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ANTAGONISM OF *Bacillus licheniformis* M2–7 AGAINST PHYTOPATHOGEN FUNGI OF *Mangifera indica* L.

Antagonismo de *Bacillus licheniformis* M2–7 contra hongos fitopatógenos de *Mangifera indica* L.

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Key words: *Aspergillus oryzae*, *Colletotrichum* sp., *Aspergillus niger, Mangifera indica*

ABSTRACT

The biological control using organisms that inhibit growth or the appearance of pests or diseases has proven to be a successful alternative for mango disease control. In this work, *in vitro* analyses were carried out with *Bacillus licheniformis* M2–7 and *B. licheniformis* LYA12 strain derived from M2–7 against the phytopathogenic fungi *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*. Besides, the effect of bacteria on the micellar structure of phytopathogenic fungi was observed and evaluated through real-time PCR, the expression of two main metacaspases (*casA* and *casB*) that triggers the process of programmed cell death in fungi. The results obtained showed that both bacterial species inhibited by 45, 40, and 35 % the fungal growth of *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*, respectively. The bacteria presence affected the mycelial appearance of the fungi because they presented fragmentation of the hyphae with intracellular inclusion bodies, swellings, malformations and scarce growth of the hyphae. The gene expression results conclude that the LYA12 and M2-7 strains against *A. oryzae* and *A. niger* have a fungicidal effect; therefore, the bacterial strains could be used in disease control in mango.

Palabras clave: *Aspergillus oryzae*, *Colletotrichum* sp., *Aspergillus niger*, *Mangifera indica*

RESUMEN

El control biológico utilizando organismos que inhiben el crecimiento o la aparición de plagas o enfermedades ha resultado una alternativa que ha tenido éxito en la prevención de enfermedades del mango. En este trabajo se realizaron análisis *in vitro* con las cepas de *Bacillus licheniformis* M2–7 y la cepa de *B. licheniformis* LYA12 derivada de la M2–7 contra los hongos fitopatógenos del mango: *Aspergillus oryzae*, *Colletotrichum* sp. y *Aspergillus niger*. Además, se observó el efecto de las bacterias sobre la estructura micelar de los hongos fitopatógenos y se evaluó mediante PCR en tiempo real, la expresión de dos principales metacaspasas (*casA* y *casB*) que desencadenan el proceso de muerte celular programada en hongos. Los resultados obtenidos demostraron que ambas especies bacterianas inhibieron en un 45, 40, y 35 % el crecimiento fúngico de *Aspergillus oryzae*, *Colletotrichum* sp. y *Aspergillus niger,* respectivamente. La presencia de las bacterias afectó la apariencia micelial de los hongos debido a que presentaron fragmentación de las hifas con cuerpos de inclusión intracelular, hinchamientos y malformaciones, además de escaso crecimiento de las hifas. Los resultados de la expresión génica concluyen que la cepa LYA12 y M2-7 frente a *A. oryzae* y *A. niger* tienen un efecto fungicida; las cepas bacterianas podrían usarse en el control de enfermedades en mango.

INTRODUCTION

Mangifera indica L., commonly known as mango, is a tropical fruit that plays an important economic and social role globally, especially in developing countries. Mexico has a global production of around 27 million tons. Hence, it ranks third in production and fourth place regarding marketing and exports worldwide (FAO 2017). Currently, due to the high production, 100 % of the national requirements are satisfied with domestic production. In 2016, Mexican exports increased by 34.75 %, which is why they have been commercialized in 27 international destinations, mainly to the United States (65.41 %), Canada (63.86 %), and Japan (47.66 %) (SAGARPA 2017). The main varieties cultivated in Mexico are Ataulfo, Manila, Haden, Kent, and Criollo. The first three are commercialized in the international market (CONASPROMANGO 2012). There are 24,793 ha cultivated, with an annual production of 353,095 tons of fruit. Mango production is an important source of employment, income, and foreign exchange generation. Therefore, the fruit's quality is critical since it determines the type of market (national or international), the consumer, and the product's price (SAGARPA 2017).

Mangifera indica L. is a species affected by different diseases of bacterial and fungal origin. Naerly 90 pathogens are known (Njuguna et al. 2016); these pathogens alter plants' physiological functions, affecting their functioning, decreasing their yields and damaging the fruit (Janisiewicz and Korsten 2000). Among the most important quality problems in mango are post–harvest diseases (Arauz 2007); losses from these diseases range from 20 to 33 % (Pantástico 1979, Serrano–Carreon et al. 2010). The most common diseases caused by fungi are anthracnose, caused by *Colletotrichum* sp., a fungus that affects the foliage, stems, flowers, and fruit. This fungus produces small or large dark brown spots that become blackish with slightly sunken injuries (Arauz 2007). Another disease in mango is the rot caused by the fungus *Aspergillus niger*. This disease is characterized by irregular areas, with a tendency to be circular, wet, brown, or brown–purple; the lesions have a wrinkle in the center, which over the days becomes black with mold rot (Verma and Kamal 1951). *Aspergillus oryzae* is a phytopathogenic agent that causes deterioration of seeds, fruits and leaves in *Mangifera indica* L. (Verma and Karmal 1951); the infections caused by this fungus are physically characterized as small black spots; being this a visual disease decreases its commercialization because it does not have the physical appearance sought by traders.

Currently, the control of the fungal diseases of *Mangifera indica* L. is based on the programmed spraying of chemical agents, especially during the production phase. The sprayings begin in the flowering when the panicles are several centimeters long, shortly before the floral opening and continue until the initiation of the fruits; in total, there are approximately 8 to 12 applications (Carrillo–Fasio et al. 2005). To avoid economic losses, farmers

need to improve the crop production processes, obtain good quality fruit, and reduce the application of chemical pesticides, thus facilitating the production of export quality fruit. An alternative that has been successful in controlling diseases in mango is the one that has employed organisms that inhibit growth or the appearance of pests or diseases. These products are called biological control agents (Galindo et al. 2015). In the biological world, there is a continuous interaction between potential pathogens and their antagonists, so that the latter contribute in most cases do not develop the disease (Fernández–Larrea–Vega 2001, Ongena and Jacques 2008). The inhibition deterioration gives microbial antagonism, or death of some species of microorganisms by another's action (Andrews et al. 1983, Fernández–Larrea-–Vega 2001, Souto et al. 2004). The antagonistic microorganisms have two modes of action that are reflected in two effects; the first can be a fungistatic effect, that is, it does not allow the growth, and the second a fungicidal effect, that is, it eliminates the pathogen by causing the programmed cell death. The latter is mediated by an apoptotic mechanism associated with the externalization of phosphatidylserine to the plasma membrane's outer shell, DNA fragmentation, the release of cytochrome C from mitochondrial activation and metacaspases expression (Fuchs and Steller 2011, Danay et al. 2009).

Metacaspases are cysteine-dependent proteases found in protozoa, fungi, and plants. Metacaspases are reported as indicators of cell death; positive expression of *casA*, and *casB* genes are participating as indicators of programmed cell death. Increased environmental stress may be associated with a shift from regulated death to uncontrolled death (Echeverri-Ruiz and Mockus–Sivickas 2010). Lee and collaborators (2008) reported metacaspase–dependent cell death using mutant strains of Yca1 yeast under oxidative stress.

The massive application and lack of knowledge of chemical substances to control agricultural diseases have caused notable negative effects on human health and damage to the environment and other organisms, becoming a global problem. On the other hand, biological control has proven to be a sustainable and ecological tool that, when well used, avoids the use and handling of chemical agents (Zavaleta–Mejía, 1999). Therefore, in the present work, we evaluated *in vitro* antagonistic activity of *Bacillus licheniformis* M2–7 (Guevara–Luna *et al*. 2018) and LYA12 (Serrano-Ángel *et al*. 2020) on *Aspergillus oryzae*, *Colletotrichum* sp, and *Aspergillus niger* phytopathogenic fungi of *Mangifera indica* L., as well as the effects of bacteria on the micellar structure and gene expression of *casA* and *casB* in fungi.

MATERIAL AND METHODS

Strains and growing conditions

The strain used was *Bacillus licheniformis* M2–7, a bacterium isolated from thermal waters in the Cocoyul municipality of San Marcos, Guerrero, and the strain of *B. licheniformis* LYA12 derived from the M2–7. The strains were grown on Luria Bertani agar (LB, 5 g/L Yeast extract, 10 g/L Casserole peptone, 5 g/L Sodium chloride). In contrast, the LYA12 strain culture medium was supplemented with spectinomycin (100 mg/mL), the Petri dishes were incubated at 30 °C for 24 hours The phytopathogenic fungi *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*, were planted on potato dextrose agar (PDA) and incubated at 37 °C for 12 days; these were preserved in 80 % glycerol.

Evaluation of the antagonism of M2–7 and LYA12 strains against *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*

In vitro bioassays were performed following the plate casting methodology to evaluate the antagonistic effects. The inoculum was prepared from bacteria of 24 hours incubation at 37 °C; later, in separate flasks with PDA medium, the inoculum of bacteria was added at an optical density (600 nm OD), 0.5-0.6 for both strains (M2–7 and LYA12) and 25 mL of culture medium was transferred into each Petri dish. In the Petri dish center, a 5 mm diameter disc of mycelium from the fungus was placed and incubated for 12 days at a temperature of 30 °C. The mycelium diameter was measured using a digital vernier, and the data obtained were used to calculate the percentage of inhibition using the equation 1 described by Tejera et al. (2012).

$$Percent\ inhibition = \left(\frac{C-T}{100}\right) \times 100 \qquad (1)$$

where C is the diameter of the fungus mycelium in the negative control (without bacteria) and "T" is the diameter of the mycelium in the plates treated with the bacteria (strains M2–7 and LYA12). As a control, a 5 mm diameter disk of mushroom mycelium was placed in the center of the Petri dish without bacterial inoculum. The tests were carried out in

triplicate. The analysis of variance of the data was performed using Sigmaplot 11.0.

Determination of hyphae damage during antagonism by Lactophenol staining

Staining was done with lactophenol (Thermo Scientific™ Remel) at a concentration of 0.5 g/100 mL. From 12 days of antagonism bioassays, slide portions of the fungus in antagonism with the bacteria and the control fungus (without bacteria) were sampled. 10 µL of were added to samples and examined in an optical microscope (Biological Binocular 40x 2500x Amscope B020c).

Quantification of *casA* and *casB* gene expression by real time PCR

Total RNA was extracted and purified from the phytopathogenic fungi *Aspergillus oryzae*, *Colletotrichum* sp., A*spergillus niger* with and without *B. licheniformis* M2–7 and B. licheniformis LYA12 following the protocol described by Rojas–Aparicio et al. (2018) with some modifications. The residual DNA was degraded with DNase I (Thermo Scientific) then the RNA was stored at –70 °C. The oligonucleotides used for *casA* were, *CasA* forward (5'TCCAACGA-ACCACATCTTCA'3) and *CasA* reverse (5'GAA-GCCAGTCTTCCAGTCG'3); for *casB* gene, were *CasB* forward (5'GCGTAGAGCCCTACTGATCG'3) and reverse (5'TGGTCCGCTTATA TGACCC'3). Fragments of the Ubiquitin gene; ubiq forward (5'GTCACCGACCCTAAACGAAA'3), ubiq reverse (5'GTCATCGTTGGTCACATTGG'3) were used as a control. For the complementary DNA (cDNA), the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and the reverse oligonucleotides were used for each problem and control genes. The reaction was as follows: 1 µg of total RNA, 20 pmol/µL of the corresponding reverse oligonucleotide of each gene, 2 µL of dNTP's (10 mM), 4 µL of 5X reaction buffer, 1 µL of RNAase inhibitor (40 U) and 0.5 µL of reverse transcriptase. The reaction was incubated at 42 °C for 1 hour and then inactivated at 70 °C for 10 min. The cDNA obtained was used as a template for the qRT-PCR reaction. The qRT–PCR was performed on a CFX96 Touch™. Each reaction contained: 1 µL of cDNA, 10 µL of Master Mix 2x (LightCycler® 480 SYBR Green I, from Roche) and 2.5 mM of corresponding oligonucleotide mixture. Each reaction was performed in triplicate. The amplification was carried out as follows: denaturation at 95 °C for 10 min., followed by 40 cycles at 95 °C for 10 seconds and 60 °C for 60 seconds for 39 cycles. The analysis of the relative expression was carried out using the method 2-ΔΔCT.

RESULTS

Bacillus licheniformis* M2–7 and LYA12 inhibit the in vitro growth of *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger

The fungus in antagonism with *B. licheniformis* M2–7 presented adecrease in its radial growth, with a whitis mycelium. It was onserver that *B. licheniformis* strain inhibited 45 ± 1 % of *Aspergillus oryzae* mycelial growth. On the other hand, without the bacteria's presence, the control fungus wholly covered the Petri dish 12 days after the evaluation, presenting an olive green coloration. In contrast, the fungus in antagonism with *B. licheniformis* M2–7 decreased radial growth with a whitish mycelium. The *B. licheniformis* LYA12 strain inhibited the mycelial growth of the fungus by 40 ± 0.8 %, with the mycelium showing a whitish color and little abundance at the edges (**Fig. 1a**).

Concerning the growth of *Colletotrichum* sp. without *Bacillus licheniformis*, a cottony mycelium with a growth of a radial form was observed; whereas, in the confrontation of *B. licheniformis* M2–7 against the fungus, inhibition of the mycelial growth of 40.4 ± 1.5 % was observed, presenting the fungus a mycelial growth of irregular form and scarce mycelium towards the edges. The mutant strain LYA12 inhibited mycelial growth of 40.4 ± 0.5 %, being observed in the mycelium of the confronted fungus a whitish cottony appearance (**Fig. 1b**). In the bioassay of antagonism of *B. licheniformis* M2–7 against the fungus *A. niger*, it was observed that the negative control and *Bacillus licheniformis* M2–7 presented a mutual antagonism during the 12 d of evaluation. However, *B. licheniformis* M2–7 inhibited the fungus by 35 ± 0.7 %. Also, in evaluating the antagonistic effect of the mutant strain of *B. licheniformis* LYA12 against *A. niger*, mutual antagonism between the mutant strain and the fungus was observed. However, *B. licheniformis* LYA12 inhibited the growth of the fungus by 34 ± 1.5 % (**Fig. 1c**).

The presence of *Bacillus licheniformis* M2–7 and LYA12 damages the structures of *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*

In order to know the viability of fungal cells in the absence and presence of *B. licheniformis* M2–7 and LYA12, the mycelial morphology of fungi was observed at 12 days of antagonism by lactophenol staining. In the bioassay, the control, *A. oryzae* without bacteria (**Fig. 2a**), presented regular growth whereas in the presence of *B. licheniformis* M2–7 (**Fig. 2b**), presented irregular growth,

damage to the hyphae was observed, with loss of cell content and malformation hyphae.

Meanwhile, the LYA12 strain against *A. oryzae* (**Fig. 2c**) caused hyphae malformation, resulting in thickening and/or acute thinning with blunt–looking tips. Regarding the structures of *Colletotrichum* sp., in the absence of the bacteria, it was found that the hyphae did not show visible damage (**Fig. 2d**). In contrast, in the *B. licheniformis* M2–7 bioassays, damages in the hyphae were observed, presenting malformations with irregular growth and fragmented and thick hyphae which had the appearance of cellular chains (**Fig. 2e**).

The presence of the LYA12 strain demonstrated more significant damage in the cellular structures of the hyphae of *Colletotrichum* sp., some showed apparent fragmentations with intracellular inclusion bodies and swellings and malformations, besides scarce growth of the hyphae (**Fig. 2f**). Similarly, in the evaluation of damage to hyphae in A. niger, the control was observed integral and well-defined hyphae (**Fig. 2g**), it was determined that *B. licheniformis* M2–7 caused cell damage, it was observed that the hyphae have a thin appearance, fragmented and undefined (**Fig. 2h**). Similarly, the LYA12 strain-induced *A. niger* damages in the hyphae bodies, being observed in fragile A. niger damages in the hyphae bodies, being observed very thin, fragile structures and inclusion bodies inside the hyphae (**Fig. 2i**).

***Bacillus licheniformis* has a fungicidal effect on *Aspergillus oryzae* and *Aspergillus niger* and a fungistatic effect on *Colletotrichum* sp.**

To determine the fungistatic or fungicidal effect of *Bacillus licheniformis* M2–7 and LYA12 on phytopathogenic fungi, the expression of two critical metacapas in signaling and triggering programmed cell death, *casA*, and *casB*, were evaluated. So the expression of one of them directs the death of the mushrooms (Colabardini et al. 2010). It was observed that *Aspergillus oryzae* overexpressed *casB* in the presence of *Bacillus licheniformis* M2–7 (**Fig. 3**) and LYA12 by 33 ± 0.7 % and 460 ± 20 %, respectively for the control (*Aspergillus oryzae* without *Bacillus licheniformis*, **Fig. 3**).

When analyzing the expression of the genes *casA* and *casB* in *Colletotrichum* sp. in the presence of

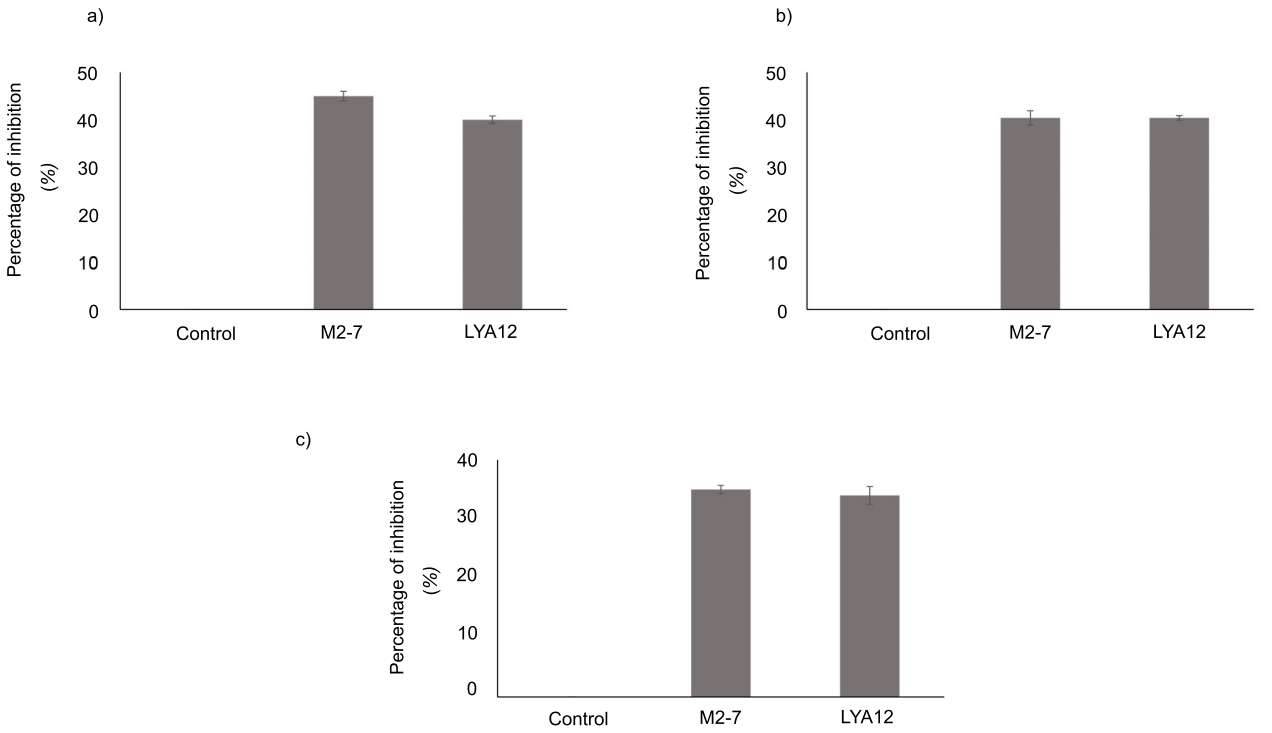

Fig. 1. Growth inhibition percentages of *Aspergillus oryzae* (a); *Colletotrichum* sp. (b) and *Aspergillus niger* (c) in the presence or absence of *Bacillus licheniformis* M2–7 and LYA12. Error bars are the standard deviations of three replicates.

Bacillus licheniformis M2–7 and LYA12 was not observed overexpression of both genes for the control (in the absence of the bacteria, data no show). Therefore, it can be concluded that the effect of *Bacillus licheniformis* on *Colletotrichum* sp. is fungistatic.

By analyzing the expression of the genes *casA* and *casB* in *Aspergillus niger* in the presence of the bacteria M2–7 and LYA12, It can be seen that the strain M2–7 manages promotes the over-expression of *casA* and *casB* by 250 ± 30 % and 190 ± 2 %, respectively (**Fig. 4a** and **b**).

While the LYA12 strain, did not promote overexpression (**Fig. 4a** and **b**), these data indicate that the *Bacillus licheniformis* strain M2–7 has a fungicidal effect on *Aspergillus niger*. In contrast, *Bacillus licheniformis* LYA12 has a fungistatic effect.

DISCUSSION

The antagonistic microorganisms that include bacteria and fungi can employ a biological control effect on different pathogenic organisms of interest.
This effect has been used to control various diseases in fruits and vegetables (Hernandez et al. 2016). The results obtained in the present study demonstrate that *Bacillus licheniformis* M2–7 and the strain LYA12 have antagonistic activity against the fungi *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*, phytopathogens of *Mangifera indica* L. in comparison, both bacterial species inhibited the growth in the same way (**Fig. 1**). These results agree with Baysal et al. (2008) report, where he mentions that the percentage of mycelium growth inhibition of *F. oxysporum* due to

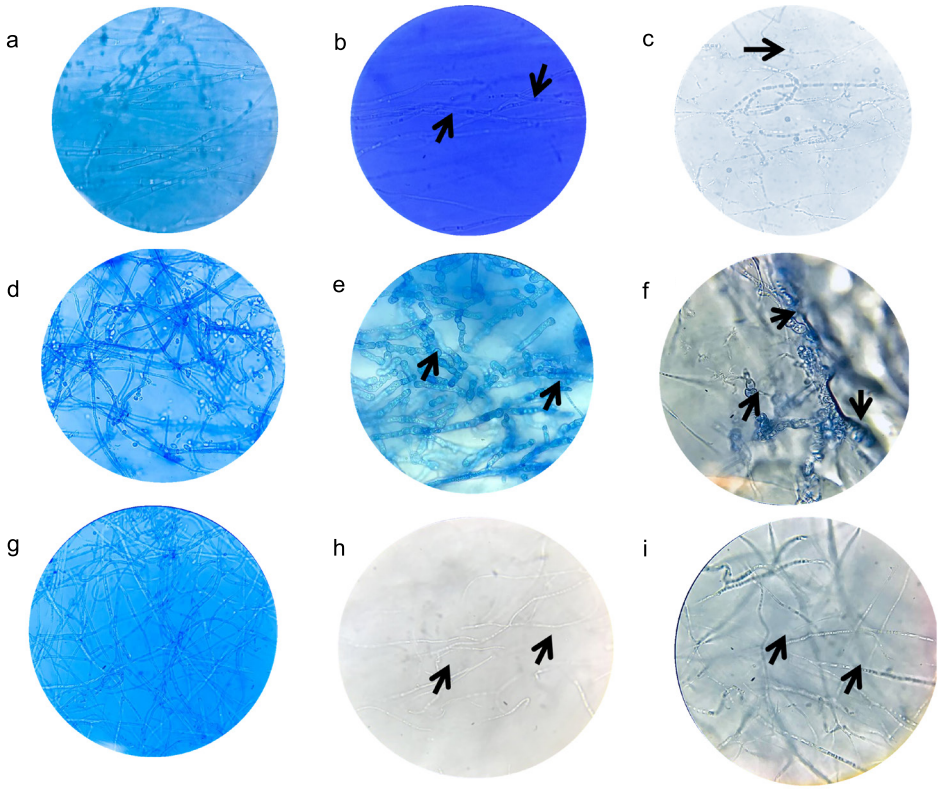

Fig. 2. Damage caused to the structures of the fungi in antagonism was observed at 40X. *Aspergillus oryzae* without bacterial inoculum (a); *A. oryzae* in the presence of *Bacillus licheniformis* M2–7 (b) and *A. oryzae* in the presence of *B. licheniformis* LYA12 (c); *Colletotrichum* sp. without bacterial inoculum (d) in the presence of M2–7 (e), in the presence of LYA12 (f) and *A. niger* without bacterial inoculum (g) in the presence of M2–7 (h) and LYA12 (i), the arrows indicate hyphae malformation.

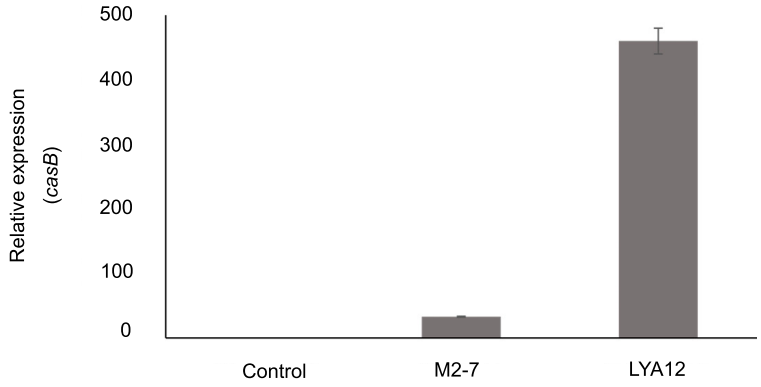

Fig. 3. Expression of *casB* of *Aspergillus oryzae* in the absence or presence of M2–7 or LYA12. Error bars are the standard deviations of three replicates.

Bacillus strains reaches up to 62 %. Drahos and West (2004) isolated from the soil a strain they identified as *Bacillus licheniformis*, performed antagonism trials against *Aspergillus*, *Sclerotinia*, *Rhizoctonia*, and *Pyricularia* strains, and demonstrated great potentials of *Bacillus* for the biocontrol of fungal diseases in tomato. However, the ability of *Bacillus licheniformis* M2–7 and LYA12 strains as biological control agents of phytopathogens has not been studied.

This work showed that the bacterial strains affected the mycelial morphology of the phytopathogenic fungi evaluated in this study because some fungi presented a decrease the color and cottony appearance in contrast to the control (**Fig. 2**). Hernández et al. (2016) explain that these observations could be due to the excretion of metabolites (cellulases, glucanases, lipases, proteases and chitinases) involved in the lysis of the hyphae cell wall, facilitating the insertion of specialized structures. Danay et al. (2009) stated that when there is a mycoparasitism, it loses the fungal cells' cytoplasmic content; the remaining cytoplasm is mainly surrounding the hyphae and therefore shows symptoms of disintegration alteration in pigmentation, which decreases its pathogenic activity. The damage observed in the structure of the hyphae morphology of the phytopathogenic fungi considered in the study showed many anomalies. Swelling and decreased cell content in the mycelium could be related to increased cell size due to a disorganization of the cytoplasm and rupture of the plasma membrane. Alteration and distortion of the cell wall at the sites of hyphae penetration of the antagonist and reach cell lysis caused by the bacteria M2–7 and LYA12.

These results confirm what was reported by Hernandez et al. (2016) and state that the staining is not uniformly appreciated due to cell content loss. Benhamou (2004) reported that in test of *Bacillus* sp. in antagonism with *Penicillium digitatum*, he observed that the pathogen's hyphae presented apparent cellular disorganization and were restricted to colonize the fruit surface. Thus, it seems that genus *Bacillus* causes alterations in mycelial morphology. The expression of *casA* and *casB* genes involved in programmed cell death in fungi indicates that *Aspergillus oryzae* expresses the *casB* gene in the presence of M2–Y and LYA12 bacteria, so they are exerting a fungicidal effect. Colabardini et al. (2010) demonstrated two genes *casA* and *casB* from *Aspergillus nidulans* metaclasses when antagonistically induced to cell death induced by stress in the endoplasmic reticulum.

On the contrary, the bacteria M2–7 and LYA12 have a fungistatic effect on *Colletotrichum* sp. because they only inhibited its growth and did not promote the expression of the genes involved in cell death (*casA* and *casB*, Data no show). Based on Santoyo et al. (2017), an antagonist agent cannot always biocontrol multiple phytopathogens, and several biocontrol agents, specific for each phytopathogen, may be required. Other biotic (phytopathogen resistance) and abiotic (environmental conditions) factors could influence biocontrol efficiency levels. Low gene expression levels were observed in the control of *Colletotrichum* sp. which according to Hernández–Salmerón et al. (2018), suggests the presence of phytopathogenic fungi modulates low gene expression. The bacterium M2–7 exerted a fungicidal effect on *Aspergillus niger*

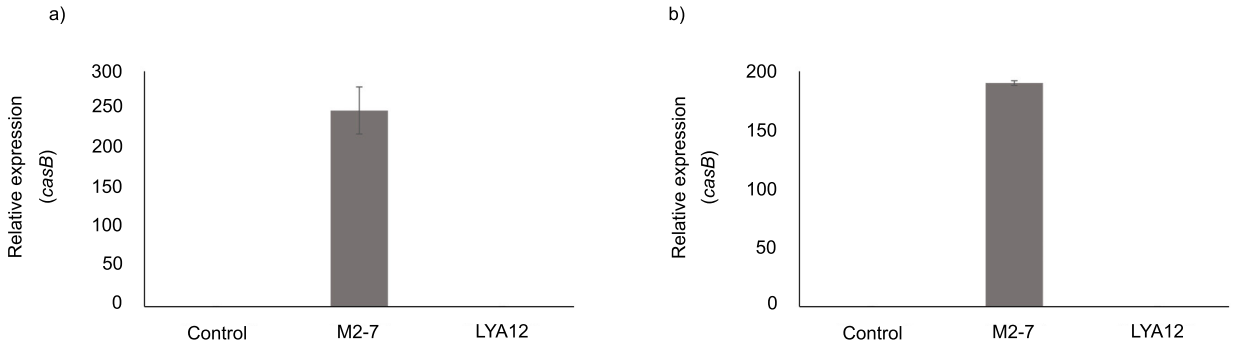

Fig. 4. Expression of *casA* (a) and *casB* (b) of *Aspergillus niger* in the absence or presence of M2–7 or LYA12. Error bars are the standard deviations of three replicates.

due to the expression of the genes *casA* and *casB* (**Fig. 4a**), while the strain LYA12 presented a fungistatic effect since the expression of the genes did not vary to the control (**Fig. 4b**). Increased stress may be associated with a shift from regulated to uncontrolled death (Phillips et al. 2003). Fungi possess signaling mechanisms that allow them to detect environmental signals and respond through changes in the expression of their genes, including those that lead not only to recognize their host but also to overcome their defenses, proliferate within their tissue, establish disease, and in some instances induce cell death (Pinto et al. 2009). These results allow us to consider *Bacillus licheniformis* M2–7 and LYA12 strains as promising biological control and perform their evaluation on phytopathogenic fungi in vivo.

CONCLUSIONS

Bacillus licheniformis LYA12 has a fungicidal effect on *Aspergillus oryzae* by promoting the expression of the *casB* gene. *Bacillus licheniformis* M2–7 strain and LYA12 strain have a fungicidal effect on *Aspergillus niger* by promoting the *casA* and *casB* genes expression. *Bacillus licheniformis* M2–7 and LYA12 strains have a fungistatic effect on *Colletotrichum* sp. by inhibiting the phytopathogenic fungus growth and not expressing any genes involved in cell death.

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