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Antifungic Activity In Vitro Of Secondary Metabolites Of Bacillus Cereus Against Colletotrichum Gloesporioides

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Abstract

The proliferation of diseases in the avocado crop has become a limiting factor in yield and production. The anthracnose disease of the avocado crop has generated large economic losses in the agricultural sector. One of the strategies commonly used by farmers to control the disease is the application of agrochemicals, but these can contaminate the environment and many microorganisms have acquired resistance. Rhizophytic bacteria are considered as a tool to control pathogens through the production of secondary metabolites. The aim of the present study was to evaluate in vitro the concentrated fraction of secondary metabolites produced by Bacillus cereus against Colletotrichum gloesporioides. The secondary metabolites produced by Bacillus cereus against Colletotrichum gloesporioides were characterized by gas chromatography coupled to mass spectrometry (GC-MS). The metabolites extracted from Bacillus cereus showed an inhibition percentage of 48% and geraniol, geranate and sulcatone were identified, which showed in vitro antagonism against C. gloesporioides, a phytopathogen of agricultural interest in avocado crops. The production of microbial secondary metabolites may be an alternative to replace agrochemicals in the future.

Key words: Bacillus cereus, Colletotrichum gloesporioides, Disease, Geraniol,

1. Introduction

Avocado is a fruit with high potential for fresh consumption due to its high nutritional content, cosmetic and pharmaceutical application (Araujo et al., 2018) and its characteristics for agro-industrial processing. In 2018, 2.4 million tonnes of avocado products were destined for export out of 6.4 million tonnes produced worldwide, generating revenues of approximately USD $5.6 billion (Munhuweyi et al., 2020). Avocado export dynamics are dominated by Mexico, which ranks first in exports, and in South American countries, Peru and Colombia lead the business in this geographical area (Arias et al., 2018). In Colombia, the departments with the highest production of avocado in Colombia are Caldas, Antioquia, Bolívar, Cauca, Risaralda, Quindío and Tolima, the latter being the department with the highest production, contributing

approximately 84,341 tonnes (Ministry of Agriculture and Rural Development, MADR, 2019).

In mountain of María grows about 40 % of the country's avocado, and it is presumed that at least 8,000 families depend on this crop as their main activity (ICA, 2014). Avocado cultivation began in the 1990s in this area in order to shade coffee crops, with the disappearance of coffee cultivation, the avocado in this area has emerged spontaneously without the implementation of planting, pruning and fertilization techniques, becoming one of the most important crops for farmers, achieving recognition in local and international markets (Vega, 2012).

However, avocado crops are very susceptible to attack by tissue-rotting pathogenic fungi responsible for important diseases such as anthracnose, which is characterized by dark, sunken, circular, ellipsoidal lesions and becomes a limiting factor in production. Disease control has been achieved through the use of fungicides such as copper oxychloride and captafol, which are not easily removed by standard packaging procedures leading to the rejection of 11.5 to 19.4 % of the fruit destined for export (Trinidad-Angel et al., 2017) as well as the application of the fungicide prochloraz which has been restricted as a priority contaminant as a probable human carcinogen (Twizeyimana et al., 2013).

To improve avocado crop production and integrated disease management, improved planting methods are currently being implemented and the search continues for innovative biological alternatives through active ingredients that allow disease control and are environmentally friendly, for which the use of beneficial microbial inoculants has been promoted (Kumar et al., 2021). Previous studies have used organisms such as plant growth promoting bacteria (PGPR), which play an important role in increasing plant biomass, carbohydrate and protein content, photosynthetic pigments, modulation in the expression of metabolites over exogenous hormones applied to improve traits associated with low yields caused by stress (Ismail et al., 2021), generating multiple benefits in plants after inoculation through various biological mechanisms, becoming an alternative to replace agrochemicals used to counteract the management of diseases and therefore generate environmental and serious damage to human health (Toghueo, 2020).

Within the PGPR, Bacillus sp have been studied and recognized as safe organisms in food application and development of plant protection products (Obianom & Sivakumar, 2018) with great metabolic versatility that allows them to carry out biological control of pests and diseases by different mechanisms (Villarreal-Delgado et al., 2018). Studies reported by Shimshoni et al. (2020) indicate that B. cereus, B. licheniformes and B. subtilis produce antibiotics and other inhibitory substances against avocado post-harvest pathogens. Microorganisms (Glomus spp., Trichoderma spp., Bacillus spp., Pseudomonas spp.) have also been inoculated in an avocado seedling production system, reporting favorable effects of the microorganisms on seedling growth and nutrient assimilation (Sotomayor et al., 2022). The objective of this study was to evaluate in vitro the fraction of secondary metabolites produced by B. cereus against the fungus Colletotrichum gloesporioides.

2. Methods

Antagonistic strain. The strain used in this study is a rhizospheric bacterium associated with avocado and banana crops with plant

growth promoting activity molecularly identified as Bacillus cereus, which was activated and purified on nutrient agar medium.

Pathogenic fungus. The fungus used for the antifungal assays was isolated from avocado (Persea americana) cultivars from Montes de María, Department of Sucre. This phytopathogens was isolated by the Agricultural Bioprospecting Research Group at the Microbiological Research Laboratory of the University of Sucre.

Antifungal activity of Bacillus cereus against Colletotrichum gloeosporioides. The ability of the bacterium to inhibit the growth of the phytopathogenic fungus Colletotrichum gloeosporioides was tested by confrontation assays and qualitative estimation. For this, the fungus was seeded on potato dextrose agar (PDA) and allowed to grow for three days at room temperature. A combination of PDA and R2A media was prepared in a 1:1 ratio to inoculate B. cereus. After 2 days of incubation of the bacteria on PDA-R2A medium at 27°C, the plant pathogenic fungus was inoculated by the direct seeding method, punch cut from the periphery of a 10-day-old colony, each isolate of approximately 6 mm diameter growth area (Perez et al., 2011). Boxes were incubated at 30°C for 7 days and an absolute control was used without any treatment.

Liquid fermentation. A pure colony of B. cereus was taken and inoculated in Luria Bertani (LB) liquid medium and left in agitation for 48 hours at 30°C. After this time, 1 ml of bacterial growth was taken and placed in 400 ml of 3s medium. The medium was left in constant agitation for 72 hours (Ortiz-Galeana et al., 2018).

Extraction and characterization of metabolites produced by B. cereus. 100 ml of the fermented medium was taken and centrifuged at 7000 rpm for 45 min. To each filtrate 80ml 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).

Antifungal activity of the extract. To carry out the antifungal activity, the agar diffusion technique was used, as proposed by Islam et al (2012). The determination of the antifungal activity of the extract was carried out in PDA medium (20 mL) using a sterile stainless steel punch, a well was made and then 60 uL of extract of each medium and strain was added, followed by the placement of two agar blocks (0.5 cm diameter) with the pathogenic fungus at the same distance. The boxes were incubated at 28°± 3°C for 14 days and the inhibitory activity of each medium and strain was expressed as the percentage inhibition of fungal growth compared to the negative control (only ethyl acetate was inoculated into the well).

The percentage inhibition of growth was determined by the following equation:

Inhibition (%) = [(Rc - Rb)/Rc x 100],

Where Rc is the radius of the control and Rb is the radius of the fungal colony interacting with the antagonistic bacteria (Rahman, 2007). Each treatment had 5 replicates.

Extraction and characterization of metabolites produced by B. cereus. 100 ml of the fermented medium was taken and centrifuged at 7000 rpm for 45 minutes. 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).

3. Statistical analysis

A completely randomized design was applied to determine the percentage inhibition of secondary metabolites. Furthermore, Duncan's multiple range test was applied to establish the difference ($p<0.05$) in the percentage inhibition against B. cereus with respect to the control. Five replicates per treatment were performed. Data were analyzed in the free version of InfoStat software.

4. Results

B. cereus showed in vitro antifungal activity against Colletotrichum gloeosporioides with an inhibition percentage of 66% compared to the control (figure 1), possibly due to the antagonistic action regulated by antibiosis, characteristic of the species.

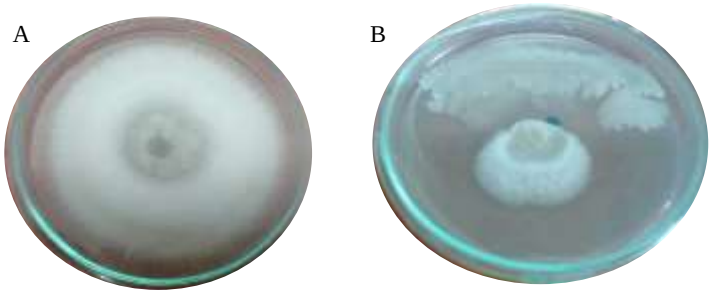

Figure 1. In vitro test of the antagonistic activity of B. cereus against C. gloeosporioides. A. Characteristics of the Colletotrichum gloeosporioides isolate (Control); B. B. cereus against C. gloeosporioides.

In the case of the metabolites produced by B. cereus (figure 2), inhibition percentages of 48% were presented, irregular growth of the phytopathogens and a change in its coloring with respect to the control were observed, indicating early cell death.

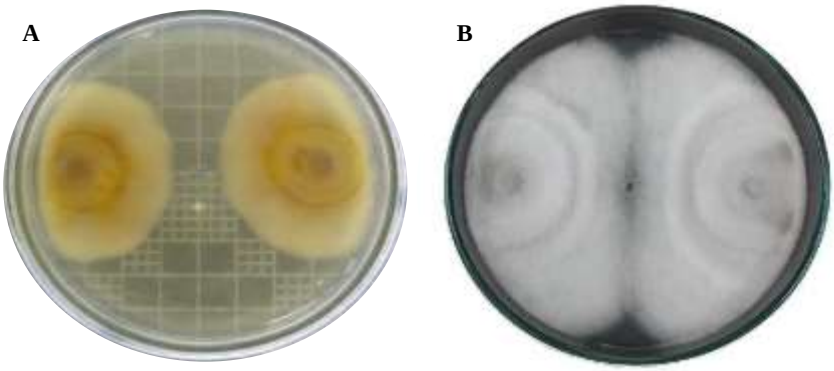

Figure 2. Growth inhibition of C. gloeosporioides. A. Metabolites produced by B. cereus; B. Control C. gloeosporioides.

On the other hand, significant statistical differences were found between the microbial metabolites with respect to the control, with B. cereus showing the highest mean inhibition of C. gloeosporioides mycelial growth with 78% (Figure 3).

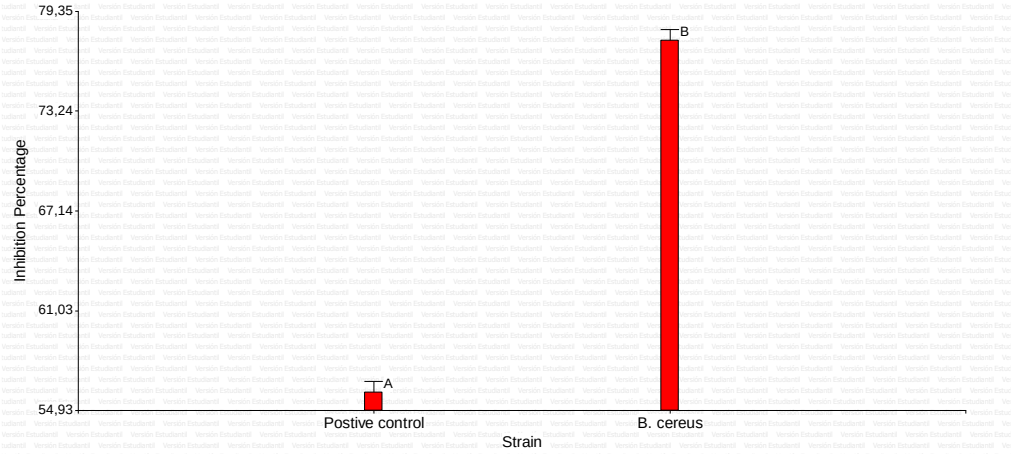

Figure 3. Percentage inhibition of microbial metabolites of B. cereus. of which geraniol showed an area percentage (30%), sulcatone (20%) and geranate (26%), these are the ones that are possibly producing the inhibitory activity against the pathogens affecting the avocado crop.

5. DISCUSSION

The genus Bacillus and its most representative species: B. subtilis, B. brevis, B. cereus, B. pumilus, B. licheniformis and B. amyloliquefaciens offer an alternative for biological control thanks to their ability to be present in the soil as they are able to produce spores that give them resistance to different conditions such as desiccation, heat, organic solvents and UV irradiation (Melnick et al., 2008; Alvarez & Sánchez, 2016). The work carried out by Santander (2012) showed that the consortium of B. subtilis and T. harzianum increased the inhibitory activity of C. gloesporioides in mango fruits (Mangifera indica L) by evaluating the percentage of inhibition and sporulation. B. cereus is also able to produce protease and chitinases enzymes with activity on the chitin component of the fungal cell wall (Wang et al., 2009), which make them suitable for use as a biological control agent for plant pathogens (Layton et al., 2011).

Most of the studies on the production of microbial metabolites have focused on the genus Bacillus, which includes four families of metabolites: bacteriocins, lantibiotics, lipopeptides and polyketides (Sansinenea & Ortiz, 2011). 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 thus reduce

the application of chemical pesticides (figure 2) (Chen et al., 2020; Daraz et al., 2021). This idea is confirmed by the production and characterization of metabolites produced by B. subtilis against Fusarium sp., the results obtained allow the detection of a controlling effect against the fungus through the production of Iturin A with an inhibition percentage of 70% (Daraz et al., 2021).

In vitro inhibitory activity of B. velezensis against Alternaria solani has been demonstrated by producing metabolites such as iturins and acetophenone, which significantly affected the hyphae by means of perforation and swelling where the microbial metabolite acted (Ariza & Sánchez, 2012). Additionally, 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. At the same time, they reported by chromatographic analysis the metabolites synthesized as surfactin and Iturin which showed inhibition against Fusarium sp. These data can give us an indication for the manufacture of effective fungicides to protect crops from any pathogen (Sidorova, et al., 2020; Jin et al., 2020; Shew et al., 2019; Kang & Hwang, 2018).

Geraniol, geranate and sulcatone are monoterpenes that act on the cell membrane of pathogens by generating an imbalance in the lipid membrane which causes an increase in membrane fluidity leading to a loss of potassium ions (Bard et al., 1988; Linde et al., 2018; Stashenko et al., 2014). Several studies have shown that geraniol is present in the extracted oil Clinopodium pulchellum (Kunth) (Lamiaceae) which showed inhibitory activity with Candida albicans (Tapia Manrique, 2018). Likewise, geraniol presented an inhibitory effect against the formation of pathogenic fungal biofilms and hyphal morphogenesis, destroying the cell wall by regulating the low ATpase activity of the plasma membrane and reducing ergosterol levels (Singh et al., 2016).

Conclusions

In this study, secondary metabolites produced by B. cereus inhibited in vitro the phytopathogenic fungus Colletotrichum gloesporioides which affects avocado crop yield. The 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 as a substitute for agrochemicals.

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