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Secretion of secondary metabolite-like compounds by Burkholderia cepacia with antifungal activity against phytopathogens from commercial plants

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ABSTRACT
The objective of the present study was to evaluate the activity of secondary metabolites excreted by the endophytic bacterium Burkholderia cepacia against Colletotrichum gloeosporioides. The antifungal capacity of metabolite-type compounds obtained in modified culture media with different nutritional sources was evaluated. The results show an average inhibition of B. cepacia of 67.07% against the phytopathogenic fungus in contrast to the chemical control which was 61.35% against the phytopathogenic fungus C. gloeosporioides in the in vitro inhibition tests. A variety of secondary metabolites with antifungal activity against Colletotrichum are present in the extracts secreted by B. cepacia C. gloeosporioides.

Keywords: Phytopathogen, inhibitory activity, metabolite, control.

1.INTRODUCTION

According to what was referenced by the works of Feng et al., (2019 and Xu et al., (2016), the phytopathogenic fungus Colletotrichum gloeosporioides Penz is an important fungus that infects more than 1000 plant species, causing severe symptoms known as anthracnose in the field. This disease occurs in many fruit and vegetable crops globally, particularly in tropical and subtropical areas (Cannon et al., 2012; Barrios-Roblero et al., 2019; Yong-Chao et al., 2018; Bautista-Rosales et al., 2013; Wen-Hsin et al., 2014). Crop varieties such as mango, citrus, papaya, yam, avocado, cocoa, among others, are susceptible to anthracnose caused by C. gloeosporioides , which has led to reduced yields and huge economic losses in the agricultural systems of the countries that produce these crops.

According to studies (Siddiqui, 2014, Zhou et al., 2016, Feng et al., 2019, Bautista-Rosales et al., 2013), the management of the disease in the field is achieved by the application of fungicides, such as benomyl, benlate, daconil, carbendazim, mancozed, maneb, thiabendazole, copper oxychloride, propineb, difenoconazole and prochloraz. Other forms of management consist of the mixed and rotational use of fungicides with different mechanisms of action to delay the development of resistance and acquire a desired control efficacy against said disease (Xu et al., 2014).

According to (Lucas, 2015, Ong, 2015), the indiscriminate use of synthetic fungicides has led to the resistance of C. gloeosporioides to chemicals, and consequently to the emergence of strains of the fungus resistant to certain fungicides; transforming this pathogen into a serious agronomic problem. Different studies carried out by (Siddiqui, 2014; Bautista-Rosales et al., 2013), show that the growing awareness of the dangers to health and environmental pollution due to the indiscriminate use of pesticides, in addition to the risk of acquired resistance by pathogens as a result of this same abuse, has forced environmental protection agencies in several developed and developing countries to prohibit the use of numerous chemicals that were frequently applied to different crops.

What was stated by (Egesi et al., 2007, Yong-Chao et al., 2018), anthracnose is one of the most widespread and economically important diseases of yam (Dioscorea) cultivation. Dioscorea alata rotundataand Dioscorea cayenensis). This pathology has devastating effects on yam production in many tropical regions where the crop contributes to food security and income generation, being considered the most important biotic restriction for this agricultural product.

Yam cultivation as identified by (Yong-Chao et al., 2018), is infected with C. gloeosporioides can develop necrosis in the leaves and stems, thus reducing the photosynthetic efficiency of the plant and seriously affecting tuber production by causing yam shoots to die back and ultimately causing the death of the plant resulting in yield losses between 80 and 90% when susceptible varieties are grown under high disease pressure and

favorable environmental conditions. (Abang et al., 2009, Egesi et al., 2007).

Most yam varieties found in Latin America are susceptible to attack by the fungus C. gloeosporioides, requiring extensive use of fungicides as the primary means of controlling the disease (Siddiqui, 2014). However, these fungicides have negative side effects, such as reduced food biosecurity, risks to human health, and environmental contamination (Barrios-Roblero et al., 2019, Zhang et al., 2019, García-Gutiérrez, 2012; Speck - Planche et al., 2012; Del Puerto, 2014).However, these pesticides continue to be widely used in agricultural systems.

In Colombia, yam cultivation is severely affected by anthracnose, causing large economic losses, making its effective control necessary. However, this has become increasingly costly both economically and environmentally because the number of chemical treatments needed has increased over time due to the rapid emergence of resistance by the pathogen to the different fungicides that have been used (Ramírez-Gil et al., 2014; Ramírez-Gil, 2017). Therefore, it is necessary to search for new alternatives that are much more economical, more effective against C. gloeosporioides, and that at the same time are not harmful to the environment and human health (Xu et al., 2017, Bautista-Rosales et al., 2014).

One of these more sustainable alternatives is biological control. In recent years, there has been an increase in the use of active ingredients extracted from microorganisms as control agents in agricultural crops (Bautista-Rosales et al., 2013; Bautista-Rosales et al., 2014).

The implementation of bacterial secondary metabolites as a biological control tool is a phytosanitary and economic option to minimize the effects of phytopathogens (Barrios-Roblero et al., 2019).

Endophytic bacteria are an important source of microbial biological control agents, many of these bacteria belong mainly to the genera Bacillus,Pseudomonas,Burkholderia, Agrobacterium, Streptomyces, etc. These are directly associated with a host plant, because they reside in plant tissues, mainly in the intercellular space and within vascular tissues without harming the plant; They are characterized by promoting plant growth, and also by inhibiting some pathogens associated with them. For this reason, endophytic bacteria and their active products are increasingly used as a biotechnological tool for the control of phytopathogens (Liu et al., 2017; Mishra & Arora, 2018, Yang et al., 2020). These bacteria have been intensively studied for their biotechnological applications in plant growth promotion, biological control of plant pests, and bioremediation (Depoorter, 2018). et al., 2016).

Throughout history, various endophytic microorganisms have shown antimicrobial activity against different phytopathogens, which demonstrates their potential as biological control agents. The mode of action of these biological agents is antibiosis (through the production of antibiotics, lytic enzymes and antagonist proteins) (Zeidanet al., 2019).

The use of secondary metabolites derived from endophytic bacteria as a biological control agent for phytopathogens has been an ecological alternative to the use of chemical pesticides, because these compounds have excellent inhibitory potential, have low toxicity, are easily degraded, so they are environmentally friendly, in addition to being economical; for this reason,they are being widely studied (Doncel, 2018).

Multiple investigations have focused on the biological control of C. gloeosporioides with the use of endophytic bacteria and/or their extracts, among these the potential of the endophyte stands out. Burkhulderia cepacia, which has shown a great antagonistic effect against said phytopathogen (Doncel, 2018, Kadir et al., 2008). Some species of the environmental Burkholderia cepacia complex (CBC) are known to produce unique antifungal antibiotics, such as pyrrolnitrin, phenazine, phenylpyrrole, occidiofungin and glidobactins (Kong & Hong, 2020, Yang et al., 2020). However, the fact that many BCC species are responsible for severe chronic infections among immunosuppressed patients, especially those with cystic fibrosis (CF), has hampered their biotechnological use, especially in agriculture (Coulonet al., 2019). Therefore, studies are needed on alternative biotechnological uses of strains belonging to the CBC, which are safer and which also allow the great antifungal potential of these bacteria to be exploited. In this sense, the use of bacterial extracts derived from these bacterial strains represents a promising method.

Many factors can directly influence the production of secondary metabolites by bacteria, which can affect both positively and negatively their antifungal potential. The nutritional composition (carbon and nitrogen) of the culture medium, the pH, the temperature and the fermentation time are some of the most important factors. Because of this, it is necessary to determine culture media with optimized conditions, which provide a higher yield and bioactivity of the secondary metabolites to be extracted (Doncel, 2018, Depoorter et al., 2016).

According to what was reported by Roja et al., (2019), the Burkholderia cepacia complex B. is made up of 22 species known as opportunistic pathogens in immuno-compromised individuals, especially those with cystic fibrosis. They are also isolated from nosocomial infections and are difficult to eradicate due to their intrinsic ability to resist a wide variety of antibiotics. In general, these species have large genomes (up to 9 Mbp) divided into 2-5 replicons.

This characteristic provides great metabolic versatility, which is considered important for inhabiting soil, water, plants, even nodules in legumes. Some species of the B. cepacia complex exhibit beneficial activities, such as bioremediation, biocontrol and plant growth promotion. However, due to its role in human infections, its use in

agriculture is restricted. The B. cepacia complex is a constant topic of study due to its impact on the health sector and its potential in agriculture. This paper examines the history of the B. cepacia complex and reviews recent information related to this group of bacteria.
Burkholderia cepacia with potential as a biological control agent was raisedagainstColletotrichum gloeosporioides by evaluating the inhibitory activity of bacterial extracts derived from different modified culture media.

2. MATERIALS AND METHODS

Isolation of pathogenic strains of fungus causing anthracnose. Sampling was carried out following the standardized protocol by(Peroza, 2016), which consisted of collecting samples of yam leaves (Dioscorea alata) with characteristic symptoms of anthracnose of crops present in rural areas of the municipality of Sincelejo. These were then taken to a humid chamber for eight days to induce spore production by the fungus. After this time, observation was carried out using a stereoscope and microscope of necrotic areas of the leaf in order to observe reproductive structures. Once the spores (conidia) were identified, they were isolated with the help of a hypodermic needle and transferred to Sabouraud and PDA culture media enriched with antibiotics. Finally, the growth of the fungus in the culture media was monitored, discarding those media that did not correspond to the characteristics of the mycelium of said pathogen in the box.

Use Burkholderia cepacia as an alternative biocontrol. Burkholderia strain cepacia identified as A3T1, preserved in the genomic bank of the Microbiological Research Laboratory of the University of Sucre (Figure 1), was taken as a reference and was subjected to activation on the surface of the nutrient broth culture medium, LB broth and King B broth. Once the strain was activated in the different broths, their growth was monitored for two days in order to observe in which medium it performed better, and then it was inoculated onto R2A Agar for later use.

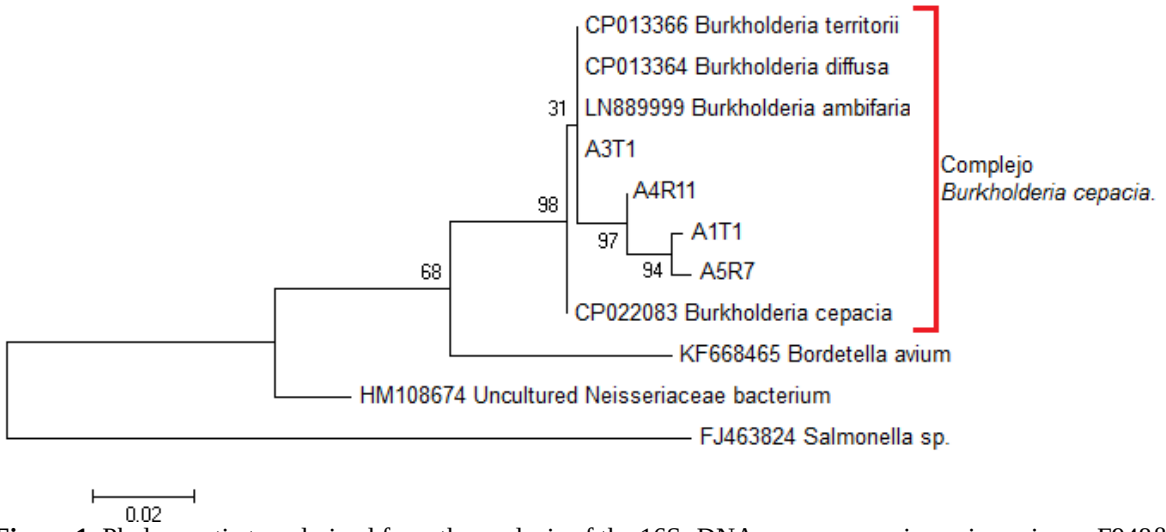

Figure 1. Phylogenetic tree derived from the analysis of the 16S rDNA gene sequencing using primers F948β and R1492 of the A1T1, A3T1, A4R11 and A5R7 morphotypes with homologous sequences obtained from NCBI. The scale of the bar is 0.02 substitutions per nucleotide position.
Source: genomic bank, Laboratory of Research in Agricultural Bioprospecting, Faculty of Agricultural Sciences, University of Sucre, 2018.

Inhibitory activity

Once the fungus C. gloeosporiodes was isolated and the bacterial strain B. cepacia A3T1 was activated, the inhibitory activity of said bacteria against the pathogen was preliminarily evaluated by means of direct confrontation. To do this, a Dual PDA/R2A culture medium was prepared, the bacteria was inoculated at the left end of the Petri dish, allowing it to grow for two days. After this, a 7 mm segment of the fungus mycelium was planted in the central part of the dish. Subsequently, fungal growth was monitored and read for seven days.

Culture media used

A bacterialpreculture was prepared in nutrient broth of the strain B. cepacia A3T1, which was allowed to grow for 24 hours; three culture media supplemented with different nutritional sources were also prepared to evaluate the production of metabolites and their antifungal activity, called M1: culture medium 1: chemical composition: (8 g Nutrient broth + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4)), M2: culture medium 2:

chemical composition: (8 g Nutrient broth + 0.79 mL Glycerol + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4)) and M3: culture medium 3: chemical composition: (8 g Nutrient broth + 1 g Molasses + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4), these were prepared in 1000 mL of distilled water, and the initial pH was adjusted to 6.8 with 0.1 N sulfuric acid. Finally, these media were inoculated with the bacterial preculture and then incubated at 28 °C with constant shaking (150 rpm) for seven days, followed by extractions (Doncel, 2018).

Extraction and concentration of bacterial extracts

Metabolite extraction was performed at 72 and 120 hours of fermentation of the modified culture media inoculated with B. cepacia. This was done by centrifuging a sample of each medium at 7000 rpm for 45 minutes, which were then sterilized separately with 0.22 µm Millipore filters and from which a small aliquot, called crude or aqueous extract, was isolated for later evaluation of inhibitory activity. The remaining part of the aqueous extracts was taken to rotary evaporation for concentration, and thus later to be evaluated.

Antifungal activity of bacterial extracts by agar diffusion method

The inhibitory activity of the different extracts obtained from fermented modified culture media inoculated with B. cepacia was evaluated against the phytopathogen C. gloeosporiodes in order to determine which of the media had the best yields in the production and bioactivity of secondary metabolites. This was done through the agar diffusion test, which consisted of preparing Petri dishes with PDA medium, for which a hole was made in the central part with the help of a hole punch, then two inoculant of the fungus (7 mm circles) were added parallel to each other at the same distance from the central hole; then this hole was filled with 60 µL of bacterial extract to be tested, and finally incubated at 28 °C for 8 days and then calculated their inhibition percentages (Islam et al., 2012).

Statistical analysis

The results of all inhibition treatments were expressed as means ± standard deviations (SD). An analysis of variance (ANOVA) was performed for each variable, previously establishing the normality criterion using the Shapiro-Wilk test (5%). A completely randomized experimental design was made with a 2 × 3 × 5 factorial arrangement where the first factor corresponds to the extraction times with two levels that are 72 and 120 hours, the second factor was the different modified culture media from which the extracts in ethyl acetate were obtained with three levels that are M1, M2 and M3, as a third factor the different concentrations of the extracts in ethyl acetate with five levels that are 5%, 10%, 20%, 40% and 80% v/v. Duncan's multiple range test was used (p-value ≤ 0.05) for significant differences. All analyses will be carried out using InfoStat software.

3. RESULTS AND DISCUSSION

The leaves of D. alata infected with the phytopathogen C. gloeosporioides were characterized by semi-sunken necrotic areas surrounded by a yellow halo, a characteristic symptomatology of anthracnose, which coincides with that described by Cannon et al. (2012). Eight days after being exposed to a humid chamber, acervuli developed, which are orange mucilaginous reproductive structures made up of short conidiophores at the apex of which are the conidia (figure 2). The conidia observed were bacillary in shape, with an average length between 8.5-16 µm, these aspects agree with that reported by Siddiqui (2014), who describes a size of the conidia of 14.35 µm. The mycelial growth of the fungus in sabouraud culture medium it presented a white-grayish color, characteristic of some strains of the fungus C. gloeosporioides (Figure 3).

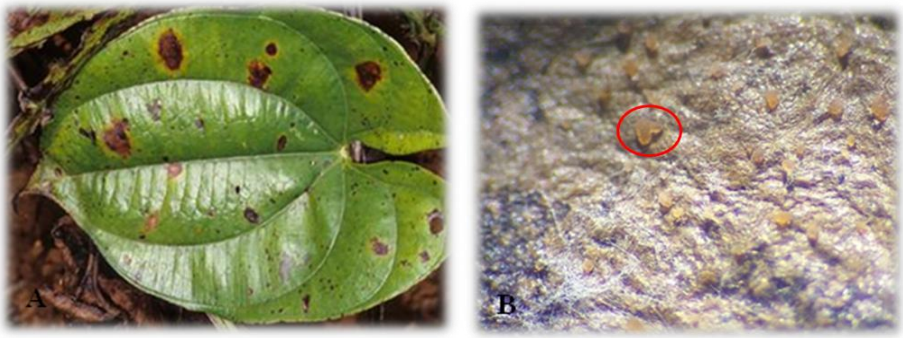

Figure 2. A: Dioscorea leaf alata with symptoms of anthracnose; B: Sporulation by acervuli (marked in red) of the fungus Colletotrichum gloeosporioides on D. alata leaves incubated in a humid chamber. Source: Rodríguez and Perez, 2021.

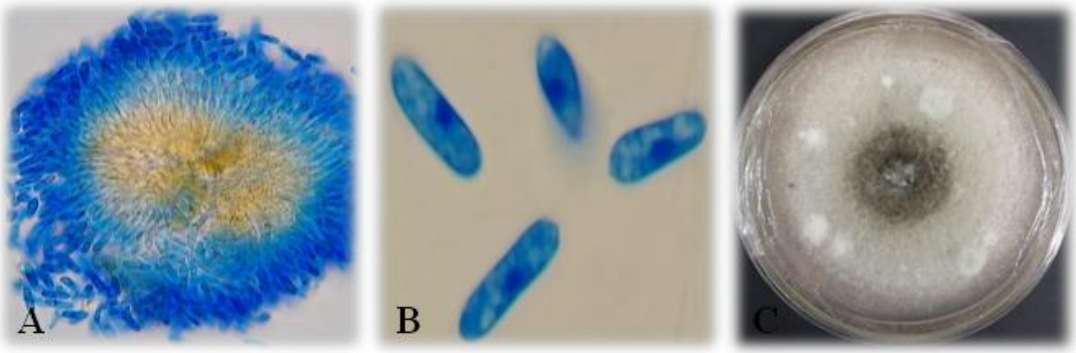

Figure 3. Reproductive structures of the fungus C. gloeosporioides. A. Acervulus stained with lactophenol blue seen under a microscope at 40x; B. Conidia of C. gloeosporioides stained with lactophenol blue seen under a microscope at 100x; C. Isolated strain of C. gloeosporioides in Sabouraud culture medium with mycelial growth characteristic of this fungus. Source: Rodríguez and Perez, 2021.

Molecular identification using sequencing techniques of the isolates of the phytopathogenic fungus confirms the taxonomic identity with Colletorichum gloeosporioides, as shown in Figure 4.

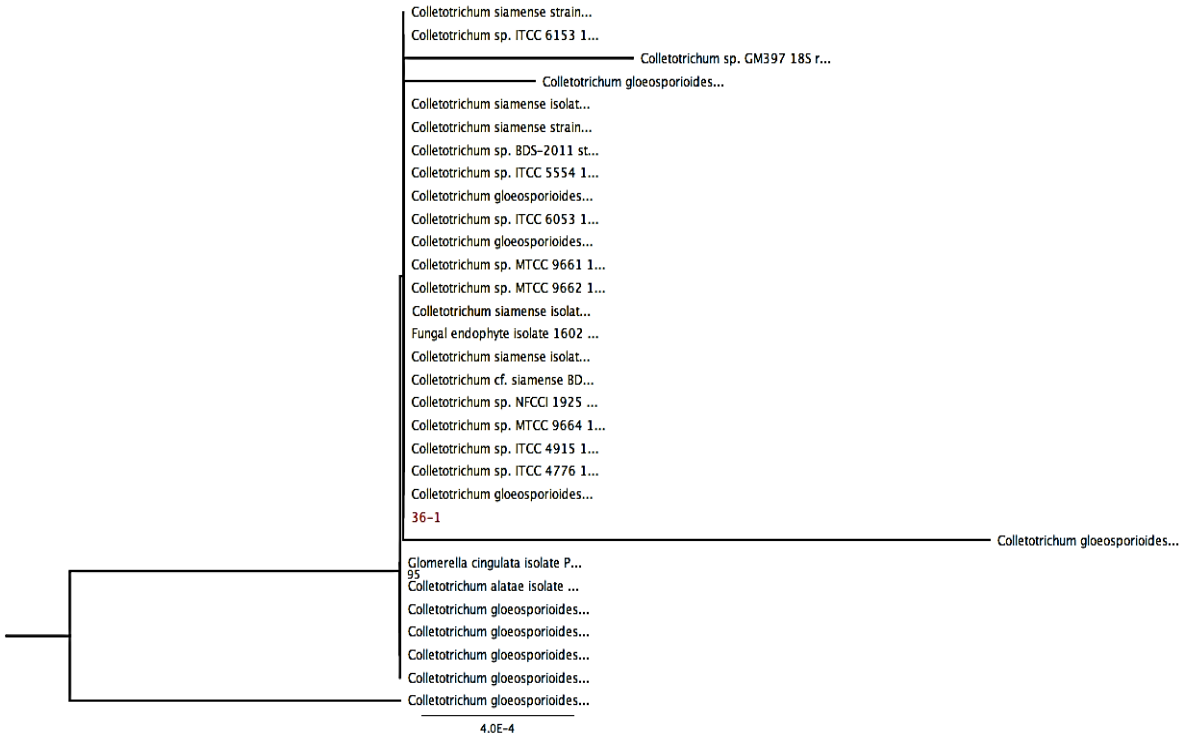

Figure 4. Distance tree constructed from the closest sequences available in the NCBI NR/NT database. Source: Genomic bank, Laboratory of Agricultural Bioprospecting Research, Faculty of Agricultural Sciences, University of Sucre, 2018.

The bacterial strain A3T1 of B. cepacia showed a greater growth in nutrient broth, this was due to the fact that in this medium there was a greater turbidity in a shorter growth time, compared to the other media used. This is evidence of what was reported by Kadir et al. (2008), where B. cepacia grew faster in nutrient broth than in other media, with a cell concentration that reached the highest level at 72 h after inoculation, an aspect that benefits the yield of the extracts in a shorter growth time.

The bacterial strain A3T1 of B. cepacia showed an antagonistic effect against the phytopathogen C. gloeosporioides in the direct cell-cell confrontation test. In this test, the formation of an inhibition zone where there was no growth of the fungus was clearly noted, demonstrating the antifungal effect of the A3T1 strain. This result agrees with what was mentioned by Depoorter. et al. (2016), Rojas-Rojas et al. (2018) and Yang et al. (2020), who highlight the antifungal potential of multiple bacterial strains belonging to the Burkholderia cepacia complex.

Figure 5 shows the results of the percentages of inhibition of secondary metabolite type extracts of B. cepacia and of the chemical control with benomyl against C.gloeosporioides. The results show an average inhibition of B. cepacia of 67.07% against the phytopathogenic fungus in contrast to the chemical control which was 61.35%.

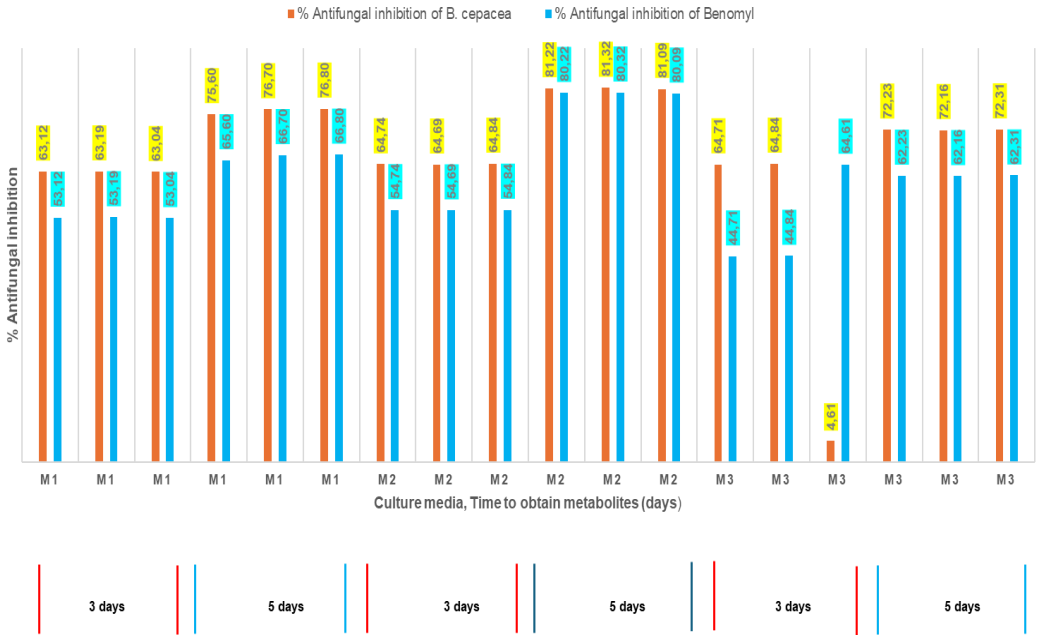

Figure 5. Antifungal activity assay of bacterial extracts of B. cepacia and benomyl against C. gloeosporioides . , called M1: culture medium 1: chemical composition: (8 g Nutrient broth + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4)), M2: culture medium 2: chemical composition: (8 g Nutrient broth + 0.79 mL Glycerol + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4)) and M3: culture medium 3: chemical composition: (8 g Nutrient broth + 1 g Molasses + Phosphate buffer (1.20 g Na_2HPO_4 and 0.25 g KH_2PO_4)

The different modified media showed good growth of the bacterial strain, with which extracts can be obtained after just 72 hours of fermentation, as reported by Kadir et al. (2008) the nutrient broth allows the bacteria to reach the stationary growth phase after 72 hours of extraction, during this phase of the bacterial growth curve secondary metabolites begin to be produced (Doncel, 2018). All the prepared media were based on nutrient broth, which justifies the good growth and obtaining of secondary metabolites after just 72 hours of fermentation.

Extractions were also carried out after 120 hours, in order to check if there is an increase in the antifungal potential of the extracts. The inhibitory activity of bacterial extracts was subsequently evaluated, with which the presence of secondary metabolites was determined. From the modified media, extracts were obtained at 72 and 120 hours of fermentation, these were sterilized and subsequently concentrated with the aim of evaluating the potential of both aqueous and concentrated extracts through inhibition tests.

The different aqueous extracts evaluated presented low percentages of inhibition, this may be due to the dilution they possess, with which there are low concentrations of bioactive secondary metabolites, reflected in a weak antifungal activity. However, a better antifungal potential could be distinguished in the extracts obtained after 120 hours of fermentation, this is due to the fact that the production of antibiotics occurs late in the stationary phase, as a result of exposure to extreme conditions, nutrient deficiency or feedback phenomena (Zeidan et al., 2019, Yang et al., 2020).

Among the three media evaluated, the M3 and M2 media showed the best inhibition percentages, which indicates that The nutritional composition (carbon and nitrogen) influenced the production of metabolites with antifungal action (Doncel, 2018), however, the M3 medium supplemented with molasses was the most efficient, thanks to the large amounts of carbohydrates that it provides, which They appear to act as precursors in the production of antifungal metabolites. The concentrated extracts, on the other hand, showed an even lower inhibition of the fungus, as a consequence of the loss and decomposition of metabolites resulting from the extreme conditions of water rotary evaporation.

Consequently, with the low percentages of inhibition obtained from the aqueous and concentrated extracts, the bioactive metabolites were extracted using organic solvents, in this case ethyl acetate. This solvent has the advantage that the extracted active ingredients can be concentrated under conditions of reduced temperature and pressure, thus avoiding the alteration of their physicochemical and biological properties. In addition, it has been

reported that this solvent allows the extraction of metabolites with antifungal activity (Kong & Hong, 2020, Doncel, 2018).

As part of the metabolic versatility that allows the B. cepacia complex to adapt to diverse environments, the production of antimicrobial compounds that inhibit the growth of various plant and human pathogens has been reported. Different peptides with antifungal activity have been described, such as cepacidins, glidobactins and altericidins produced by B. cepacia (Kirinuki et al., 1984; Lee et al., 1994; Schellenberg et al., 2007), a non-ribosomal oligopeptide, and occidiofungin, produced by B. contaminans (Gu et al., 2009; Chen et al., 2013). Additionally, the production by B. cepacia of siderophore-type compounds and pigments of the phenazine family is known. with activity against phytopathogenic fungi (Cartwright et al., 1995; De los Santos-Villalobos, 2012)

4. CONCLUSIONS

The bacterial strain A3T1 of B. cepacia had marked antifungal activity against C. gloeosporioides in direct confrontation tests. The different modified culture media showed good growth of the bacterial strain, and bioactive compounds could be obtained 3 and 5 days after the microbial extracts were extracted. The bacterial extracts extracted with ethyl acetate showed good inhibitory activity, with those obtained from the M2 medium after 5 days of fermentation having an average antifungal activity of 72.98% compared to an average of 67.47% of the chemical control with benomyl.

5. Acknowledgements

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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. All authors have read and approved the manuscript.

7. Conflict of interest

All the authors of the manuscript declare that they have no conflict of interest.

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