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Antifungal Activity of Secondary Metabolites Produced by *Burkholderia cepacia* Against *Colletotrichum gloesporioides*

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Abstract

Avocado is a fruit with numerous benefits for the pharmaceutical and agro-industrial sectors. In recent years, anthracnose caused by *Colletotrichum gloesporioides* has significantly affected the crop, leading to reduced production and yield. Chemical control methods have been effective however have resulted in the development of resistant strains. This study aimed to evaluate the inhibitory activity of secondary metabolites produced by *Burkholderia cepacia* against *C. gloesporioides* and its potential to promote plant growth *in vitro*. Various culture media with different carbon sources were tested to optimize the production of *B. cepacia* metabolites and assess their activity against *C. gloesporioides* on PDA-R2A culture medium. Additionally, the promotion of plant growth was evaluated through the production of siderophores, phosphate solubilization, and nitrogen fixation. The results demonstrated that the rhizospheric bacterium *B. cepacia*, isolated from avocado cultivar soils, exhibited antagonistic effects on the growth of *C. gloesporioides*. For all four media tested whey, glycerol, honey and molasses, significant statistical differences ($p<0.05$) were observed. The culture medium supplemented with molasses exhibited the highest antifungal activity (77.56%) against the pathogen *in vitro*. The GC-MS analysis identified pyrrole [12-a] pyrazine-14-dione hexahydro-3-(2-methylpropyl), a compound capable of deteriorating the cell wall of the pathogen. Furthermore, the bacterium showed the ability to produce siderophores, solubilize phosphate, and fix nitrogen *in vitro*. This study highlights the versatility of *B. cepacia* in controlling pathogen growth *in vitro* and suggests its application could protect crops against diseases by producing volatile secondary metabolites and promoting plant growth, thus reducing the need for chemical fertilizers.

Keywords – avocado – phytopathogenic fungus – rhizospheric inhibition – secondary metabolites

Introduction

Avocado is an important crop cultivated in tropical and subtropical regions worldwide (Bill et al. 2014). Its popularity has surged in recent years, mainly due to it is rich in monounsaturated fats, minerals, fiber, and antioxidants (Pedreschi et al. 2019). Additionally, avocado is used in traditional medicine for treating cardiovascular diseases, controlling blood pressure, and for its high

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antimicrobial potential against pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Jesus et al. 2015). Persea, the genus to which avocado belongs, has around 150 species. However, only three main species are commonly cultivated and consumed: *Persea americana* var. americana (Antillean avocado), *Persea americana* var. drymifolia (Mexican avocado), and *Persea americana* var. guatemalensis (Guatemalan avocado). These varieties are key to global avocado production (Hurtado et al 2018). In Colombia, about 75% of the land used for avocado cultivation is dedicated to Creole or Antillean varieties. The regions of Antioquia, Bolívar, Caldas, Cauca, Risaralda, Tolima and Quindío account for 86% of the country's total production (MADR 2020).

However, Avocado production in the country faces several challenges, including anthracnose disease caused by *Colletotrichum gloesporioides*, which can lead to up to 50% loss of production due to fruit abortion and postharvest rot (Gañán et al. 2015, Kimaru et al. 2020). The disease manifests as dark, sunken necrotic lesions with large orange conidial masses, and affected fruits develop brown or black spots that expand, leading to rot, thus limiting marketability and export (Fuentes et al. 2020, Moral et al. 2021). Chemical fungicides have been employed to manage anthracnose, but their use has raised environmental concerns and the development of resistant strains (Lobo et al. 2000).

To address these concerns, the use of rhizospheric microorganisms capable of inhibiting *Colletotrichum gloesporioides* growth has been proposed as a control strategy. Among these, *B. cepacia* is recognized for promoting plant growth, competing for nutrients in the rhizosphere, and releasing iron-chelating substances (Elshafie & Camele 2021). *Burkholderia* species demonstrate plant growth-promoting properties, including the synthesis of indole acetic acid (IAA) phosphate solubilization, and siderophore production (Pandey et al. 2005). Moreover, *Burkholderia* species possess a strong capacity for biological control against phytopathogens through the release of secondary metabolites, including bacteriocins, alkaloids, and polypeptides (Elshafie & Camele 2021).

In vitro studies have highlighted the potential of *Burkholderia cepacia* in controlling the growth of *Fusarium oxysporum* by releasing enzymes, such as endoglucanases and chitinases that degrade the pathogen's cell wall (Galeano et al. 2020). Additionally, *B. cepacia* has shown antifungal activity against *Fusarium culmorum*, *Aspergillus carbonarius,* and *Penicillium verrucosum*, causing mycelial malformations in these pathogens (Zeidan et al. 2019). Due to its biotechnological potential as a source of antibiotics and bioactive secondary metabolites, *B. cepacia* can be utilized in agriculture to control diseases and reduce the environmental impact caused by agrochemical use. (Depoorter et al. 2016, Kunakom & Eustáquio 2019).

Materials & Methods

Antagonist strain and Pathogenic fungus

The *Burkholderia cepacia* strain was isolated from avocado cultivation soils in the municipalities of Chalán and Ovejas, located in the Montes de María region, Sucre Department, soil samples were sieved to separate the roots, after which a homogenization process was carried out. Serial dilutions were prepared from each sample and plated on R2A agar using the diffusion technique, followed by incubation at 28°C for 72 hours. Bacterial density was estimated by direct colony counting, observing and selecting colonies based on shape, surface characteristics, color, and size. The selected morphotypes were purified and maintained on R2A agar for subsequent *in vitro* evaluation.

Isolates of the fungus *Colletotrichum gloeosporioides* were obtained from avocado fruits in the same region. To isolate the fungus, small samples of cladodes showing anthracnose symptoms were plated on Petri dishes containing potato dextrose agar (PDA). Once the first colony was obtained, subculturing was performed on new Petri dishes with PDA to obtain pure colonies for further analysis.

In vitro Analysis of Burkholderia cepacia Antagonistic Interaction with Colletotrichum gloeosporioides

A pure colony of *B. cepacia* was inoculated onto one end of a PDA-R2A culture medium using a sterile bacterial loop. After 24 hours of incubation at 32°C, a 0.5 cm block of mycelium of seven-day-old with four days of growth was inoculated at the opposite end. The dishes were incubated at 32°C for 14 days to observe the inhibition effect of the bacterium on the fungus (Palaniyandi et al. 2011, Doncel & Pérez 2017).

Isolation and characterization of microbial metabolites produced by *Burkholderia cepacia* against *Colletotrichum gloesporioides*.

The process was carried out in bioreactors supplemented with four different media, each containing distinct carbon sources. The media used were as follows: M1 (glycerol, nutrient broth, Na_2HPO_4, KH_2PO_4), M2 (nutrient broth, honey, Na_2HPO_4, KH_2PO_4), M3 (nutrient broth, Na_2HPO_4, KH_2PO_4, 1.0 g/L whey), and M4 (nutrient broth, Na_2HPO_4, KH_2PO_4, 1.0 g/L molasses). To each medium, 1,000 µL of the liquid bacterial culture was added. Each bioreactor was agitated at 150 rpm for 7 days (Cho et al. 2007, Doncel & Pérez 2017). From each medium, 100 mL were taken and centrifuged at 7,000 rpm for 45 minutes. The supernatant was collected and carefully filtered using 0.2 µm acrodiscs. The filtered culture was then mixed with 80 mL of ethyl acetate and agitated at 150 rpm for 15 minutes to ensure homogenization (Cho et al. 2007, Parra et al. 2009). After this, the mixture was separated, and the organic phase was recovered and concentrated using a rotary evaporator. The concentrate was preserved in microtubes at -20°C for future analysis using gas chromatography-mass spectrometry (GC-MS).

Inhibition assay of microbial secondary metabolites of *Burkholderia cepacia* against *Colletotrichum gloeosporioides*.

A well was drilled in the PDA medium, which had been previously inoculated with the fungus, and 60 µL of the concentrate from each medium was added. Ethyl acetate was used as a negative control. The plates were incubated at 32°C for 7 days. The inhibitory activity of each medium was calculated using the formula proposed by Rahman et al. 2007.

$$\% \, Inhibition = \frac{Rc - Rb}{Rc} \, X \, 100$$

where *Rc* represents the radius of the control colony and *Rb* denotes the radius of the fungal colony in the presence of the antagonistic bacterium. Each treatment was tested in five replicates. The presence of inhibition zones signifies that the bacteria exhibit positive inhibitory activity against the pathogen.

Biological nitrogen fixation, phosphate solubilization and siderophore production

Bacteria were directly seeded onto selective BURK agar medium and incubated at 30°C for 7 days. A positive result was indicated by the presence of bacterial growth (Park et al. 2005). The ability to solubilize phosphate was determined using SRS medium (Sundara-Rao & Sinha 1963). The bacteria were incubated for 3 days at 30°C. A color change from pink to yellow was considered a positive indication of phosphate solubilization activity. Qualitative assessment of siderophore production was carried out using chromium azurol-S (CAS) agar medium (Schwyn & Neilands 1987).

Statistical analysis

A completely randomized design was used to evaluate the inhibitory activity of *Burkholderia cepacia* against *Colletotrichum gloeosporioides*. Statistical differences ($p < 0.05$) in inhibition percentages across culture media were determined using Duncan's multiple range test. Data were analyzed with the student version of InfoStat.

Results

The results demonstrate that the rhizopheric bacterium *Burkholderia cepacia* isolated from avocado cultivar soils has an antagonistic effect on the growth of the pathogen *Colletotrichum gloeosporioides* (fig. 1).

Fig. 1 – A *In vitro* growth control of *C. gloesporioides* by *B. cepacia* on PDA-R2A medium. B growth of *C. gloesporioides* on PDA medium.

The analysis of secondary metabolite production by *Burkholderia cepacia* in media supplemented with different carbon sources showed statistically significant differences ($p < 0.05$) (Fig. 2). The medium supplemented with molasses exhibited the highest percentage of antifungal activity (77.56%) against the pathogen *in vitro* (Fig. 3). Furthermore, *Burkholderia cepacia* promoted plant growth *in vitro* by producing siderophores, solubilizing phosphate, and fixing nitrogen, highlighting its potential for improving crop yields (Fig. 4). The ability of *B. cepacia* to promote plant growth through nitrogen fixation, phosphate solubilization, and siderophore production makes it a promising candidate for enhancing agricultural productivity.

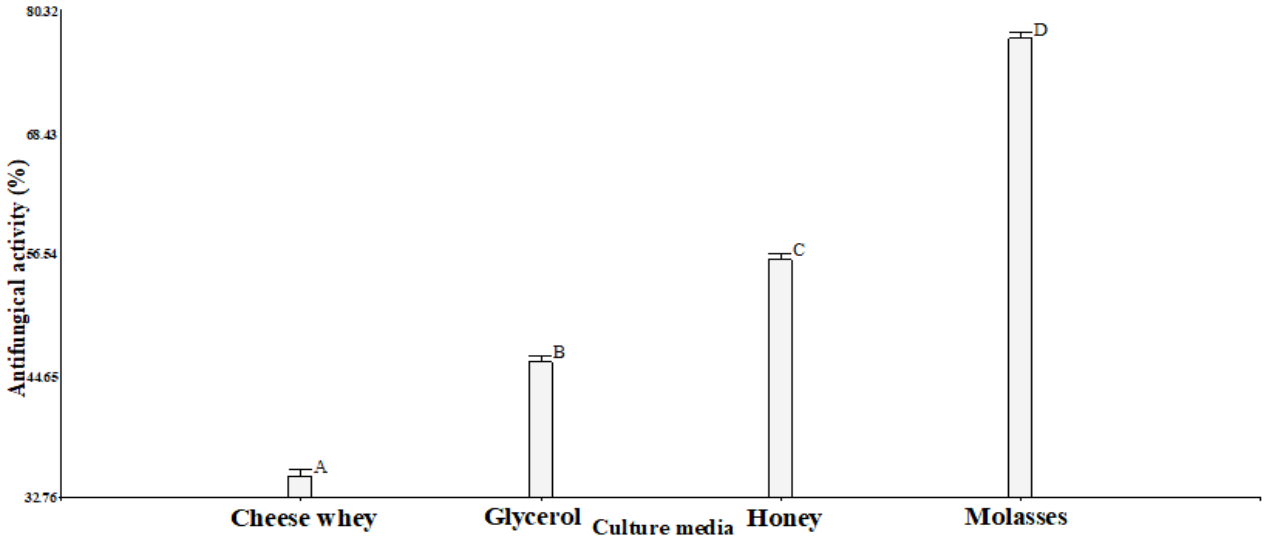

Fig. 2 – Percentage of antifungal activity of culture media with different carbon sources. Means with a common letter are not significantly different ($p>0.05$).

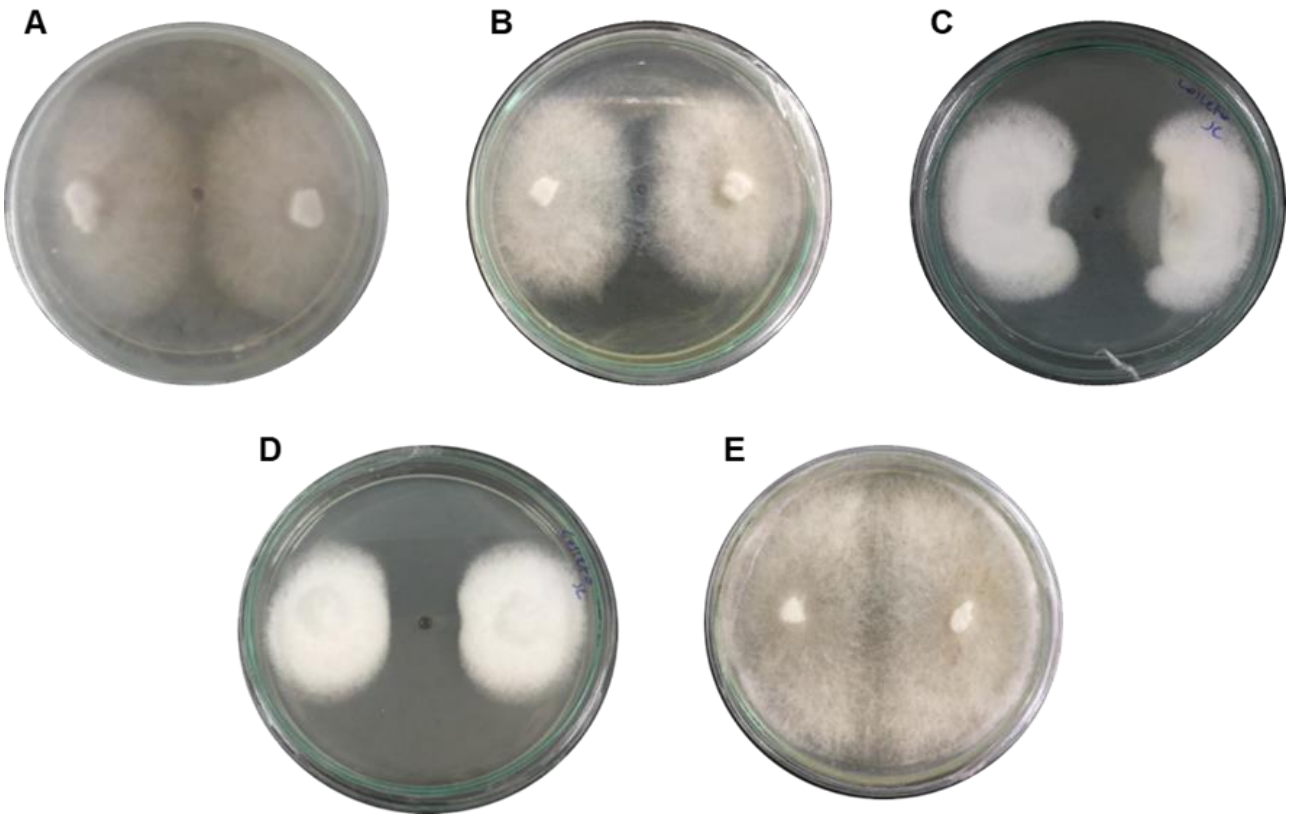

Fig. 3 – Antifungal activity of concentrated extracts of the different culture media for the production of secondary metabolites of *B. cepacia* against *C. gloesporioides*. A medium supplemented with whey. B medium supplemented with glycerol. C medium supplemented with honey. D medium supplemented with molasses. E negative control with ethyl acetate.

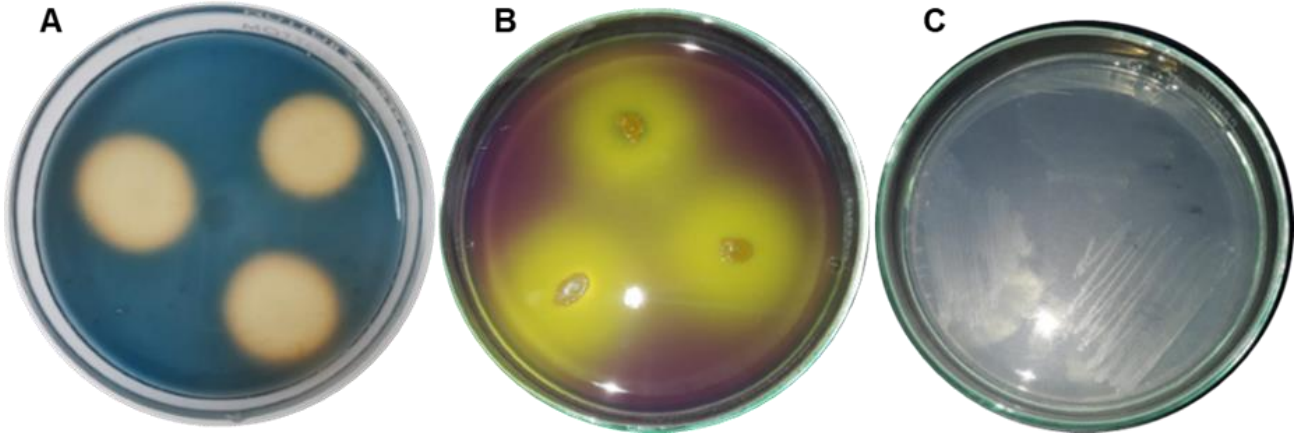

Fig. 4 – *In vitro* plant growth promotion of *B. cepacian.* A siderophore production. B phosphate solubilization. C nitrogen fixation.

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified the compound pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), with a 90.5% probability in the medium supplemented with molasses (M4). In the medium supplemented with honey (M2), the compound showed a 70.7% probability, 67.9% in glycerol (M1), and 30.5% in whey (M3), as compared to the NIST library. This metabolite is likely one of the key agents responsible for inhibiting the growth of *C. gloeosporioides*.

Discussion

Burkholderia cepacia is recognized as a valuable biotechnological tool due to its various mechanisms for controlling pathogen growth, including the release of volatile secondary metabolites and enzymes that degrade pathogen cell walls. This aligns with findings by Toledo et al. 2002, who reported a 73.68% inhibition of *Fusarium* species growth *in vitro* by *B. cepacia*, along with a 90% reduction of pathogen growth in *Gladiolus* plants. Similarly, Parra et al. 2009 isolated four *B. cepacia* strains (SR2, ER3, LG12, and N10) that inhibited *Trichoderma viride* and fully suppressed the growth of *Fusarium moniliforme*, *Fusarium solani*, *Aspergillus niger*, and *Penicillium expansum*.

Kiliani-Feki & Jaoua 2011 identified the *B. cepacia* Cs5 strain through 16S rDNA analysis, showing inhibition against *Botrytis cinerea* and protecting *Vitis vinifera* plants through secondary metabolite production and siderophore release. Galeano et al. 2020 further demonstrated the efficacy of *B. cepacia* in inhibiting *Fusarium oxysporum in vitro* by producing chitinases and endoglucanases. The antibiotic mechanisms of *B. cepacia* also contribute to its pathogen control capabilities. Rahman et al. 2007 attributed its antifungal activity to the production of pyrrolnitrin, which disrupts cytoplasmic integrity, leading to irregular mycelial growth.

Melo et al. 2013 identified the endophytic fungus *Mortierella alpina* using 18S rRNA, which exhibited strong antioxidant and antagonistic activity against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. The antibiotics pyrrolo[1,2-a]pyrazin-1,4-dione derivatives were responsible for pathogen inhibition. Similarly, Manimaran et al. 2017 isolated a *Streptomyces* species strain from the Pichavaram mangroves in India, which produced the same pyrrolo[1,2-a]pyrazine compound with antibacterial activity against *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella paratyphi*, and *Bacillus cereus*.

Metabolite production can be influenced by the composition of the culture medium. Factors such as pH and the availability of nitrogen and carbon sources play crucial roles in optimizing secondary metabolite production (Ismet et al. 2004). For example, Keum et al. 2009 found that using glycerol as a carbon source significantly increased the production of *B. cepacia* secondary metabolites like pyrrolnitrin. El-Banna & Winkelmann 1998 confirmed that glycerol led to higher metabolite production compared to glucose, mannose, and fructose. In our study, molasses, which has a high sucrose content essential for microbial growth and metabolism (Gómez et al. 2008), demonstrated excellent inhibition against the pathogen. This aligns with Doncel & Pérez 2017, who reported that a glucose-containing medium led to optimal inhibition of *C. gloeosporioides* due to pyrrolnitrin production in *B. cepacia*. Thus, we can infer that molasses could be an effective carbon source for optimizing *B. cepacia* metabolite production.

The plant growth-promoting capabilities of *B. cepacia* are consistent with the findings of Devi et al. 2012, who showed that *B. cepacia* enhanced French bean growth by solubilizing inorganic phosphate, producing siderophores, and releasing indole acetic acid. This contributed to increased plant fresh weight and significantly reduced wilt disease caused by *Fusarium* species and *Rhizoctonia solani*. Castanheira et al. 2016 identified *B. graminis*, *B. fungorum*, and *B. cepacia* complex via 16S rRNA, which inhibited the oomycete *Phytophthora cinnamomi* through secondary metabolite release and glucanase production. These bacteria also solubilized inorganic phosphate, produced siderophores, stimulated root development, and increased leaf lipid content in *Lolium multiflorum*.

Conclusion

Burkholderia cepacia, isolated from avocado soils, effectively inhibits *Colletotrichum gloeosporioides* growth, with molasses-supplemented media showing the highest antifungal activity. Additionally, *B. cepacia* promotes plant growth through siderophore production, phosphate solubilization, and nitrogen fixation. These findings suggest its potential as a sustainable alternative for managing anthracnose in avocado cultivation while reducing chemical fertilizer use.

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