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ANTIFUNGAL ACTIVITYOF MICROBIAL METABOLITES AGAINST *Colletotrichum gloesporioides*

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ABSTRACT
The objective of the present study was to evaluate *in vitro* the concentrated fraction of secondary metabolites produced by the *Bacillus* bacteria *cereus* against the phytopathogenic fungus *Colletotrichum gloesporioides*. Using the agar diffusion technique and the characterization of secondary metabolites using gas chromatography coupled to mass spectrometry (GC–MS) technique. **Results:** The microbial metabolites extracted from *Bacillus cereus* presented a 48% inhibition percentage *and* geraniol, geranate and sulcatone were identified, which presented *in vitro antagonism* against *C. gloesporioides* phytopathogen of agricultural interest at regional, national and international levels. **Conclusion.** The production of microbial secondary metabolites in the future may be an environmentally friendly biological alternative to replace agrochemicals.
Keywords: Bacteria, microbial extract, inhibition, phytopathogen.

1. INTRODUCTION

According to Whitelaw et al., (2007), the genus Colletotrichum causes diseases in virtually all agricultural crops in the world. The typical symptoms of infection by Colletotrichum are called 'anthracnose', which is characterized by necrotic sinking of the tissue where masses of conidia are produced within an acervulus (Freeman et al., 2000). Anthracnose occurs in developing and mature plant tissues, affecting fruits during their development in the field, as well as mature fruits during storage (Prusky et al., 2000).
However, avocado crops are very susceptible to the attack of pathogenic fungi that rot tissues responsible for important diseases such as anthracnose, characterized by dark, sunken, circular, ellipsoidal lesions, becoming a limiting factor in production. The disease has been controlled through the use of fungicides such as copper oxychloride and captafol, which are not easily eliminated by standard packaging procedures, leading to the rejection of 11.5 to 19.4% of the fruit destined for export (Trinidad-Ángel*et a*., 2017), as well as the application of the fungicide prochloraz, which has been restricted for being a priority contaminant as a probable human carcinogen (Twizeyimana *et al* ., 2013).

For this, 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, modulating the expression of metabolites, outperforming exogenous hormones applied to improve characteristics 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 disease management and consequently cause environmental and serious damage to human health (Toghueo , 2020) . Within the PGPR, *Bacillus have* been studied *sp* recognized as safe organisms in food application and development of phytosanitary products (Obianom&Sivakumar, 2018) with great metabolic versatility that allows it 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 post-harvest pathogens of avocado. Likewise, microorganisms (*Glomus spp* ,*Trichoderma spp* ., *Bacillus spp* ., *Pseudomonas spp* .) in an avocado seedling production system reporting favorable effects of 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 Colletotrichumgloesporioides* .

2. MATERIALS AND METHODS

Antagonist 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 tests was isolated from avocado crops (*Perseaamericana*) in Montes de María, Department of Sucre. This phytopathogen was isolated by the Agricultural Bioprospecting Research Group in the Microbiological Research Laboratory of the University of Sucre.

Inhibitory activity of *bacterial extracts* against *Colletotrichum gloeosporioides.* The ability of the bacteria to inhibit the growth of the phytopathogenic fungus *Colletotrichum gloeosporioides*, was carried out using confrontation tests and qualitative estimation.For this purpose,the fungus was sown on potato dextrose agar (PDA) and allowed to grow for three days at room temperature. A combination of PDA and R_{2A} media was prepared in a 1:1 ratio to inoculate *B. cereus*. After 2 days of incubation of the bacteria in the PDA-R_2 A medium at 27 ° C, the phytopathogenic fungus was inoculated by the direct sowing method. Each isolate was cut with a punch from the periphery of a 10-day-old colony, approximately 6 mm in diameter in growth area (Pérez *et al.,* 2011). The dishes were incubated at 30 ° C for 7 days and absolute control was used without any type of treatment.

Liquid fermentation. *B. cereus* was taken,which was inoculated in Luria Bertani (LB) liquid medium and left shaking 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 under constant shaking 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 minutes. 80 ml of ethyl acetate was added to each filtrate, 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, according to that proposed by Islam et al (2012). The determination of the antifungal activity of the extract was carried out in PDA medium (20 mL) with the help of a sterile stainless-steel punch. A well was made and then 60 uL of extract from each medium and strain was added, followed by the placement of two agar blocks (0.5 cm in diameter) with the pathogenic fungus at the same distance. The dishes were incubated at 28° ± 3° C for 14 days and the inhibitory activity of each medium and strain was expressed as the percentage of inhibition of fungal growth, compared to the negative control (only ethyl acetate was inoculated in the well).

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

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

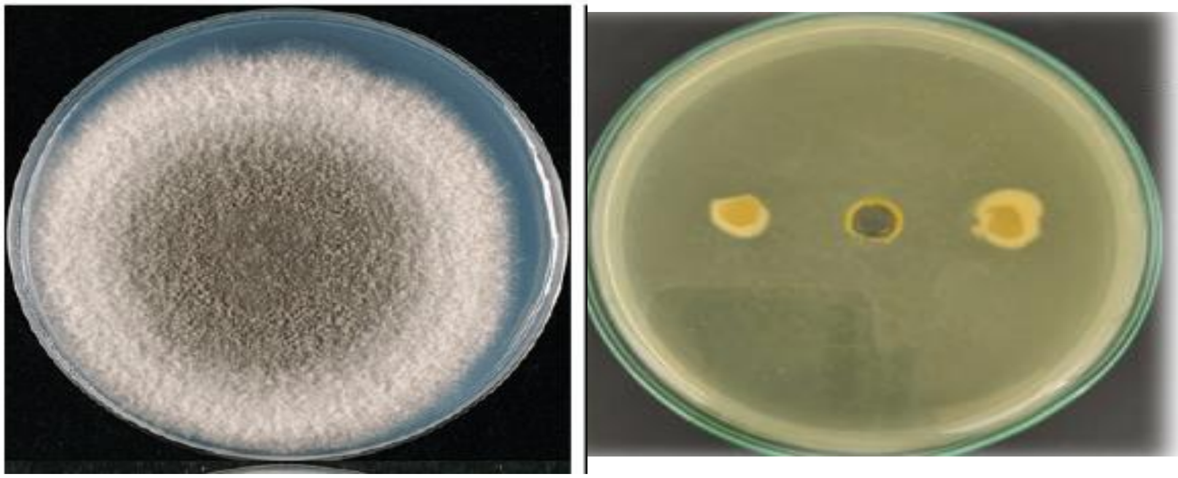
Where Rc is the radius of the control and Rb is the radius of the fungal colony interacting with the antagonistic bacteria (Rahman, 2007). Each treatment had 5 replicates.

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).

Statistical analysis. A completely randomized design was applied to determine the percentage inhibition of secondary metabolites. Also, Duncan's multiple range test was applied to establish the difference ($p<0.05$) in the percentage of 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.

3. RESULTS AND DISCUSSION

Figure1 shows the inhibitory activity of Bacillus cereus cell suspension against *Colletotrichum gloeosporioides* with an inhibition percentage of 86,19% with respect to the control (figure 1), possibly due to the antagonistic action regulated by antibiosis, characteristic of the bacterial strain against pathogens.



Colletotrichumgloeosporioides (Control) **Bacteria extractfrom*Bacilluscereus***

Figure 1. In vitro, test of the inhibitory activity of bacterial extracts of B. cereus against C. gloeosporioides.

Figure 2 shows the inhibition efficiency of 15 treatments of secondary metabolite-type microbial extracts produced by B. cereus. The inhibition ranges of the secondary metabolite-type extracts ranged from 19.41 ± 86.19 %, showing irregular growth of the plant pathogen (*C. gloeosporioides*). It was also observed that the lower the concentration of the microbial extract (5.0 %), the lower the percentage of fungal inhibition (19.41 %), compared to the treatments with concentration (80 %), the higher the fungal inhibition (80.09, 86.14 and 86.19 %) (Figure 3).

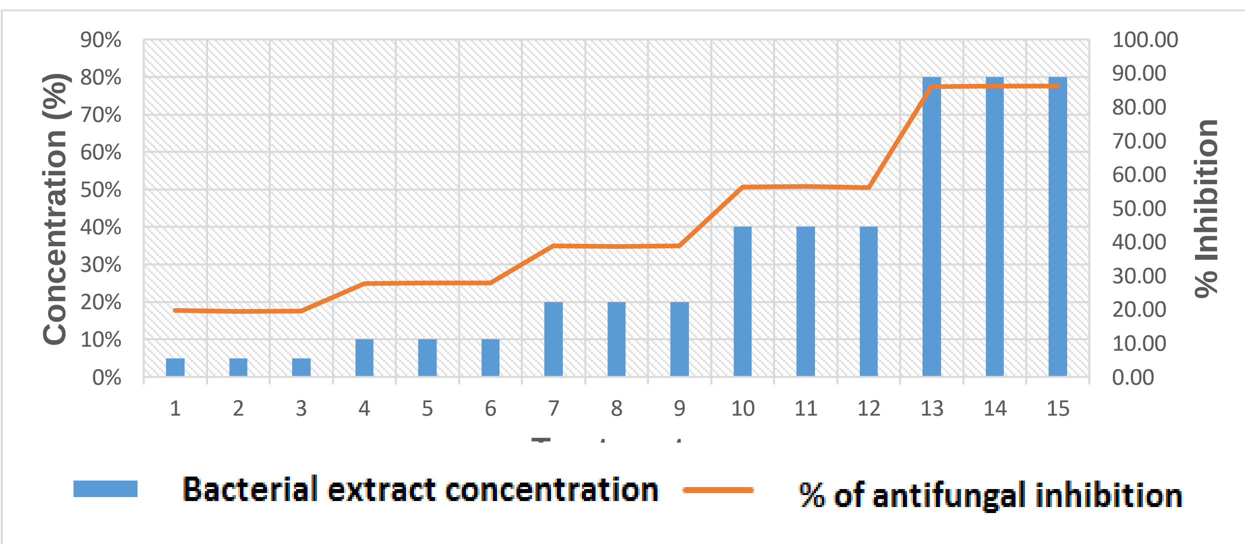


Figure 2. Percentage inhibition of secondary metabolite-like microbial extracts of Bacillus cereus against *Colletotrichum gloesporioides.*

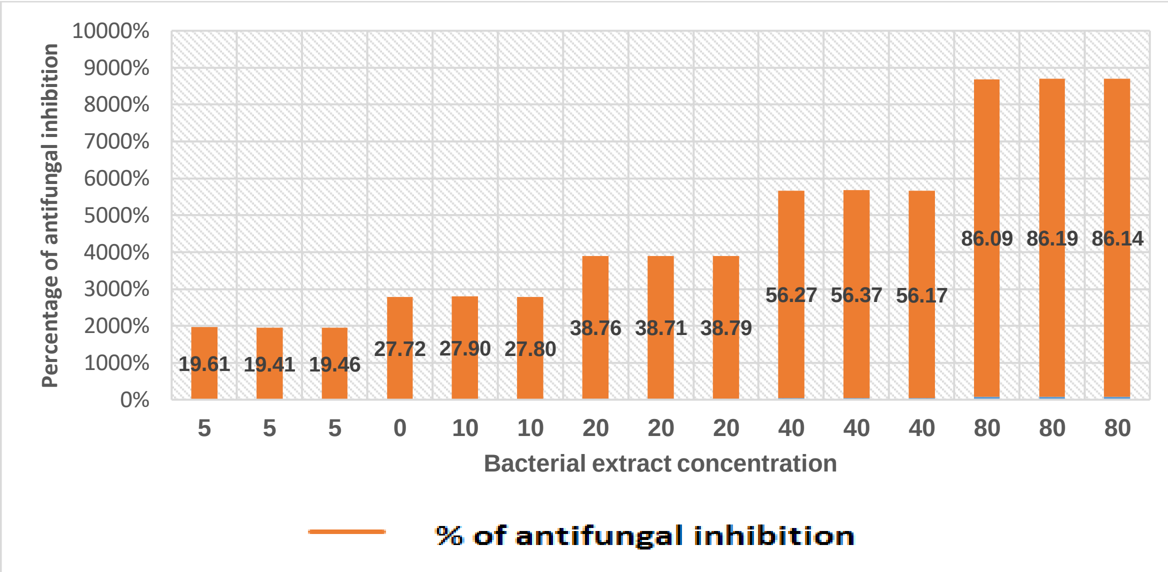


Figure 3. Percentage antifungal inhibition of different concentrations of secondary metabolite-type microbial extracts of Bacillus cereus against *Colletotrichum gloesporioides.*

The *Bacillus cereus* group contains closely related bacteria exhibiting a broad range of properties associated with their diverse lifestyles (Jensen et al., 2003). Some members have known biotechnological applications, while others may pose a potential health risk (Ehling-Schulz et al., 2019).

According to the results of the analysis of the bacterial extracts from Bacillus cereus by chromatography technique, 42 compounds were identified, of which geraniol showed an area percentage (30%), sulcatone (20%) and geranate (26%), these are the ones that are possibly producing the inhibition activity against pathogens such as *Colletotrichum gloesporioides*.

The biological control activity exhibited by Bacillus strains is possibly often due to the production of volatile organic compounds (VOCs), which can directly protect plants against plant pathogens and/or indirectly through the induction of plant resistance (Lim et al., 2017; Xing et al., 2018). These compounds were chemically classified into groups of esters, alkanes, alkenes, alkynes, alcohols, terpenoids, aldehydes, ketones and sulphur-containing compounds (Avalos, 2018). Previous studies have illustrated the antimicrobial activity of violacein produced by Bacillus cereus against various pathogens (Im et al., 2017; Cauz et al., 2019).

The genus Bacillus and its most representative species: *B. subtilis*, *B. brevis*, *B. cereus*, *B. pumilus*, *B. licheniformis* and *B. amyloliquefaciens* offer an alternative 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 (*Mangiferaindica L*) by evaluating the percentage of inhibition and sporulation. B. cereus is also able to produce protease and chitinase 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).

Sansinenea & Ortiz, (2011), report that part of the studies on the production of microbial metabolites have emphasized the genus Bacillus, within the metabolites there are four families: bacteriocins, lantibiotics, lipopeptides and polyketides. Many of the species belonging to this genus have become biotechnological tools in order to control pathogens that affect the yield of crops of agricultural interest and also to reduce the application of chemical pesticides (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).

According to Ariza& Sánchez, (2012), the bacterial phylotype identified as Bacillus velezensis has demonstrated the ability to inhibit Alternariasolani in vitro by producing metabolites such as iturins and acetophenone, which significantly affected the hyphae by means of perforations and swelling where the microbial metabolite acted. In addition, optimal metabolite production by B. subtilis is found at a pH between 6.0-8.0 and at a temperature between 25°C and 30°C. The results presented by Sidorova, et al., (2020); Jin et al., (2020); Shew et al., (2019); Kang & Hwang, (2018) according to the chromatographic analysis reports the synthesized metabolites as surfactin and Iturin which showed inhibition against Fusarium sp. This data can give us an indication for the manufacture of effective fungicides to protect crops from any pathogen.

According to studies by Bard et al., (1988); Stashenko et al., (2014); Linde et al., (2018), geraniol, geranate and sulcatone are monoterpenes that 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. On the other hand, Singh et al, (2016). Several studies have shown that geraniol is present in the extracted oil Clinopodiumpulchellum (Kunth) (Lamiaceae) which showed inhibitory activity with Candida albicans (Tapia Manrique, 2018), as well as an inhibitory effect against the formation of pathogenic fungal biofilms and hyphal morphogenesis, destroying the cell wall by regulating the low ATPase activity on plasma membrane and reducing ergosterol levels.

4. CONCLUSIONS

In this study, secondary metabolites produced by Bacillus cereus inhibited Colletotrichum gloesporioides, which affect avocado crop yield, in the range of 19,41 ± 86,19% in vitro. The large-scale production of metabolites from the genus Bacillus can be considered a biological alternative for the management of different diseases in the field and the environmental sustainability of agricultural ecosystems.

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6. AUTHOR CONTRIBUTION. Alexander Perez Cordero: experiment execution, data analysis.Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. Allauthors havereadandapprovedthemanuscript.

7. CONFLICTOFINTEREST.Alltheauthorsofthemanuscriptdeclarethattheyhavenoconflictofinterest

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