle disease.

MATERIALS AND METHODS

Study area

Rice varieties were collected from the large experimental farm La Victoria of the Fondo Nacional del Arroz, located in the municipality of Mocarí-Córdoba-Colombia. This area is characterized by an average temperature of 29 °C, relative humidity of 80%, average annual rainfall of 1200 mm and altitude of 20 m.

Collection of plant material

A zig-zag sampling was carried out collecting 3 complete rice plants of the varieties F473, F2000 and FMocarí. The samples were labelled with their respective variety and date of collection. The samples were stored and preserved in plastic boxes at 4 °C for transport to the Microbiological Research Laboratory of the University of Sucre and processed within 24 hours after collection.

Isolation of endophytic bacteria

For the disinfection process, the methodology proposed by Cordero et al. (2010a) was followed, which consists of washing the root, stem, leaf and panicle tissues previously cut to 1 cm. After the disinfection process, each tissue was placed in a porcelain dish and macerated until a homogeneous mixture was obtained, which was then inoculated in 9 ml of peptone and left in agitation for 24 hours at a temperature of 34 °C. From the homogenate, serial dilutions were made and seeded by diffusion on R2A agar surface and incubated at 28 °C for 72 hours. In addition, B. glumae was activated on King B agar medium. The selected morphotypes were purified and maintained on R2A agar for further in vitro evaluation against B. glumae.

In vitro antimicrobial activity of endophytic bacteria against B. glumae

Filter paper discs were impregnated with 20µl of bacterial suspension and placed on King B agar medium previously inoculated with B. glumae. Once inoculated, the Petri dishes were incubated at 32°C for 3 days. Oxolinic acid was used as positive control and sterile water as negative control (Sierra et al., 2012). The percentage inhibition was calculated using the formula (Da/C) *100. Where Da corresponds to the inhibition of the bacteria in each treatment and C corresponds to the inhibition of the control. The assay was performed in triplicate. Bacterial isolates that showed inhibitory activity were selected for molecular identification (Cordero, 2013b).

Molecular identification of endophytic bacteria

For DNA extraction from endophytic bacteria and amplification of the 16S rRNA gene by PCR, the methodology proposed by Oliveira et al. (2013) was followed. The amplified products were purified and sent for sequencing to Macrogen. The sequences obtained were compared with those stored in Genbank. Base alignment was performed in the Clustal W program; phylogenetic inferences were obtained by Neighbor Joining method based on the kimura-2-parameter model with bootstrap 1,000 test in the MEGA X program.

Statistical analysis

A completely randomized design was applied for the differences between the population density (CFU/g tissue) of endophytic bacteria present in rice varieties and for the inhibitory activity of endophytic bacteria against B. glumae. Likewise, the Duncan multiple range test was used to establish significant differences (p-value < 0.05) in the percentage of inhibition of endophytic bacteria against the pathogen and between communities of endophytic bacteria (CFU/g tissue) present in the tissues of the rice varieties. Three replicates per treatment were carried out. Data were analysis in the student version of InfoStat software.

RESULTS AND DISCUSSION

A total of 148 endophytic bacteria were isolated from different rice plant tissues. The population density according to rice variety and colonized tissue (CFU/g tissue) showed significant statistical differences (p-value < 0.05). The rice variety with the highest population density was F2000 (4.65x10^2 CFU/g), followed by F473 (3.74 x102 CFU/g) and finally the variety with the lowest density was FMocarí (2.95x102 CFU/g) (figure 1).

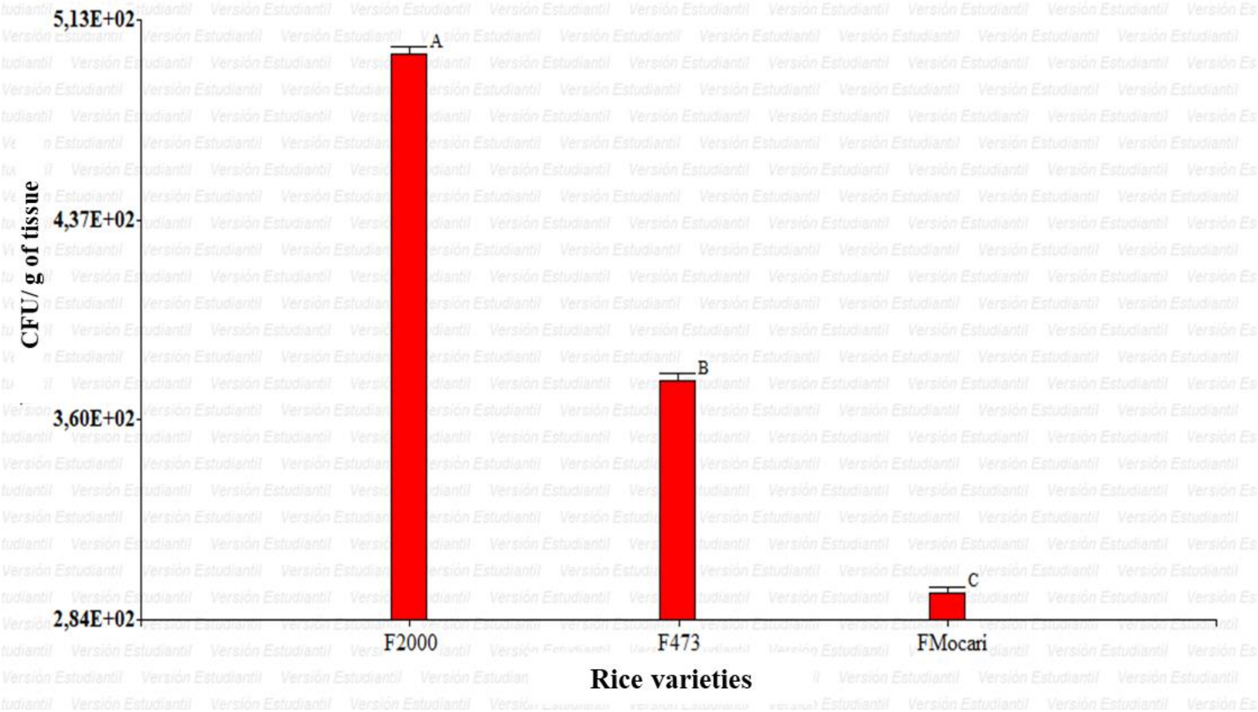

Figure 1. Population density of endophytic bacteria in CFU/g tissue of the different rice varieties. Means with a common letter are not significantly different (p-value > 0.05).

Likewise, the tissues of the rice varieties showed significant differences (p-value < 0.05), with the highest colonization being observed in the root (5.30x10^2 CFU/g tissue), followed by the stem (4.40x102 CFU/g tissue), leaf (2.95x10^2 CFU/g tissue) and panicle (2.81x10^2 CFU/g tissue) (figure 2).

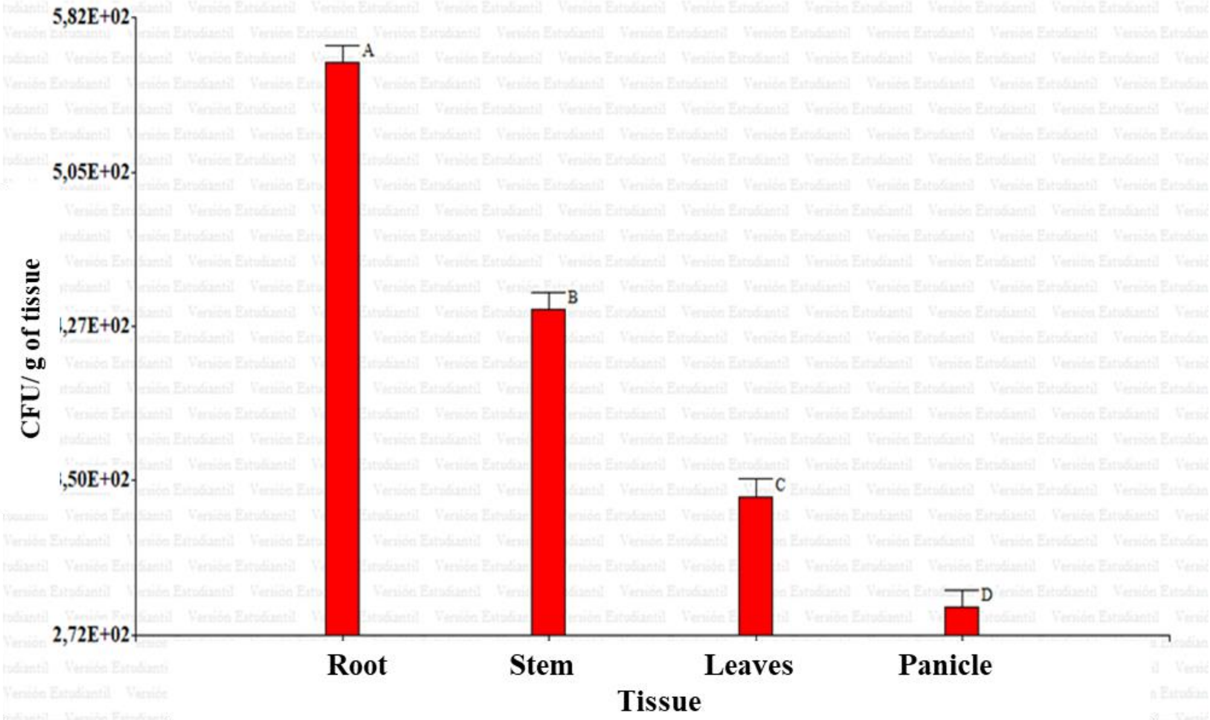

Figure 2. Population density of endophytic bacteria in CFU/g tissue as a function of the different tissues of rice varieties. Means with a common letter are not significantly different (p-value > 0.05).

The diversity of endophytic bacteria varies according to the rice plant tissue. The main reason why there is a higher colonization in the root as opposed to other plant tissues may be due to the fact that roots are in intimate contact with the environment which has a high diversity of microorganisms (Senthilkumar et al., 2011). Bacilio et al. (2001), indicated that the density is higher in the intercellular junction zone of the root epidermis, this is probably due to the space and the possibility of mobility provided by these regions, because the mucilaginous layer that is found covering the epidermis has less tension in this area. Molecular studies on bacterial diversity indicate that the root has a higher population density compared to the stem and leaves. This is due to the great diversity of bacteria in the soil and the site of entry of bacteria into the plant through this organ (Mano & Morisaki, 2008). At the same time, the concentration of carbon as an energy source is higher in this area, which favors the growth of microorganisms (Bennett & Lynch, 1981). Studies by Cordero et al. (2013b) identified high population densities of endophytic bacteria in rice tissues, and that these densities differed significantly depending on the tissue where they were located and the variety from which they were isolated.

Naik et al. (2009) determined the diversity of endophytic bacteria from rice plants at two sites in the Bhadra River area of Karnataka, southern India in two different seasons and them in vitro antagonistic properties. The results obtained were that in the winter season the plant tissues showed a colonization percentage of 40.3% for roots and 25.83% in leaves; while for the summer season the colonization percentage for roots was 20.15% and 8.6% in leaves. Streptomyces sp. showed in vitro inhibition against rice plant pathogens. This suggests that rice plants harbor in their tissues organisms that have antagonistic properties against pathogens.

In the tests of inhibitory activity of endophytic bacteria, the morphotypes H2M1LIM isolated from the variety F2000 and P4M2LIM isolated from the variety FMocarí did not present significant statistical differences (p-value > 0.05) with respect to the control; while R1M2LIM, R3M3LIM and T5M7LIM isolated from the variety F473 presented significant statistical differences (p-value < 0.05) with respect to the control and with the morphotypes of the variety F2000 and FMocarí. This inhibitory effect may be caused by the release of secondary metabolites that possibly have the ability to diffuse in the culture medium (figure 3). Likewise, these morphotypes were identified as R1M2LIM and R3M3LIM as Bacillus subtilis, T5M7LIM as Bacillus cereus, P4M2LIM and H2M1LIM as Burkholderia cepacia (figure 4).

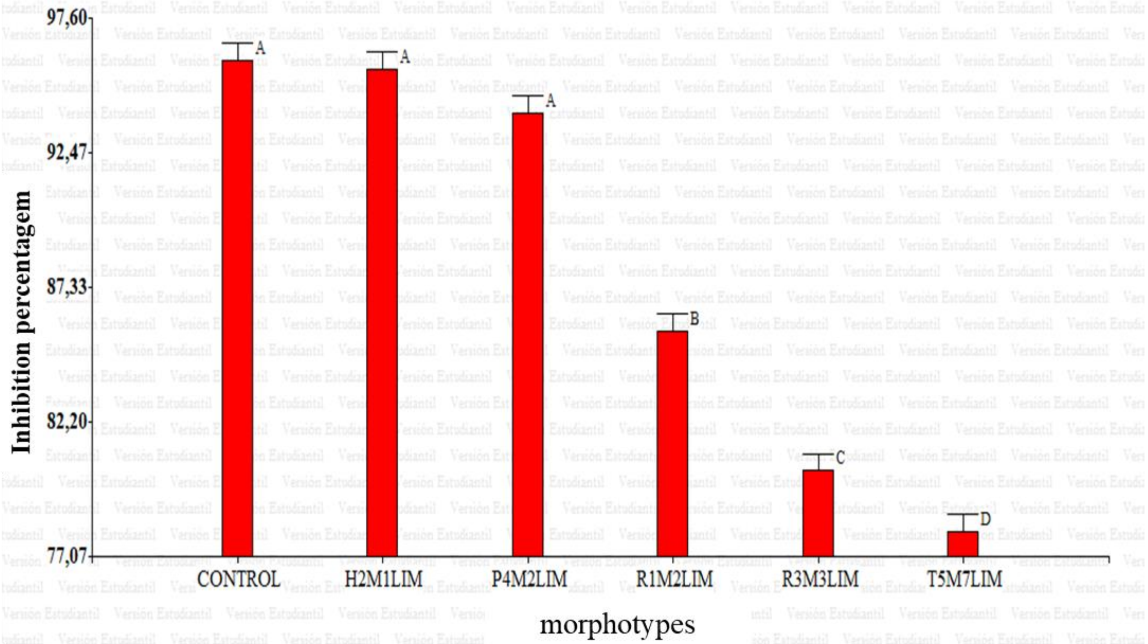

Figure 3. Percentage inhibition of endophytic bacterial morphotypes isolated from tissues of rice varieties. Means with a common letter are not significantly different (p-value > 0.05).

According to the inhibition results, the B. cepacia species isolated from leaf and panicle of the FMocarí and F2000 varieties, is characterized by antagonistic properties against plant pathogens, plant growth promoter, degradant agents of toxic substances and able to bioremediate Cadmium contaminated soils (Chiarini et al., 2006; Song et al., 2019; Zhang et al., 2019). Trujillo et al. (2007) evaluated the antagonistic activity and inhibitory effect of active metabolites produced by rhizobacteria against plant pathogens affecting maize and rice crops. The results showed that B. cepacia and P. fluorescens bacteria exhibited in vitro inhibitory effect against Curvularia sp. and Alternaria alternata through the production of secondary metabolites.

Kilani et al. (2011) reported a strain identified as B. cepacia Cs5 by 16S rDNA gene search and homology. This bacterium exhibited a broad spectrum of fungicidal activities against plant pathogens such as Alternaria alternata, Aspergillus niger, Fusarium culmorum, F. graminearum, F. oxysporum and Rhizoctonia solani. Identified in liquid and solid culture conditions.

Doncel and Pérez, (2017) isolated B. cepacia from Dioscorea sp. yam plants (Dioscoreaceae). The test results showed that this species had the ability to inhibit the fungus Colletotrichum gloesporioides in vitro by producing pyrrolnitrin which significantly affected the growth of the pathogen. Zeidan et al. (2019) explored the antifungal potential of a Qatar strain of B. cepacia. The authors reported that this species exhibited in vitro growth inhibition against Aspergillus carbonarius, Fusarium culmorum and Penicillium verrucosum in PDA medium. The findings of the present study indicate that B. cepacia is a suitable biocontrol agent against toxigenic fungi, due to the inhibitory activity of its thermostable metabolites.

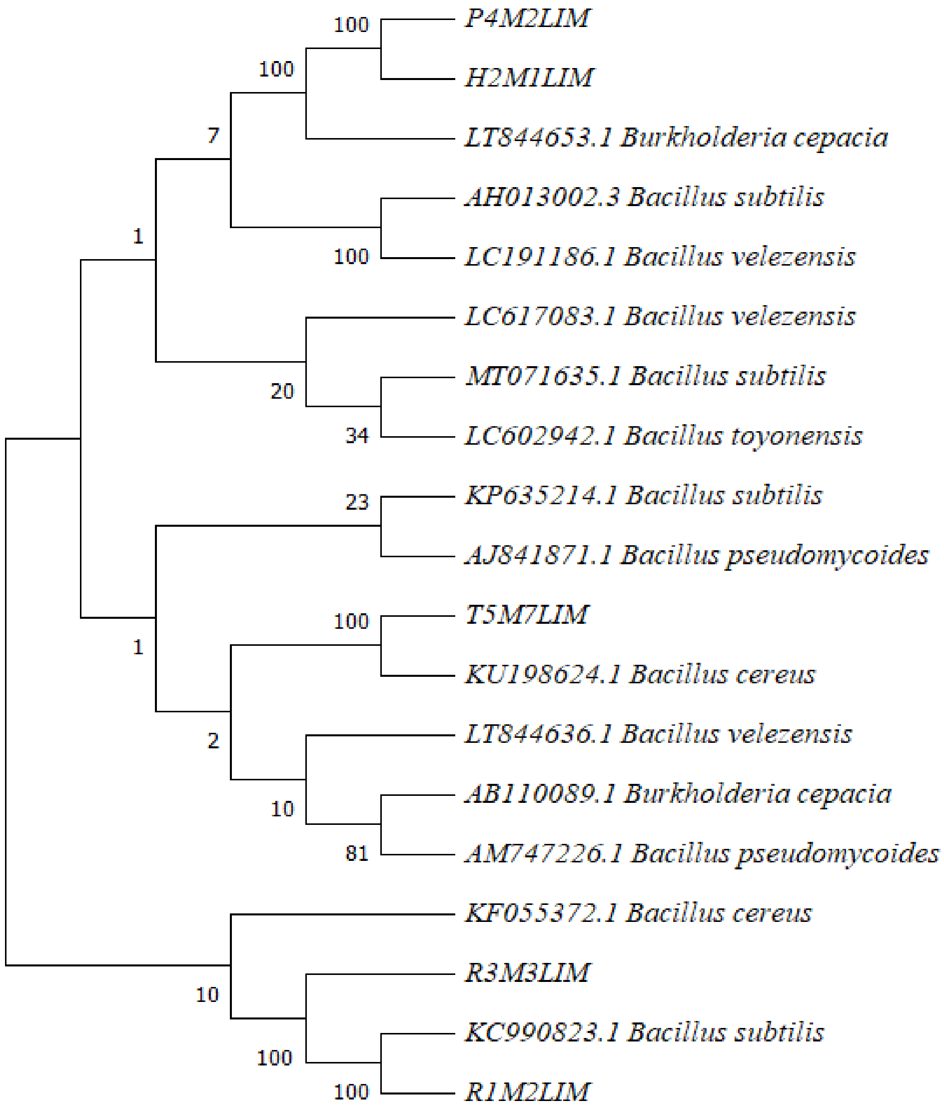

Figure 4. Dendogram of endophytic bacteria using the Neighbor-joining method from 16S rRNA gene sequences. H: leaf; P: panicle; T: stem; R: root; M: morphotypes; LIM: Microbiological Research Laboratory.

On the other hand, the genus Bacillus is one of the most studied and widely used groups as a biocontrol agent and for its ability to generate plant growth to its host (Singh & Dubey, 2018). For example, Alvarez and Sanchez, (2016) evaluated the growth of different species of the genus Bacillus in minimal salt medium. The results determined that Bacillus cereus, B. licheniformis, B. subtilis, B. pumilus presented in vitro inhibition against Fusarium sp. It is worth noting that the species B. subtilis was the species that presented the highest antagonistic capacity (79.73%PICR) and was characterized by its higher growth rate.

Ariza and Sanchez, (2012) evaluated the inhibitory activity of secondary metabolites obtained by liquid fermentation produced by B. subtilis against Fusarium sp. The metabolites produced by the bacteria were analysis by HPLC. The results showed that the metabolite identified as Iturin A has a controlling effect within a percentage of inhibition between 70% and 100% against the pathogen. Likewise, Sarti and Miyazaki, (2013) evaluated the crude extract of B. subtilis against Glycine max L. (Fabaceae), showing that the species reduced mycelial growth in vitro against Fusarium solani (50 %) and Pythium sp. (47 %) with respect to the control. In addition, it synthesized a metabolite of proteinaceous nature and others with biosurfactant capacity with inhibitory capacity.

Bacillus cereus is reported as an endophytic bacterium in rice plants Oryza sativa L. (Poaceae), which has the ability to promote plant growth (Khaskheli et al., 2020). This species is used for bioremediation processes with Cd-contaminated soils in order to decrease the metal in rice grain (Wang et al., 2019). Likewise, Etesami and Alikhan, (2017) characterized endophytic bacteria from the root of the rice plant. The results obtained by molecular identification by 16S rDNA gene were closely related B. cereus and B. mojavensis which showed in vitro inhibition against rice pathogens. In addition, this species has also been isolated from potato crops which helps in biological nitrogen fixation (Walitang et al., 2017). Recently, B. cereus has been isolated from the plant Lippia origanoides Kunth. (Verbenaceae) which promoted plant growth in vitro by producing ACC deaminase, biological nitrogen fixation and phosphate solubilization (Chamorro et al., 2020).

CONCLUSIONS

The results of this study show that rice harbors a great diversity of endophytic bacteria with great potential for the control of diseases of agricultural importance. Likewise, the application of microorganisms with high potential in the production of secondary metabolites and promotion of plant growth may become a great alternative to replace the application of agrochemicals and chemical fertilizers, which cause serious damage to ecosystems.

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Conflict of interest.

All authors made significant contributions to the paper and agree with its publication and declare that there are no conflicts of interest in this study.

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