Actinobacteria from avocado rhizosphere: antagonistic activity against *Colletotrichum gloeosporioides* and *Xanthomonas* sp.
Actinobacterias de la rizosfera del aguacate: actividad antagónica contra *Colletotrichum gloeosporioides* y *Xanthomonas* sp.

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SUMMARY

Actinobacteria from the rhizosphere of agricultural crops are a potential source of antagonists against phytopathogenic microorganisms. Thus, the objective of this study was to characterize the antagonistic activity *in vitro* of actinobacteria from the rhizosphere of avocado trees (*Persea americana*) against *Colletotrichum gloeosporioides* and *Xanthomonas* sp. Actinobacteria were isolated from rhizosphere soil samples of avocado trees var. Hass collected from an orchard from Ziracuaretiro, Michoacán. The isolated strains were assessed against *C. gloeosporioides* and *Xanthomonas* sp. by means of *in vitro* confrontation tests. The antagonistic activity was determined by measuring the radius and diameter of the growth inhibition zone of *C. gloeosporioides* and *Xanthomonas* sp., respectively. A total of 41 actinobacterial strains were isolated, of which 44% inhibited growth of at least one of the two plant pathogens. Specifically, 15% of the isolates inhibited growth of *C. gloeosporioides*, 22% of *Xanthomonas* sp. and 7% of both phytopathogens. The mycelial growth inhibition radius of *C. gloeosporioides* fluctuated from 6.6 to 15.5 mm, while in *Xanthomonas* sp., the inhibition zone was from 15.5 to 62.7 mm. These results indicated the importance of rhizosphere actinobacteria as antagonists of phytopathogenic microorganisms, some of which could be used as potential biological control agents against anthracnose and bacterial leaf spot.

Index words: *actinomycetes, antagonist, biological control, Persea americana, phytopathogens, rhizobacteria, Streptomyces.*

RESUMEN

Las actinobacterias de la rizosfera de cultivos agrícolas son una fuente potencial de antagonistas contra microorganismos fitopatógenos. El objetivo de este estudio fue caracterizar la actividad antagónica *in vitro* de actinobacterias de la rizosfera de árboles de aguacate (*Persea americana*) contra *Colletotrichum gloeosporioides* y *Xanthomonas* sp. Las actinobacterias fueron aisladas de muestras de suelo rizosférico de árboles de aguacate var. Hass colectadas de una huerta

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en el municipio de Ziracuaretiro, Michoacán. Las cepas aisladas fueron evaluadas contra *C. gloeosporioides* y *Xanthomonas* sp. mediante ensayos de confrontación *in vitro*. La actividad antagónica se determinó midiendo el radio y diámetro de la zona de inhibición del crecimiento de *C. gloeosporioides* y *Xanthomonas* sp., respectivamente. Se aislaron un total de 41 cepas de actinobacterias, de las cuales el 44% inhibió el crecimiento de al menos uno de los dos fitopatógenos. En específico, el 15% de los aislados inhibió el crecimiento de *C. gloeosporioides*, el 22% a *Xanthomonas* sp. y el 7% a ambos fitopatógenos. El radio de inhibición del crecimiento miceliar de *C. gloeosporioides* fluctuó de entre 6.6 a 15.5 mm, mientras que en *Xanthomonas* sp. la zona de inhibición fue de entre 15.5 a 62.7 mm. Estos resultados indican la importancia de las actinobacterias de la rizosfera como antagonistas de microorganismos fitopatógenos, algunas de las cuales podrían ser utilizadas como potenciales agentes de control biológico contra la antracnosis y la mancha bacteriana.

Palabras clave: *actinomicetos, antagonistas, control biológico, Persea americana, fitopatógenos, rizobacterias, Streptomyces.*

INTRODUCTION

Plant diseases caused by microorganisms are one of the limitations during production, storage, and marketing of cereals, fruits, and vegetables. Loss in annual crop production at world level due to phytopathogen microorganisms has been estimated around 12% (Oerke, 2006; Pimentel, 2007). The use of agrochemicals as the only measure of plant disease control is not an alternative anymore. Phytopathogens have generated resistance to these products and their excessive use has resulted harmful for the environment and human health (Aktar *et al*., 2009; Areas *et al*., 2018). Antagonist microorganisms are an alternative to the use of agrochemicals for disease control because they are specific for certain phytopathogens, harmless for non-target species, innocuous for man, and eco-friendly (O'Brien, 2017).

Soil is a reservoir for a complex microbiome of prokaryotic organisms (eubacteria and archaebacteria) and eukaryotes (fungi and algae) that play an important role in biogeochemical processes (Kaviya *et al*., 2019). In soil, actinobacteria, commonly called actinomycetes, belong to the phylum *Actinobacteria*; they participate actively in biological processes, such as nitrogen fixation, phosphate solubilization, and dead organic material degradation (Bhatti *et al*., 2017). Additionally, as part of the rhizosphere microbiome, they promote plant growth and protect the crop against phytopathogens (Sharma and Salwan, 2018). This bacterial group is found widely distributed in land and aquatic environments. It is also a prominent source of natural bioactive products of importance to biotechnology, medicine, and agriculture (Bérdy, 2005; Goodfellow, 2015).

Actinobacteria are an alternative to the use of agrochemicals for phytopathogen control. Some commercial products contain *Streptomyces lydicus* WYEC 108 (Actinovate®; Valent USA) and *Streptomyces* sp. K61 (Mycostop®; Verdera, Oy, FI) spores as active ingredient, which have demonstrated the capacity to control some diseases caused by phytopathogen fungus and oomycetes (Lahdenperä, 1987; Crawford *et al*., 1993). Some actinobacterial species associated to the rhizosphere have demonstrated their role as antagonists against phytopathogens, such as *Alternaria solani*, *Xanthomonas oryzae* pv. *oryzae* and *Bacillus pumilus* (Dicko, 2013). Similar studies Intra *et al*. (2011) have demonstrated the antagonistic activity of actinobacterial strains isolated from the rhizosphere soil of different fruit trees against *C. gloeosporioides* and *C. capsici*. Recently, Suárez-Moreno *et al*. (2019) isolated actinobacterial strains from rhizosphere soil samples of rice crops (*Oryza sativa*), some of which showed very important antibacterial and antifungal activity against *Burkholderia glumae* one of the most important phytopathogens in rice crops. Similarly, Dai *et al*. (2019) isolated actinobacteria with antagonistic activity against the phytopathogen fungus *Valsa mali* from rhizosphere soil of wild apple trees.

Colletotrichum gloeosporioides is a phytopathogen fungus widely distributed that causes anthracnosis in avocado in pre- and post-harvest stages, as well as in other economically important crops (Dean *et al*., 2012). On the other hand, *Xanthomonas* species causes bacterial leaf spot of pepper, leading to severe damage in crop production (EFSA-PLH, 2014). Therefore, the objective of this study was to characterize the antagonistic *in vitro* activity of rhizosphere actinobacteria from avocado trees (*Persea americana*) against *Colletotrichum gloeosporioides* and *Xanthomonas* sp.

MATERIALS AND METHODS

Soil Sampling in an Avocado Orchard in Michoacán

Rhizosphere soil samples were collected during 2017 in the avocado orchard "El Zarco" in the Municipality of Ziracuaretiro, Michoacán, México (19° 23' 26.5'' N; 101° 51' 42.8'' W). The orchard of avocado var. Hass had a combined conventional and organic agronomic management. Four healthy avocado trees were selected randomly, and two soil samples per tree were taken in the drip irrigation area at a depth no greater than 15 cm. Soil samples were stored in high density polyethylene bags, taken to the laboratory at room temperature, and then air-dried for one week. Soil samples were sieved in a 1-mm mesh; those samples coming from the same tree were mixed in a 1:1 (w/w) ratio to obtain a compound sample and stored at 4 °C to be used later.

Actinobacteria Isolation

Actinobacteria were isolated with the spread plate technique starting from a compound sample of rhizosphere soil for each tree. From each tree, 10 g of soil were taken, deposited independently in dilution bottles with 90 mL of sterile water, and mixed for five min. Subsequently, serial dilutions were prepared in sterile distilled water until a 10^{-4} dilution was obtained for each soil suspension. A 100 µL aliquot of soil suspension of 10^{-2} up to 10^{-4} dilutions were inoculated in the isolation medium and disseminated with a Digralsky metal handle. The culture medium for isolation was potato-dextrose-agar (PDA); 2 g L^{-1} yeast extract (YE) were added, and the pH was adjusted to 7.0 with NaOH 3 M. The culture medium (PDA-YE) was supplemented with 12.5 mg L^{-1} of nalidixic acid and 50 mg L^{-1} of cycloheximide (Intra *et al*., 2011). Petri plates were incubated at 28 °C from 7 to 14 days. The emerging actinobacterial type colonies with different morphological characteristics (Kämpfer, 2015) were selected. Spores and/or mycelia of the isolated actinobacteria were preserved with 25% glycerol at -80 °C (Shepherd *et al*., 2010).

Phytopathogens: Culture Media and Growth Conditions

Colletotrichum gloeosporioides isolated from papaya fruit with typical anthracnosis symptoms was cultured in a routine manner in PDA medium at 28 °C for 12 days in darkness. A fungal mycelial disk of 7 mm in diameter was used for the antagonism assay. *Xanthomonas* sp. BV801 previously isolated from a poblano *Capsicum annuum* pepper with typical symptoms of the bacterial leaf spot (López-Vielma *et al*., 2016) was cultured routinely in nutrient-yeast extract glycerol (NYG) liquid or solidified agar (NYGA) medium (5 g L^{-1} bacto peptone, 3 g L^{-1} yeast extract and 20 mL L^{-1} glycerol; the same solid medium contained 15 g L^{-1} agar) at 28 °C for 16 or 48 h, respectively (Daniels *et al*., 1984).

Bioassays of *in vitro* Antagonistic Actinobacterial Selection

Antagonistic activity against *Colletotrichum gloeosporioides*. The selection of actinobacteria with antagonistic activity against *C. gloeosporioides* was done by spot inoculation method (Shomura *et al*., 1979) with slight modifications. The actinobacteria strains isolated were streaked on Petri plates (45 mm in diameter) with PDA-YE medium, incubated at 28 °C for seven days and obtained mycelium disks (7-mm in diameter) from each strain using a cork borer. Of the four strains randomly selected, one mycelium disk was inoculated in each one of the four points equidistant to the center of the Petri plate onto PDA medium to then inoculate one active mycelium disk of *C. gloeosporioides* in the center (Figure 1). The same procedure was repeated with all the isolated strains. As growth control, one fungus mycelium disk was inoculated in the center of the Petri plates with PDA medium. All cultures were incubated at 28 °C for 12 days. The antagonistic activity was determined by measuring the inhibition zone radius starting from the center of the disk of each actinobacteria until the phytopathogen fungus growth border was reached. All the actinobacterial strains and the control were performed in triplicate.

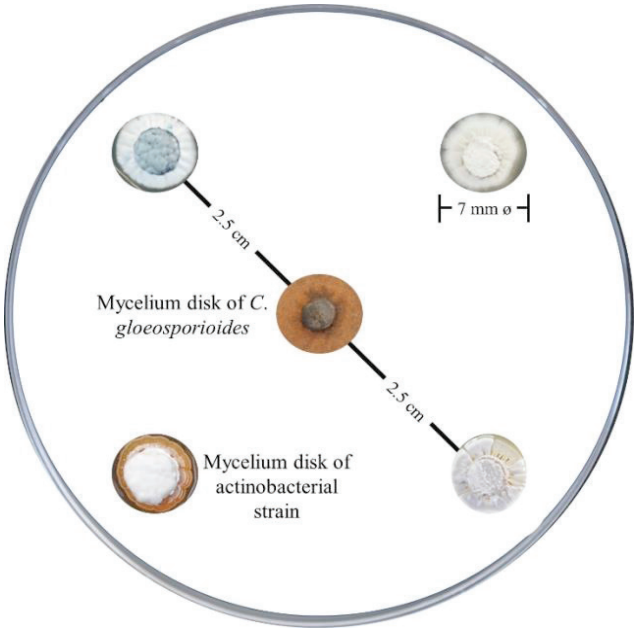

Figure 1. Graphic representation of the *in vitro* antagonistic activity assay for actinobacterial strain selection against *Colletotrichum gloeosporioides.*

Antagonistic activity against *Xanthomonas* sp. BV801. The antagonistic actinobacterial selection was performed by the double-layer agar method previously described by Salamoni *et al*. (2010) with slight modifications. The disks of each strain were obtained and inoculated as described previously in Petri plates (90 mm in diameter) in PDA medium and incubated at 28 °C for five days before confrontation. *Xanthomonas* sp. BV801 grew 20 mL of the NYG medium at 30 °C in agitation at 200 rpm for 16 h. The bacterial culture was adjusted to a OD_{600nm} = 1 with the fresh NYG medium. The overlay agar (top layer) was prepared by mixing 400 µL of the bacterial culture in 4 mL of soft NYGA (0.6% agar) tempered in a thermo-water bath at 48 °C and poured over the Petri plates that contained the actinobacterial mycelium disks. The same procedure was performed for the control group in Petri plates with PDA without actinobacteria. All the cultures were incubated for two days at 28 °C. The antagonistic activity was determined by measuring the radius of inhibition zone of *Xanthomonas* sp. BV801 starting from the center of the disk of each actinobacteria until the inhibition border was reached. The data were expressed as inhibition zone diameter. All the actinobacterial strains and the control were performed in triplicate.

Experimental Design and Statistical Analysis

A complete randomized experimental design was used where each actinobacteria corresponded to one treatment. The inhibition data of each actinobacterial strain were obtained from three independent biological replicates. The response variables of inhibition diameter and radius were analyzed by a one-way analysis of variance (ANOVA) and a comparison of means with Tukey's test (P = 0.05) utilizing the statistical package StatGraphics Centurion XV (StatPoint Inc., 2005).

RESULTS AND DISCUSSION

Actinobacterial Antagonistic Activity Against *Colletotrichum gloeosporioides* and *Xanthomonas* sp. BV801

A total of 41 actinobacterial strains of different phenotypes were isolated from avocado tree soil. Of all the strains isolated, 44% resulted antagonistic against *C. gloeosporioides* or *Xanthomonas* sp. BV801 (Figures 2 and 3). Of this percentage, 15% (six isolates) corresponded to actinobacteria that only antagonized *C. gloeosporioides* growth; 22% (nine isolates) showed antimicrobial activity against *Xanthomonas* sp. BV801; 7% (three isolates) showed inhibition activity against both phytopathogen microorganisms.

The actinobacterial strains showed *C. gloeosporioides* growth inhibition radii, which fluctuated from 6.6 to 15.5 mm (Figure 2). Several strains showed outstanding activities; BVEZA1 02 strain recorded the greatest *C. gloeosporioides* mycelial growth inhibition radius, with significant differences with respect to the other strains (Tukey's, P = 0.05). Moreover, the BVEZA2 17, BVEZA2 27, and BVEZA3 30 strains stood out because of their antifungal and antibacterial activities (Figures 2 and 3). With respect to the strains that showed antibacterial activities against *Xanthomonas* sp. BV801, the results indicated that the inhibition zones fluctuated from 15.5 to 62.7 mm in diameter (Figure 3). Three strains called BVEZA2 24, BVEZA4 34, and BVEZA4 38 stood out showing significant differences with respect to the inhibition diameters of the rest of the actinobacteria (Tukey's, P = 0.05).

Avocado rhizosphere is a potential source of antagonistic microorganisms against phytopathogen fungi. Ramírez *et al*. (2015) reported the antagonistic

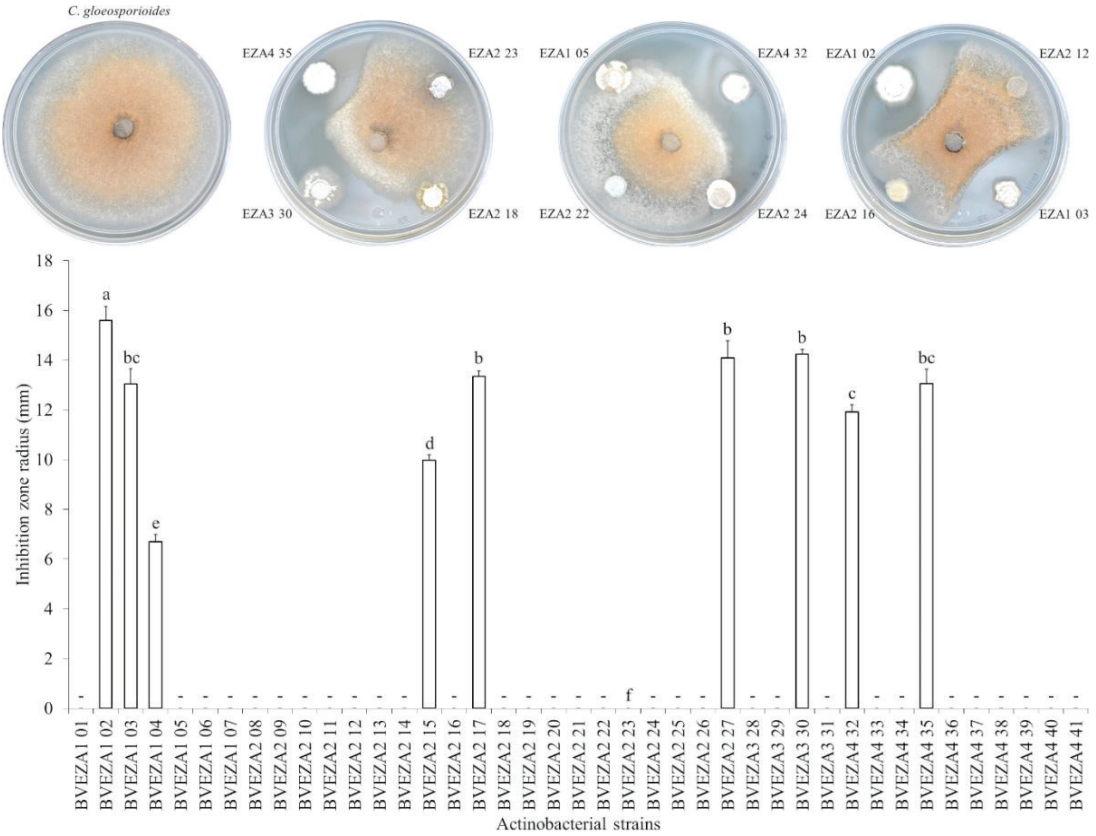

Figure 2. *In vitro* antagonistic activity of actinobacterial strains isolated from the rhizosphere of avocado trees on the growth of *Colletotrichum gloeosporioides*. The bars above the rectangle represent the standard deviation (n = 3). - = no inhibition. Different letters indicate significant differences according to Tukey's test ($P = 0.05$).

activity of 76 endophyte bacteria from the root and the rhizoplane of healthy avocado trees, of which 36.8, 32.8, and 68.4% showed *in vitro* growth inhibition in different degrees (inhibition halos from 1 to 40 mm) of *Phytophthora cinnamomi*, *Colletotrichum* sp. and *Phomopsis* sp., respectively. In another study, Méndez-Bravo *et al*. (2018) isolated avocado tree rhizobacteria that demonstrated to promote plant growth (seven isolates) and when they characterized the antagonistic activity of these isolates, A4d and A8a inhibited *P. cinnamomi* mycelial growth in direct confrontation. Guevara-Avendaño *et al*. (2018) demonstrated the antagonistic potential of bacteria that belonged to the genus *Bacillus*, isolated from avocado tree rhizosphere against the phytopathogen fungus *Fusarium euwallaceae*. Of the 168 rhizobacteria isolated, 42.8% inhibited *F. euwallaceae* radial mycelial growth from 15 to 46% in direct confrontation.

Recently, studies have reported activity of antagonistic microorganisms associated to avocado tree rhizosphere, which belong to the genus *Bacillus* spp., *Streptomyces* spp. and *Trichoderma* spp. against *C. gloeosporioides* and *F. oxysporum* (Vega-Torres *et al*., 2019; Guerrero-Barajas *et al*., 2020). The results in this study indicated that the rhizosphere of avocado trees is a source of actinobacteria capable of inhibiting phytopathogen microorganisms' growth, such as *C. gloeosporioides* and *Xanthomonas* sp. Further studies are necessary to determine the mechanism by which actinobacteria antagonize growth of these phytopathogens. Different species of *Streptomyces*, isolated from the rhizosphere of agricultural crop have been reported as potential biocontrol agents against *Colletotrichum* spp., antagonizing their growth *in vitro* and decreasing disease severity (Palaniyandi *et al*., 2011; Sadeghian *et al*., 2016; Shu *et al*., 2017; Thilagam and Hemalatha, 2019). Thus, antagonistic actinobacteria BVEZA1 02, isolated from the rhizosphere of avocado trees – could be useful as a potential biocontrol agent of *C. gloeosporioides*.

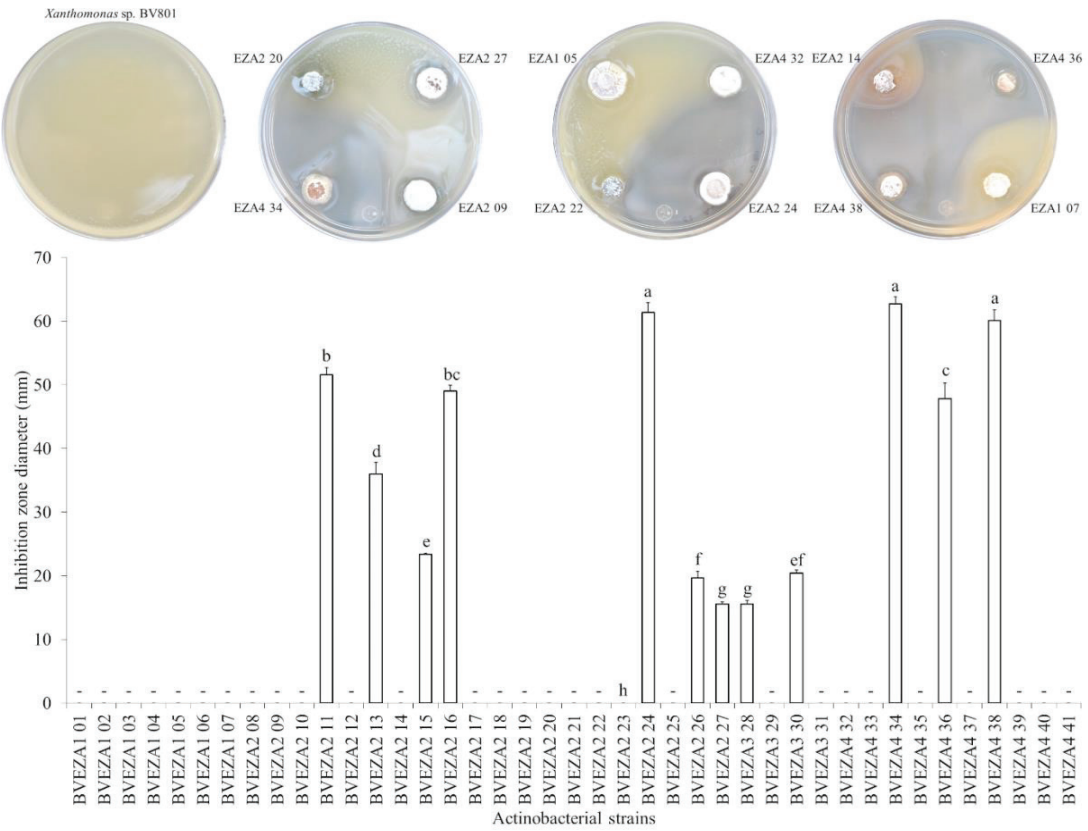

Figure 3. *In vitro* antagonistic activity of actinobacterial strains isolated from the rhizosphere of avocado trees on *Xanthomonas* sp. BV801 growth. The bars above the rectangle represent the standard deviation (n = 3). - = no inhibition. Different letters indicate significant differences according to Tukey's test ($P = 0.05$).

On the other hand, plant rhizosphere is a source of antagonistic actinobacteria against phytopathogen bacteria. *Streptomyces* spp. antagonists isolated from the rhizosphere of healthy potato plants have been reported against soft rot caused by *Pectobacterium* spp. (Mansour *et al*., 2008; Baz *et al*., 2012). Rincón-Enríquez *et al*. (2014) isolated 80 actinobacteria from agave rhizosphere, of which 17.5% inhibited growth *in vitro* of *Pseudomonas syringae* pv. *phaseolicola*, which causes halo blight in bean. Suárez-Moreno *et al*. (2019) reported that 60 actinobacteria, isolated from the rhizosphere of rice crops, only 5% showed antibacterial activity against *B. glumae* and *Pseudomonas fuscovaginae* rice phytopathogen bacteria. Muangham *et al*. (2015) reported a great proportion of rice and rubber tree rhizosphere actinobacteria; of the 210 isolates, 57.1% showed antagonistic activity against *X. oryzae* pv. *oryzae* with inhibition zones from 1 to 18.5 mm. In another study, Mingma *et al*. (2014) demonstrated that rhizosphere and roots of leguminous plants were a source of antagonistic actinobacteria of the genera *Streptomyces* and *Amycolatopsis* against *X. campestris* pv. *glycine*, which causes the bacterial pustule of soybean. In this study, the actinobacterial strains BVEZA2 24, BVEZA4 34, and BVBZA4 38, isolated from avocado tree rhizosphere, showed the greatest antagonistic activity against *Xanthomonas* sp. These strains showed the potential as biocontrol agents of the bacterial spot. Further studies are needed to determine the identity of the actinobacterial strains, as well as perform studies on secondary metabolite production and their role as growth inhibitors.

CONCLUSIONS

This study characterized the antagonistic activity of 41 actinobacterial strains isolated from avocado tree rhizosphere against *C. gloeosporioides* and *Xanthomonas* sp. BV801; some of them; BVEZA2 15, BVEZA2 27, and BVEZA3 30, showed antifungal

and antibacterial activity. These results showed the importance of rhizosphere actinobacteria as antagonists of phytopathogen microorganisms and suggest that the strains BVEZA1 02, BVEZA2 24, BVEZA4 34, and BVEZA4 38 could be used in a near future as biological control agents of anthracnosis and the bacterial spot diseases caused by *C. gloeosporioides* and *Xanthomonas* sp., respectively.

ETHICS STATEMENT

Not applicable.

CONSENTMENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY

The sets of data used or analyzed during this study are available through the corresponding author upon reasonable request.

CONFLICT OF INTERESTS

The authors declare no competing financial interests.

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AUTHORS' CONTRIBUTION

Conceptualization: J.R.T.C. and E.E.Q.A. Methodology: J.R.T.C., E.E.Q.A., Z.E.M., and G.R.E. Formal analyses: J.R.T.C. and G.R.E. Research: J.R.T.C. Resources: E.E.Q.A. and G.R.E. Data curation: J.R.T.C. Original draft preparation and translation: J.R.T.C. and E.E.Q.A. Review and edition: J.R.T.C., E.E.Q.A., G.R.E., and Z.E.M. Supervision: E.E.Q.A., G.R.E., Z.E.M., C.G.G., J.N.E.V. and L.L.P. Project administration and funding acquisition: E.E.Q.A.

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