Journal of Namibian Studies, 32 (2022): 217-225 ISSN: 2197-5523 (online)

In Vitro Antimicrobial Activity Of Secondary Metabolites Of Bacillus Cereus Against Rice Phytopathogens

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Abstract

Rice is one of the most important foodstuffs in the family food basket. In recent years, rice production has decreased due to diseases that have caused great economic losses. In order to control the diseases, agrochemicals are necessary. As an alternative to replace agrochemicals, the application of beneficial microorganisms has been chosen, which provide protection against pathogens and promote plant growth. It is necessary to search for beneficial microorganisms capable of controlling phytopathogens that cause diseases in crops, in order to be able to substitute the application of agrochemicals, which cause environmental problems. To evaluate the in vitro antagonistic activity of Bacillus cereus against Burkholderia glumae and B. plantarii. Lot1-LIM, Lor2-LIM and Lor3-LIM morphotypes isolated from Lippia organoids, which were activated and purified on R2A medium, were used. Each morphotypes was inoculated on 3s medium for 7 days for the production of secondary metabolites. After this time, the bacterial extract was concentrated and evaluated on filter paper discs in King B culture medium previously plated with the pathogens. Morphotypes were molecularly identified from the 16S rRNA gene. Analysis of variance and Duncan rank multiple testing were applied for the inhibitory activity of the morphotypes against the pathogens. According to phylogenetic analyses, the morphotypes evaluated showed 100% homology with Bacillus cereus. Furthermore, they had the ability to control pathogen growth by releasing secondary metabolites such as sulcate, geraniol and geranate. These species are characterized by the production of secondary metabolites

that control pathogen growth and in some cases promote plant growth through the release of siderophores and phosphate solubilization. The application of beneficial microorganisms with antagonistic potential may become a great alternative to substitute the application of agrochemicals in the future.

Keywords: Bacillus cereus, Burkholderia glumae, Burkholderia glumae, Performance.

1. Introduction

According to (Perfetti et al., 2013), agriculture is considered an economic activity in developed countries, providing a source of employment and income for the rural sector. In Colombia, coffee, maize and rice have been the most important productive crops in the last three decades due to the area cultivated (Agronet, 2017). Rice is the most important crop in the world; approximately 50% of the world's population depends on this grass as a staple food. Because this species is considered a staple food crop and because of its high consumption, rice is grown under different agro-ecological conditions, especially in the Caribbean region where the environment has an adequate influence on the growth and development of cultivars (Sripongpangkul et al., 2000; Tatis et al., 2011).

The proliferation of diseases caused by phytopathogens has become a limiting factor in rice production and yield, and these are mainly caused by bacteria including Burkholderia glumae and B. plantarii (Mitchell & Teh, 2005). These phytopathogens cause the disease known as rice bacterial blast, whose incidence has increased in recent years causing a decrease in production, as well as gaining tolerance to agrochemicals, the responsible entities have established agronomic zones throughout the country seeking to control the disease through integrated crop management (Beltrán et al., 2013; Quesada & García, 2014; Zhou, 2014).

Currently, the search continues for innovative biological alternatives through active ingredients that allow disease control and are environmentally friendly, which is why emphasis has been placed on the use of beneficial organisms, mainly those called endophytic microorganisms. Endophytic bacteria are defined as an important group of symbionts that live in plant tissues without causing disease symptoms (Pelaez & Londoño, 2017; Pérez et al., 2018; Porras & Bayman, 2011).

Several studies have shown that endophytic bacteria, especially the genus Bacillus promote plant growth through the production of siderophores, production of enzymes such as phosphatases and organic acids that have the ability to dispose phosphorus to forms assimilable by the plant (Ramírez & Urbano, 2014; Rojas et al., 2017; Villareal et al., 2018).

The production of secondary metabolites from endophytic bacteria has become an alternative to replace agrochemicals because they can cause environmental damage and serious consequences on human health (Montoya et al., 2014; Rojas & Bedoya, 2013; Segura, 2015). The aim of the study was to evaluate in vitro the fraction of secondary metabolites produced by B. cereus against the phytopathogenic bacteria B. glumae and B. plantarii.

2. Materials and methods

2.1. Study microorganisms. Three morphotypes isolated from the plant Lippia origanoides and the rice pathogenic bacteria B. glumae and B. plantarii were used. These are part of the strain bank collection of the Bioprospección Agropecuaria group of the University of Sucre. For the activation and purification of the bacteria, R2A culture medium was used for the morphotypes and King B medium for the pathogenic bacteria.

2.2. Liquid fermentation of the morphotypes. With the help of a previously sterile bacterial loop, a pure colony of each morphotypes was taken and inoculated into Luria-Bertani medium (10 g polypeptone, 5 g yeast extract and 5 g NaCl per litre) adjusted to pH 6.8. The culture medium was left in agitation for 24 h at a temperature of 35°C with constant stirring in order to ensure bacterial growth. After this time, 1mL was taken from each morphotypes and placed in 3s medium (30 g polypeptone, 10 g glucose, 1 g KH2PO4 and 0.5 g MgSO4*7 H20 per litre) at pH 6.8 and left in constant agitation for 7 days at 35°C (Ariza, 2012).

2.3. In vitro inhibitory activity of the morphotypes against B. glumae and B. plantarii. 100 mL of the fermented medium was taken and centrifuged at 7000 rpm for 45 min. To each filtrate 80 mL of ethyl acetate was added, then the organic fraction was collected and concentrated using a rotary evaporator. The concentrate was analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Once the bacterial extract was concentrated, its inhibitory activity was evaluated using the agar disc

diffusion technique, which consisted of impregnating sterile filter paper discs with 20µL of the concentrated extract and then inoculating them onto the surface of King B medium previously inoculated with B. glumae and B. plantarii. The boxes were incubated for 3 days at 34°C (Cuellar & Hussein, 2009). The percentage inhibition was determined using the following formula (Barraza et al., 2017):

%IB= 1-(Aai/Acc)*100 (equation 1)

Where: Aai: average growth of treatment, Acc: average growth of control test.

2.4. Molecular identification of morphotypes. Genomic DNA was performed according to the protocol proposed by Oliveira et al. (2013). Universal primers that amplify the small subunit of 16S rRNA were used. 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 1,000 bootstrap test in the MEGA X program.

Statistical analysis

Data were analyzed by one-way analysis of variance. Also, the Duncan rank multiple test was applied to establish significant statistical differences ($p<0.05$) in the percentage of inhibition of morphotypes against pathogens. The data were analyzed in the student version of the InfoStat programme, and the statistical programme R 3.4.1 was used to edit the graphs.

3. Results and discusión

From the chromatographic analysis, 42 compounds were identified of which geraniol showed an area percentage (90%), sulcatone (70%) and geranate (86%), these are possibly producing inhibition activity against the pathogens affecting the rice crop (figure 1). On the other hand, Duncan's test showed that there are no significant statistical differences in terms of percentage inhibition ($P>0.05$) (figure 2).

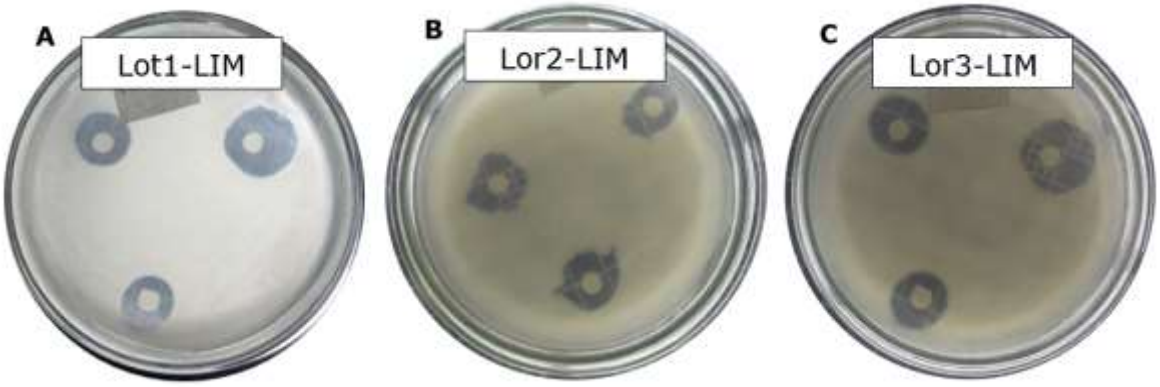

Figure 1. In vitro inhibition of (A) Lot1-LIM, (B) Lor2-LIM, (C) Lor3-LIM morphotypes against B. glumae and B. plantarii.

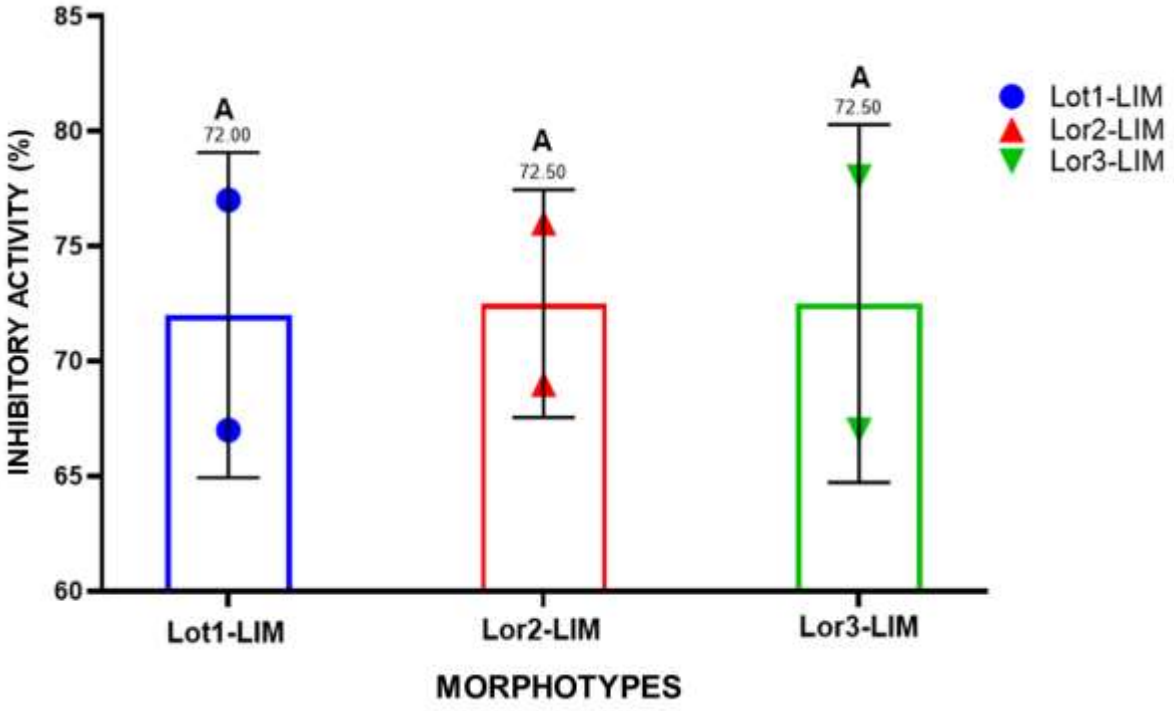

Figure 2. Percentage inhibition of morphotypes against B. glumae and B. plantarii. Letters in common are not statistically different ($p>0.05$).

According to the results of the phylogenetic analysis of the morphotypes under study, they showed similarity with the sequences stored in GenBank, which presented 100% homology with the Bacillus cereus species (figure 3).

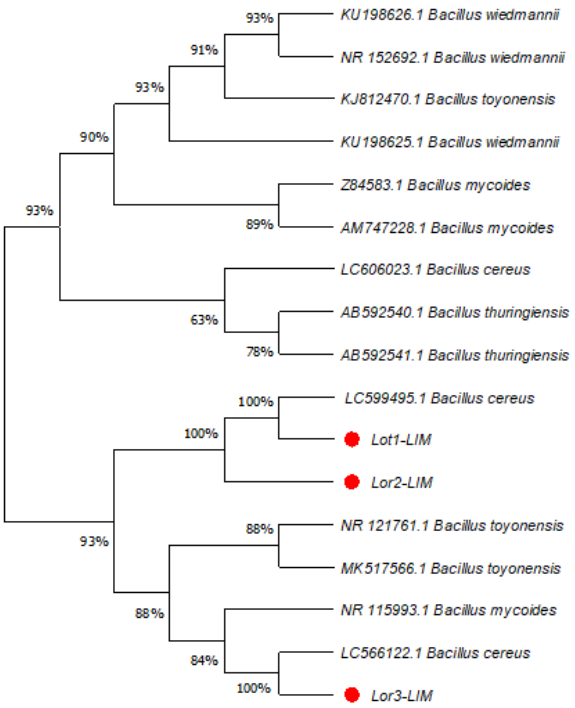

Figure 3. Sequence dendogram from the 16S rRNA gene of the morphotypes under study.

As reported by Tapia (2018), he showed that geraniol was present in the extracted oil Clinopodium pulchellum (Kunth) (Lamiaceae) which showed inhibitory activity with Candida albicans. It has been shown that many species of endophytic bacteria produce molecules that are similar to their host, suggesting that they have possibly co-evolved (Wang & Dai, 2011). For example, in this study B. cereus was isolated from Lippia spp, species belonging to this genus have a great diversity of essential oils including geraniol, geranate and sulcatone, these monoterpenes act on the cell membrane of pathogens generating an imbalance in the lipid membrane which causes an increase in the fluidity of the membrane causing a loss of potassium ions (Bard et al., 1988; Linde et al., 2016; Stashenko et al., 2014).

Lorenzi et al. (2009) reported that many of the bacterial species of clinical interest have developed some resistance to the antibiotics commonly used to inhibit them. For this reason, they performed a combination of antibiotics with geraniol to counteract growth in vitro. The results obtained were that combining the essential oil with phenylalanine arginine β-naphthylamide produced synergistic activity against

Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa and Acinetobacter baumannii.

Much of the research on microbial metabolite production has focused on the genus Bacillus. Many of the species belonging to this genus have become biotechnological tools to control pathogens that affect the yield of crops of agricultural interest and to reduce the application of chemical pesticides (Chen et al., 2020; Draz et al., 2020). This idea is confirmed by the study conducted by Ariza and Sánchez (2012), which aimed to characterize the metabolites produced by B. subtilis against Fusarium sp. The results obtained were a controlling effect against the fungus through the production of Iturin A with an inhibition percentage of 70%.

On the other hand, Zhang et al. (2020) demonstrated that B. velezensis can inhibit Alternaria solani in vitro by producing metabolites such as iturins and acetophenone which significantly affected the hyphae by means of perforation and swelling where the microbial metabolite acted. Additionally, Sidorova et al. (2020) stated that optimal metabolite production by B. subtilis is found at a pH ranging between 6.0-8.0 and at a temperature between 25°C and 30°C. They also reported chromatographic analysis of the metabolites synthesized as surfactin and Iturin A, which showed inhibition against Fusarium sp. These data can provide an indication for the manufacture of effective fungicides to protect crops from any pathogen.

4. Conclusion

In this study, secondary metabolites produced by B. cereus inhibited in vitro the plant pathogens B. glumae and B. plantarii which affect rice yield. Large-scale production of metabolites from the genus Bacillus can be considered as an alternative for the management of different diseases in the field and to replace agrochemicals.

5. Acknowledgements

The authors of this manuscript extend their thanks to the Microbiological Research Laboratory, belonging to the Bioprospección Agropecuaria research group of the University of Sucre.

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