Article

Biological control of *Lasiodiplodia theobromae* and *Fomitopsis meliae* causing the regressive death of citrus

Laura Glenys Polanco-Florián[1,2]
Omar Guadalupe Alvarado-Gómez[1]
Emilio Olivares-Sáenz[1]
Ramiro González-Garza[3]
Orquídea Pérez-González[1§]

[1]Autonomous University of Nuevo León. University Av. s/n, University City, San Nicolás de los Garza, Nuevo León. CP. 66455. (omaralvarado085@gmail.com; emolivares@gmail.com). [2]Dominican Institute of Agricultural and Forestry Research. Street Rafael Augusto Sánchez No. 89, Ensanche Evaristo Morales, Santo Domingo, Dominican Republic. (polancolaura7@gmail.com). [3]Bioscience SA de CV. Monterrey, Nuevo León. (biociencia01@prodigy.net.mx).

[§]Corresponding author: orquideapg@hotmail.com.

Abstract

The research was carried out in the state of Nuevo León, Mexico, during 2018, with the aim of isolating and identifying strains of soil microorganisms and evaluating their antagonistic capacity *in vitro* against citrus pathogens *Lasiodiplodia theobromae* and *Fomitopsis meliae*. For the isolation of the microorganisms, 17 rhizosphere samplings were carried out in different locations and types of vegetation; the samples were heat treated and spread by extension in the potato dextrose agar culture medium added with 0.3% yeast extract and 0.3% malt extract (PDLMA). The isolates obtained were preliminarily evaluated by observation and growth comparison. Of 70 isolates, 15 presented antagonistic activity, which showed inhibition percentages of 39-91%. The actinomycete strains M4 R and M1-101, and the fungus M104 (*Penicillium citrinum*) caused 84, 91 and 85% inhibition respectively against *L. theobromae* and 83, 91 and 91% respectively against *F. meliae*. Isolation of bacteria was achieved, actinomycetes and fungi from the soil capable of inhibiting the growth of *L. theobromae* and *F. meliae* fungi causing regressive citrus death.

Keywords: *Fomitopsis meliae*, *Lasiodiplodia theobromae*, antagonism, inhibition.

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Introduction

Citrus fruits (*Citrus* spp.) are considered the universal fruit since they are present in more than 100 countries and are the most economically important group of fruits, covering 20% of the world fruit market. Currently, the majority of the citrus harvest comes from 10 countries that contribute 77% of production, of which China and Brazil produce 42% and Mexico occupies the fourth position after the USA (Lerma *et al*., 2015).

Diseases are a limiting factor in the production of these crops in the producing areas of Mexico, caused by around 50 pathogens that cause various symptoms such as gummy, cracking, fruit drop, greasy spot, scab, death of branches, among others. (Orozco *et al*., 2013). The fungus *Lasiodiplodia* spp. has been reported as causing regressive death and other symptoms in citrus and in a wide range of crops in various countries (Adesemoye *et al*., 2014; Rodríguez *et al*., 2016).

Recently Polanco *et al*. (2019) reported to *Lasiodiplodia theobromae* and *Fomitopsis meliae* as the fungi that cause the regressive death of citrus in trees in orchards in the states of Nuevo León and Tamaulipas, Mexico. In general, the method of controlling this fungus is through applications of synthetic fungicides. Chemical fungicides can produce side effects such as resistance to pests and diseases, the reduction of populations of beneficial microorganisms and contamination of the environment (Adeniyi *et al*., 2013; Tovar *et al*., 2013; Alvárez, 2015).

The agents used for biocontrol significantly reduce the populations of phytopathogens and with their use it is intended to balance the ecological disturbances caused by agrochemicals (Badii and Abreu, 2006) through various mechanisms such as the production of toxins (Carisse *et al*., 2001), the competition for nutrients and space (Mutawila *et al*., 2011), inhibition of mycelial growth of the pathogen and mycoparasitism (Cheng *et al*., 2012).

Among the groups of microorganisms used for biocontrol are actinomycetes, which have been extensively studied for their ability to produce antibiotic and antifungal compounds (Lee *et al*., 2002), especially against plant pathogens of agricultural importance (Sreevidya and Gopalakrishnan 2012; Abdallah *et al*., 2013; Dávila *et al*., 2013). Actinomycetes are gram positive bacteria, generally found in soil, rivers, lakes, decomposing organic material, among other places. Many are saprophytes and some species are pathogenic (Quiñones *et al*., 2016), as is the case of *Streptomyces scabiei* causing common scabies from potato cultivation.

For several decades, the number of actinomycetes used in agriculture has been constantly increasing as a means of controlling or reducing plant pathogen populations (Dávila *et al*., 2013). Although the microorganisms have been extensively studied in the control of phytopathogenic fungi, few works have been carried out in the control of *Lasiodiplodia theobromae* and *Fomitopsis meliae* fungi. Sajitha and Florence (2013); Kamil *et al*. (2018), evaluated the antagonistic capacity of actinomycetes for the control of *Lasiodiplodia theobromae* causing damage to rubber tree wood and the regressive death of mango plants, respectively, with good results; in addition Caro *et al*. (2019) working with actinomycetes obtained promising results for the control of *Lasiodiplodia* sp., in potato cultivation.

Even so, there is little that is known of control works of the main fungi that cause the regressive death of critics, *Lasiodiplodia theobromae* and *Fomitopsis meliae*, which are normally treated with the use of chemical products or sanitary practices of the culture (pruning). Therefore, the objective of this work was to isolate strains of soil microorganisms and evaluate their antagonistic capacity *in vitro* against the phytopathogens *Lasiodiplodia theobromae* and *Fomitopsis meliae*, causing the regressive death of citrus fruits.

Materials and methods

Phytopathogenic fungal strains

The fungal strains *Lasiodiplodia theobromae* and *Fomitopsis meliae* were provided by the Faculty of Agronomy of the Autonomous University of Nuevo León (FAUANL).

Isolation of microorganisms from the soil

17 soil samplings with different types of vegetation were carried out in areas of the state of Nuevo León (Table 1), samples of the rhizosphere were collected at a depth of 10 to 15 cm, after removing the surface layer. For sampling, five different points were randomly selected to form a sample. Samples were placed in plastic bags, sealed, and stored in the laboratory in cool weather conditions.

Table 1. List of sampling locations and predominant vegetation type.

Location	Coordinates	Type of vegetation
FAUANL, Marin *Campus*, Marin Municipality	25° 52’ 25.76” north latitude; 100° 3’ 21.25” west latitude	Citrus cultivation and wasteland
FAUANL, La Ascensión *Campus*, Aramberri municipality	24.32° north latitude; 99.91° west latitude	Garlic cultivation
Mina municipality	25° 52’ a 26° 44’ north latitude; 100° 26’ a 101° 13’ west latitude	Wild plants

One gram of each sample was dissolved in 9 mL of sterile distilled water. The dilutions were heat treated (50 °C) for 10 min. Serial dilutions 1:100, 1:1 000 and 1:10 000 were prepared from each suspension. From each dilution 100 µL were transferred to Petri dishes with potato dextrose agar added with yeast extract (0.3%) and malt extract (0.3 %) (PDLMA) using the extension method. Petri dishes were incubated at 25 ±2 °C. Microorganisms exhibiting inhibition halos were isolated and purified (Oskay *et al*., 2004).

Morphological and genetic characterization of actinomycetes and bacteria with antagonistic activity

Actinomycete strains were identified according to their macroscopic growth characteristics (shape, color of aerial mycelium, texture, and pigment production in the culture medium). Additionally, microscopic observations were made using Gram stain. For the identification of the bacteria, the shape, color and texture of the colonies were considered, for the microscopic visualization the Gram stain was used.

For molecular identification of actinomycetes, DNA was extracted using the DNeasy Plant Mini Kit™ (Qiagen Inc.) and amplified by PCR using primers 8F (5'-agagtttgatcctggctcag-3') and 1492R (5'-ggttaccttgttacgactt-3') (Sacchi *et al*., 2002). Amplification reactions were carried out in a Thermo™ thermocycler, which consisted of denaturation at 94 ºC for 1 min, followed by 35 cycles at 94 ºC for 30 s, ringing at 48 ºC for 50 s, and extension at 72 ºC. for 80 s and a final extension at 72 ºC for 4 min. Products were visualized by 1% agarose gel electrophoresis stained with 0.5 ng μl^{-1} of ethidium bromide. The ladder-100™ (Axygen) molecular weight marker was used.

Morphological and genetic characterization of fungi with antagonistic activity

The morphological identification of the isolates obtained was based on the characteristics of the colonies (color, type and shape) in addition to the morphology of conidia and conidiophores using an optical microscope (Urbaez *et al*., 2013). In addition, lamella mounts were made using the cotton blue lactophenol dye as a solution, and the particular characteristics of the structures were described. Fungi were identified following the criteria of Barnett and Hunter (1998); Phillips *et al*. (2013).

For genetic characterization, genomic DNA was extracted from mycelium following the instructions of the manufacturer of the DNeasy Plant Mini Kit™ (Qiagen Inc.). DNA was quantified with a Take3™ spectrophotometer (Bioselec). The ITS-1 region, the 5.8S ribosomal gene and ITS-2 were amplified by PCR using the ITS-1fu 5'-tccgtaggtgaacctgcgg-3' and ITS-4 5'-tcctccgcttatttgatatgc-3' primers (White *et al*., 1990). Amplification reactions were carried out in a Termo™ thermocycler, which consisted of denaturation at 94 ºC for 1 min, ringing at 55 ºC for 25 s, polymerization at 72 ºC for 50 s, with 35 cycles, followed by a final extension at 72 ºC for 4 min. The PCR products were sequenced and compared with GenBank sequences to confirm the identity of the strains at the species level.

***In vitro* antagonism test**

A preliminary test was carried out to select the strains that presented the greatest inhibitory effect on pathogenic fungi. The confrontations were carried out with each strain of the microorganisms isolated against the phytopathogenic fungi *L. theobromae* and *F. meliae*. A hoe of each of the microorganisms was seeded at the four cardinal points (3 cm distance from the center, in Petri dishes) in the PDLMA medium. Forty-eight hours later, a 0.5 cm growth explant from each plant pathogen fungus was placed in the center. The boxes were incubated at 25 ±2 ºC for 5 days. Each microorganism was taken as a treatment with three repetitions, considering each Petri dish as one repetition and a negative control with each fungus without antagonist. The test was carried out twice.

The inhibitory capacity of the microorganisms was quantified by measuring the diameter of the fungal growth with a vernier, using two cardinal diameters previously drawn on the bottom of each Petri dish as a reference.

The percent inhibition was calculated using the following formula: (%) inhibition = [(A1-A2)/A1] x 100. Where: A1= growth of the phytopathogen colony (control); A2= growth of the fungal colony of the phytopathogen growing against the actinomycete.

Based on the results of the preliminary tests of the *in vitro* confrontations, the M1-101 and M104 strains were selected for a second test because they presented the highest inhibition values. The antagonist strains were grown in potato dextrose broth added with yeast extract and 0.3% malt extract (CPDLM) and incubated at 25 ±2 ºC and 125 rpm for 10 days. Subsequently, the content was centrifuged, filtered and placed in sterile bottles.

The inhibition activity of each antagonist was evaluated at concentrations of 25, 50 and 100% of the filtrate. The technique consisted of spreading 100 µl of the antagonist by extension. Subsequently, a 5 mm disk of the mycelial growth of the plant pathogen was placed in the center of the Petri dish. In addition, each antagonist was confronted with the two phytopathogenic fungi at the same time, for which 100 µl of the antagonist were spread by the box extension method and immediately a disc of mycelial growth of each fungus of 5 mm diameter was placed at a 4 cm distance between them. Daily observations were made for five days, at the end of which the mycelial growth of the fungi was measured, when the growth of the control completely covered the Petri dish.

Inhibition of germination of conidia and mycelium

Based on the preliminary results, the M1-101 strain was selected for the conidia and mycelium germination inhibition test of the fungi *F. meliae* and *L. theobromae*, respectively. The M1-101 strain was seeded in 50 ml of CPDLM broth by adding a square of the strain on agar of approximately 1 cm^2 for each Erlenmeyer flask 250 mL fired. The flasks were incubated with shaking at 125 rpm and 25 ± 2ºC for 10 days. At the end of the incubation period, the culture was filtered and the filtrate was diluted to concentrations of 25, 50 and 100%.

For the preparation of the inoculum of the phytopathogenic fungi, strains incubated for 6 days in potato dextrose agar (PDA) were used. 10 ml of sterile distilled water was added to each Petri dish to resuspend *F. meliae* conidia and *L. theobromae* mycelium by scraping the surface of the box with a bacteriological loop. The inoculum was preserved in 15 ml conical tubes.

Germination inhibition activity of the antagonist was evaluated at concentrations of 25, 50 and 100%. The technique consisted of spreading, with the help of a glass handle, 100 µl of each concentration in each Petri dish. After absorption, 100 µl of the inoculum of the plant pathogens was added to the previously prepared solution (conidia and mycelium) of the plant pathogens. Three replicates of each concentration were evaluated and the Petri dishes were incubated at 25 ±2 ºC. Observations were made every 24 h to see the inhibition of conidia germination and possible changes in mycelial morphology.

The count of germinated and non-germinated conidia was performed using a compound microscope (Leica DM500). 100 conidia cm^{-2} were counted, 500 conidia in total (germinated and non-germinated). The following formula was used to determine the percentage of germinated conidia: % germination = (a/a + b) x 100. Where: a= number of germinated conidia; and b= number of conidia without germinating. With *L. theobromae*, only the changes in mycelial morphology induced by the presence of the antifungal compounds produced by the antagonist were observed at a microscopic level.

Results

70 strains of microorganisms were isolated, of which 15 had inhibitory activity, and of these, 8 had typical characteristics of actinobacteria, 5 bacillary bacteria and 2 fungi (Table 2).

Table 2. Microorganisms isolated from soil from different areas of the state of Nuevo León with antagonistic activity.

Strains	Sampling location	Classification
A7	Mina	Bacillus
A14	Mina	Bacillus
A18	Mina	Bacillus
A20	Mina	Bacillus
A23	Mina	Bacillus
CH 102	La Ascensión	Actinomycete
CH 103	La Ascensión	Actinomycete
M 104	Marin	Fungus
M1-101	Marin	Actinomycete
M2 R1	Mina	Actinomycete
M2 R2	Mina	Actinomycete
M3	Marin	Actinomycete
M4	Marin	Actinomycete
M4 R	Mina	Actinomycete
M5-102	Marin	Fungus

Morphological and genetic characterization of actinomycetes and bacillary bacteria

The colonial growth characteristics of the actinomycetes in culture were dry, powdery aerial masses with irregular edges, of various pigmentations, showing shades of yellow, gray and others of orange. Under the microscope (40X) they showed the typical ramifications of actinomycetes. Gram staining revealed Gram positive sporulated streptobacilli.

The bacteria showed variable shades such as yellow and cream (Figure 1), with a watery texture and regular edges. Gram staining showed Gram positive and Gram negative rods. Molecular identification of actinomycete strains was not achieved due to a lack of PCR amplification possibly attributed to the fact that primers or primers have been used more with other bacteria and may not have sufficient specificity to bind to actinomycete sequences.

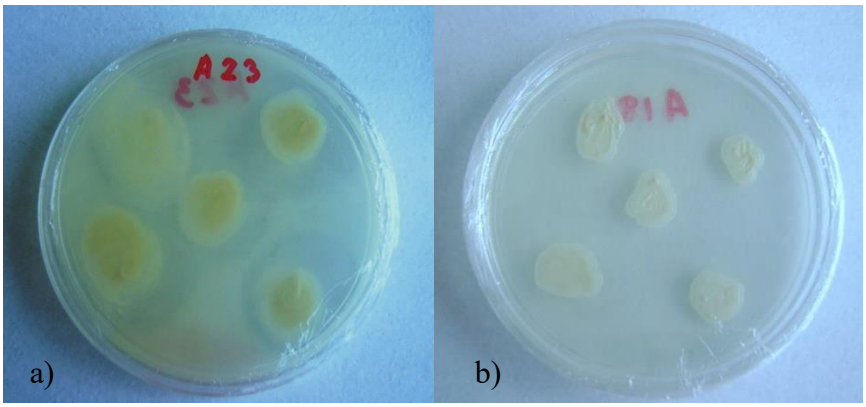

Figure 1. Bacteria isolated from the soil. a) strain A23 showing halo of inhibition; and b) strain A18.

The obtained bacillary bacteria were amplified by PCR and part of the 16S gene was sequenced. The consensus sequences of the bacillary bacteria were compared with sequences deposited in GenBank through the Blast program; strain A18 corresponded to *Achromobacter xylosoxidans* with 96% homology and A23 showed 96% similarity to *Delftia* sp.

Morphological and genetic characterization of fungi with antifungal activity

Colonies of strain M104 in culture medium were white, compact and with irregular edges, and the mycelium of strain M5-102 was brown (Figure 2). The M104 strain was identified as *Penicillium* sp. The characteristics of the conidiophore showed brush shape and hyaline conidia, unicellular in a cylindrical shape. The M5-102 strain presented conidiophores and aspergillary heads, hyaline, unicellular and cylindrical conidia; this strain was identified as *Aspergillus* sp. Morphological identification was confirmed by its DNA sequence, corresponding to *Penicillium citrinum* and *Aspergillus* sp.

In vitro antagonism test

Acromobacter xylosoxidans reduced the mycelial growth of *L. theobromae* 63% and *F. meliae* 59%. On the other hand, *Delftia* sp., inhibited both plant pathogenic fungi by 39%. *Aspergillus* sp., reduced the growth of *L. theobromae* and *F. meliae* by 83%. The strain identified as *P. citrinum* was able to reduce *L. theobromae* mycelial growth by 83% by keeping it inhibited for more than 20 days. The highest inhibition values were obtained with the fungus *P. citrinum* (M104) and the acinomycetes M4R, and M1-101, which showed percentages of inhibition of 85, 84 and 91% against *L. theobromae* and 91, 83, and 91%, against *F. meliae* respectively.

In the inhibition test with the different concentrations of the actinomycete and *P. citrinum* filtrates (25, 50 and 100%), there were statistically significant differences ($p< 0.001$) in the percentage of inhibition of the pathogens. The control showed the highest mycelial growth at 5 days, after the fungi were placed on PDA. *L. theobromae* and *F. meliae* fungi had a similar growth, showing no significant differences between them. The treatments that showed greater inhibition of the pathogens were *L. theobromae* and *F. meliae* against *P. citrinum* at 100%, followed by *L. theobromae* + *P. citrinum* and *F. meliae* + *P. citrinum* with the concentration at 100%. There were no differences between the three concentrations of the M101 strain against *F. meliae*. In the

inhibition test with the different concentrations of the filtrates of actinomycete and *P. citrinum* (25, 50 and 100%), there were statistically significant differences (p= 0) in the percentage of inhibition of the pathogens.

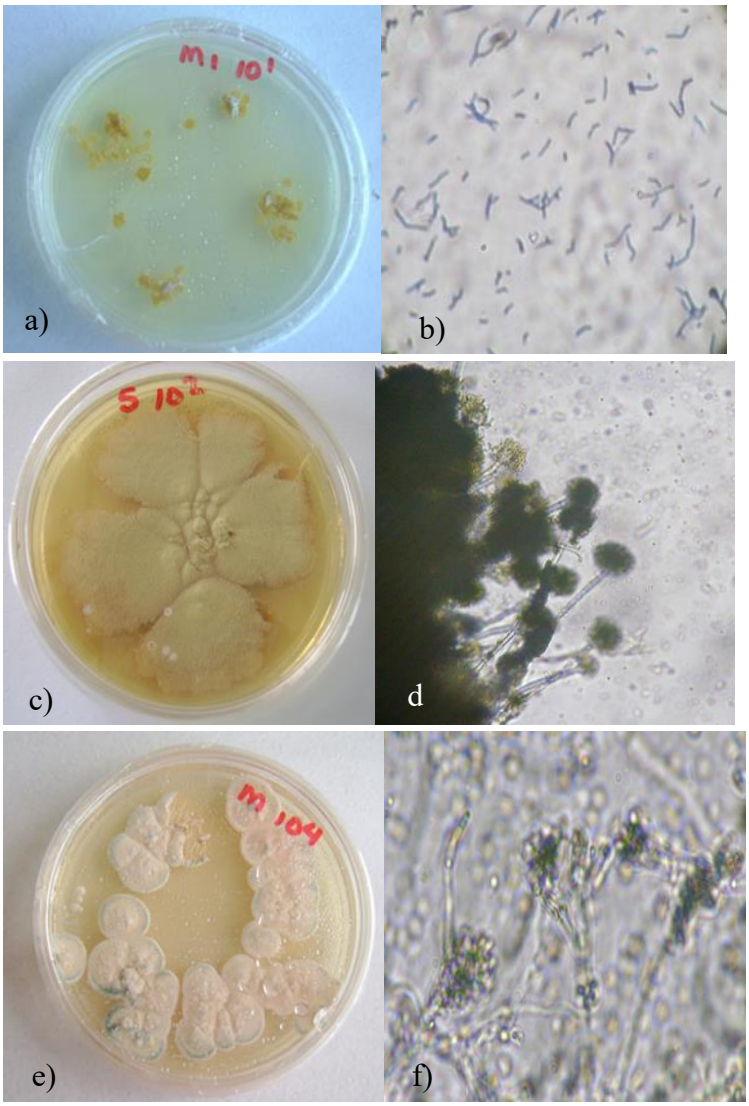

Figure 2. Microorganisms with antifungal activity. a) colonies of strain M1-101 in PDA; b) Gram positive bacillus observed under a microscope; c) strain M5-102 in PDA; d) conidiophores and aspergillary heads of *Aspergillus* sp.; e) strain M104 in PDA; and f) conidiophores, phialid and conidia of *P. citrinum*.

Inhibition of germination of conidia

L. theobromae at 96 h of incubation completely covered the Petri dish without obtaining any reproductive structure. However, in the presence of the antagonist, *L. theobromae* produced dark brown chlamydospores and conidiogenous cells (Figure 3). With the confrontation, the conidia of *F. meliae* are inhibited and were not able to germinate, the conidia were observed to clump and thicken, tending to form chlamydospores (Figure 3).

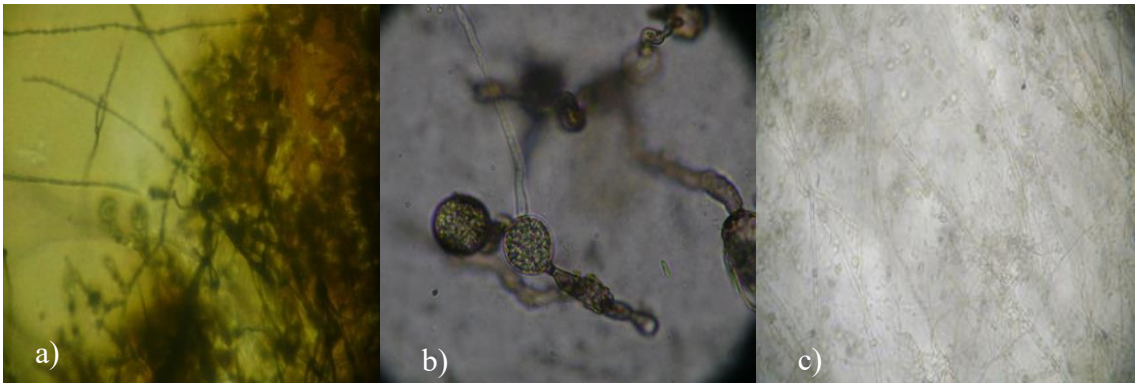

Figure 3. Growth of *L. theobromae* under confrontational conditions. a) conidiogenous cells; b) and c) chlamydospores.

Discussion

In this study the bacteria *A. xylosoxidans* was effective in reducing the in vitro growth of *L. theobromae* and *F. melie*. The ability of this bacterium to biocontrol these pathogens is comparable to the results reported by Moretti *et al*. (2008), who used this bacterium to control *Fusarium oxysporum* causing tomato wilt. Bagheri and Ahmadzadeh (2016) also reported that *A. xylosoxidans* can inhibit *Curvularia lunata* and *Bipolaris sorikinian* present in wheat crops.

On the other hand, some species of the *Delftia* genus have been reported by Han *et al*. (2005) inhibiting *Xanthomonas oryzae* pv. *oryzae*, *Rhizoctonia solani* and *Pyricularia oryzae*. Additionally, these bacteria play an important role in bioremediation technologies (Ubalde *et al*., 2012). Jergensen *et al*. (2009) cite *Delftia* with the ability to break the glycosidic bonds in chitin and peptidoglycan, which could show greater inhibition capacity of phytopathogenic fungi like those used in this study.

In general, species of the genus *Aspergillus* are considered as saprophytic or opportunistic fungi (Cortes and Mosqueda, 2013). Some races of *Aspergillus* sp. are considered as atoxigenic because they do not produce aflatoxins and have been registered as biocontrolling strains, whose mechanism of action is competitive exclusion (Cotty, 1994). Due to these qualities, an *Aspergillus* strain was formulated and has been successfully marketed as a biocontrol agent for the aflatoxin-producing species *Aspergillus flavus* (Dorner, 2009).

In this study, *Aspergillus* sp. was able to inhibit the growth of *L. theobromae* and *F. meliae*. These results are confirmed by the findings obtained by Adeniyi *et al*. (2013) who reported *A. niger* as a biocontrol agent for *L. theobromae*, although they suggest that its potential in other phytopathogens should be exploited and possible adverse effects in crops should be managed.

Despite the fact that the fungus *P. citrinum* can cause diseases in field crops and post-harvest products (Marquardt, 1996), according to our results, some *Penicillium* species also have the potential to inhibit pathogens. These results can be explained according to Quiroz *et al*. (2008), who reported that different species of fungi have both antagonistic and pathogenic mechanisms.

Of the 15 strains that were isolated and evaluated, actinomycetes showed the highest inhibition values (M4 R and M1-101), with M1-101 being the strain with the highest antagonistic activity, with percentages of inhibition against *L. theobromae* and *F. meliae* of 84 and 91%, respectively. Sánchez *et al*. (2011); Wang *et al*. (2015) isolated actinomycetes with the ability to inhibit agriculturally important plant pathogens such as *Fusarium equiseti*.

The results obtained in this study demonstrate that the ability of microorganisms to inhibit pathogens can be increased or decreased, depending on the conditions of the survival structures that the latter can produce and that the antagonist manages to reduce their growth by the production of antifungal compounds or competitive exclusion.

Conclusions

Isolation of actinomycetes was obtained from soil samples, capable of inhibiting the growth of pathogens that cause the regressive death of citrus *L. theobromae* and *F. meliae*. The M4R and M104 strains presented percentages of inhibition greater than 84% against the previous phytopathogens.

Aspergillus sp. and *P. citrinum* were able to reduce the growth of *L. theobromae* and *F. meliae*. Due to the antagonistic capacity demonstrated in the present study, the actinomycete strains M4R and M104 and the fungi *Aspergillus* sp. and *P. citrinum* could be considered as potential candidates to be used in *L. theobromae* and *F. meliae* control programs, but it is necessary to continue with the studies of this antagonistic capacity *in vivo*.

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