Capítulo 8.3. *Bacillus licheniformis* M2-7 inhibits the growth and affects the structure of phytopathogenic fungi of Zea mays

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Abstract

The tar spot complex is one of the fungal diseases of the most extraordinary economic importance. Root rot and the red rot of the roots are diseases that deteriorate the vascular bundles causing irreparable losses in maize crops. The genus Bacillus produces of multiple metabolites capable of inhibiting other organisms' development and normal growth. In the present study, the antagonistic activity of the bacterium *B. licheniformis* M2-7 on the phytopathogenic fungi *Microdochium* sp., *Curvularia lunata, Phoma* sp., *Microsphaeropsis* sp. and *Fusarium oxysporum*, associated to the tar spot complex and others diseases in maize was evaluated, and the damage to the hyphae was observed during antagonism. The antagonism test was performed using the plaque emptying method, and to determine the level of damage by *B. licheniformis* M2-7 staining was performed with lactophenol blue. As a result, we obtained that *Bacillus licheniformis* M2-7 inhibits the growth of all the species of phytopathogenic fungi of the tar spot complex and Root rot used in the experiment, and it was also capable of causing structural damage to the hyphae such as rupture, lysis, abnormal growth and low spore production, in all cases. This allows us to consider *Bacillus licheniformis* M2-7 as a controlling organism of the fungi associated with these diseases.

Keywords: Antagonist bacteria; Biological control; Root rot; tar spot complex.

Introduction

Maize is one of the most important produces in the national and international markets, surpassing any other crop (FIRA, 2016), due to the energy content it provides, its cultural importance and the utilization of all its components, from corncob to produce dough derivatives, to fodder for livestock feed (González et al., 2013). However, the production and quality of the product are affected by various pests and diseases caused mainly by fungi (Marcianò et al., 2021). In corn, *Phoma maydis* is reported as a pathogen that produces the white spot of *Phoma* (Urbano-Castillo et al., 2019), while *Phoma terretris* causes the red rot of the roots in corn-producing areas of Argentina, as well as important losses by the lodging, necrosis and wilting of the root tissue (Boa, 2001). *Fusarium* is one of the genera that have the largest number of host plants; this species causes blockages in the vascular bundles of plants causing wilting and death of the plant by hindering the flow of nutrients, causing root rot and aerial parts (Arbeláez, 2000; Jones et al., 1997). *Microsphaeropsis* sp. is a saprophyte fungus adapted to winter conditions that affect crop diversity (Kerdraon et al., 2019). Tar spot complex (TSC) is the fungal disease most affecting maize and represents the greatest limiting factor for production in Mexico and Latin America (Ríos et al., 2017) and, recently, for the United States, which is the primary producer and exporter of maize worldwide (McCoy et al., 2018; Robertson, 2016; Ruhl et al., 2016). This disease is mainly caused by the interaction of two phytopathogenic fungi, *Pyllachora maydis* and *Monographella maydis*, which are associated with *Coniothyrium phyllachorae* (A hyperparasite) (Hock et al., 1995). However, the interaction of these pathogens with maize genotypes is not yet well described (Hock et al., 1995), New associations are still being discovered with other organisms involved in the full development of the disease, as a fourth fungus was recently reported to be associated with this complex, *Curvularia lunata* (Ríos et al., 2017). In previous work, we isolated fungi associated with leaves with the symptoms of the disease, which were identified as *Phoma, Microsphaeropsis* and *Fusarium,* which could be related to the disease, as well as *Microdochium* that is anamorphic to *Monographella maydis* (Unpublished data).

The intensity of the disease and the degree of economic damage depends on the environmental conditions of each growing area and the susceptibility of the maize genotypes (Pereyda et al., 2009); since fungi are favored in places with altitudes above 1,300 meters, temperatures below 25 ºC and high relative humidity (Gonzaléz et al., 2008). The control and management of this disease have traditionally been with chemical fungicides (Gómez et al., 2013). Given the current conditions of international market demands for food safety and reporting of many of these pesticides as mutagenic and therefore the effects on human health and the environment (Monterroso S, 2014; Quiroga et al., 2017), in addition, to the possible emergence of some race resistant to the active substances (Quiroga et al., 2017); it has been necessary to seek viable alternatives for the control of fungal diseases. One of these alternatives using natural enemies or biological control, using microorganisms present in the soil, such as plant growth-promoting bacteria (Hang et al., 2005). These bacteria, in addition to helping plants in the absorption of nutrients, also provide other benefits, such as decreasing or nullifying the action of pathogens through the production of siderophores, lytic enzymes, antibiotics, fluorescent pigments and cyanide, increasing the systemic resistance of plants

(Hernández et al., 2006). One of the best-referenced families of bacteria in the literature that has been shown to have antagonistic activity against various plant pathogenic microorganisms of crops, including only rice and various fruit trees (Galindo et al., 2015; Li et al., 2015; Wang et al., 2014) is the *Bacillaceae* family (Layton et al., 2011). The strain M2-7 of *Bacillus licheniformis* was used in formulations specific to increase the germination percentage, the production and yield of *Capsicum annuum* (Bolaños-Dircio et al., 2021). *Bacillus* is a producer of multiple metabolites, capable of inhibiting the normal development and growth of other organisms (Izzeddin and Medina, 2011) and forming spores that withstand adverse conditions, allowing the formulation of viable long-term products (Tucuch et al., 2018). Such as from a strain of *Bacillus subtilis* (QST713), containing more than 30 biochemical compounds of the lipopeptide type (McSpadden and Fravel, 2002). Similarly, *B. subtilis* 83 (Fungifree AB) has been used to control fungal diseases in more than 20 crops (Galindo et al., 2015). Another species of this genus with an antagonistic activity against phytopathogenic fungi is *Bacillus licheniformis*, which presents antagonistic effect against *Sclerotinia*, *Rhizoctonia, Bipolaris*, *Aspergillus* and *Pyricularia* (Drahos and West, 2001). Although there are many studies on bacterial antagonism on phytopathogenic fungi, there are no reports on *Phoma*, *Microsphaeropsis* and *Fusarium* and pathogens of tar spot complex, therefore, the objective of this work was to evaluate the antagonistic activity and the effect on the fungal structure exerted by *B. licheniformis* M2-7, on the phytopathogenic fungi *Phoma*, *Microsphaeropsis*, *Fusarium*, *Microdochium* and *Curvularia*.

Materials and methods

Biological material. *B. licheniformis* M2-7 and the fungal strains were taken from the strain bank of the Laboratory of Molecular Microbiology and Environmental Biotechnology of the Universidad Autónoma de Guerrero. GenBank accession numbers for 18S gene rDNA sequences are *Microdochium* (MK990150), *Curvularia lunata* (MK990136)*, Phoma sp* (MK990141), *Microsphaeropsis sp* (MK990140) and *Fusarium oxysporum* (MK990134).

Culture media and conditions. *B. licheniformis* M2-7 was cultured on Luria Bertani (LB) medium, composition in g/L (10 g casein peptone, 10 g NaCl, 5g yeast extract) for liquid medium (supplemented with 15 g/L agar for medium solid) at 37 ° C. For the antagonistic tests, the medium was inoculated 250 mL with *B. licheniformis* M2-7 of a 16-hour culture of the bacteria was grown in LB medium at 37 ° C and 2,000 rpm, in 50 mL of fresh LB medium, allowing it to grow for six h under the same conditions of temperature and agitation.

Evaluation of the antagonism of the M2-7 strain against *Microdochium, Curvularia*, *Phoma, Microsphaeropsis* and *Fusarium*. The antagonism test was performed using the plaque emptying method, in triplicate with each phytopathogen in PDA medium (Potato Dextrose Agar) added with *B. licheniformis* M2-7 (3.0 x 10 UFC/mL). To estimate the mycelial growth of the fungus, 4mm were placed on each plate in the center of the plate with each fungus, using the fungus as a negative control in PDA medium without bacteria. They are incubated at 37 ºC for seven days. To determine the percentage of inhibition (I), the following formula was used: I = (RCC - RTC) / RCC x 100, where RCC: Radio of the control colony and RTC: Radio of the treated colony (Acosta et al., 2007).

Determination of hyphal damage during antagonism by Lactophenol staining. To determine the level of damage by *B. licheniformis* M2-7, thin slices of mycelial growth were placed in the presence or absence of antagonism. Staining was performed with lactophenol blue (Thermo Scientific™ Remel) at a concentration of 0.5 g /100 mL. They were observed under an optical microscope (Velab- VE-B300).

Statistical analysis. A paired t-test was used to compare the growth of each fungus species in the presence and absence of *B. licheniformis* M2-7, using as a response variable the average growth radius of the colony of each fungus in each experiment. The SPSS version 20.0.0 program was used to compare the inhibition efficiency of *B. licheniformis* M2-7 among fungus species, a one-way analysis of variance test was used, using the inhibition percentage as the response variable and the fungus species as the independent variable. Bonferroni post-hoc tests were used to assess significant differences among fungal species.

Results and Discussion

Bacillus licheniformis M2-7 inhibits the growth of *Phoma, Curvularia, Microspheropsis, Microdochium* and *Fusarium*.

Bacillus licheniformis M2-7 inhibits the growth of some species of phytopathogenic fungi of the tar spot complex used in the experiment. Significant differences in plate growth were found in the presence and absence of *B. licheniformis* M2-7 from *Microdochium* sp. (t= 569.0, P= 0.002), *Curvularia lunata* (t= 16.0; P= 0.004), *Phoma* sp. (t= 91.6; P= 0.001), *Microsphaeropsis* sp. (t= 8.5; P= 0.014) and *Fusarium oxysporum* (t= 21.6; P= 0.002), growing in the absence and presence of *B. licheniformis* M2-7 (Figure 1 and figure 2).

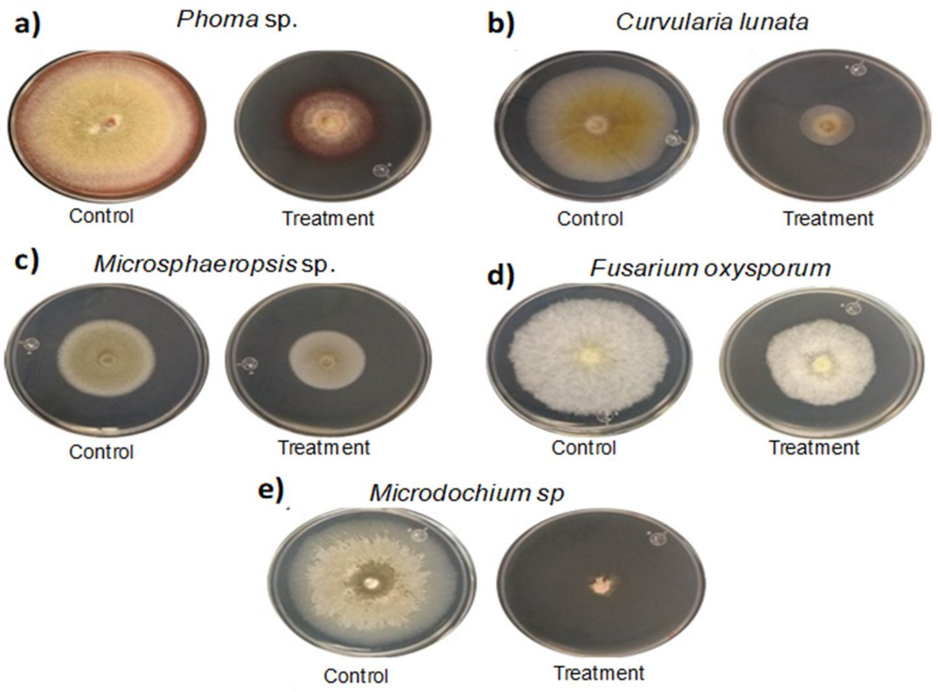

Figure. 1. Antagonism test of *Bacillus licheniformis* M2-7 against *Phoma* sp. (a), *Curvularia lunata* (b), *Microspheropsis* sp. (c), *Fusarium oxysporum* (d) and *Microdochium* sp. (e) fungi. The trials were conducted in triplicate

The inhibition of *Bacillus licheniformis* M2-7 on phytopathogenic fungi was of different magnitude for the species (F4,15= 128.01, P<0.001). On average, this bacterium inhibited the growth of *Microdochium* sp. by 81.1%, *Curvularia lunata* by 63.7%, *Phoma* sp. by 48.3%, *Fusarium oxysporum* by 28.2% and *Microsphaeropsis* sp. by 22.6% (Figure 2).

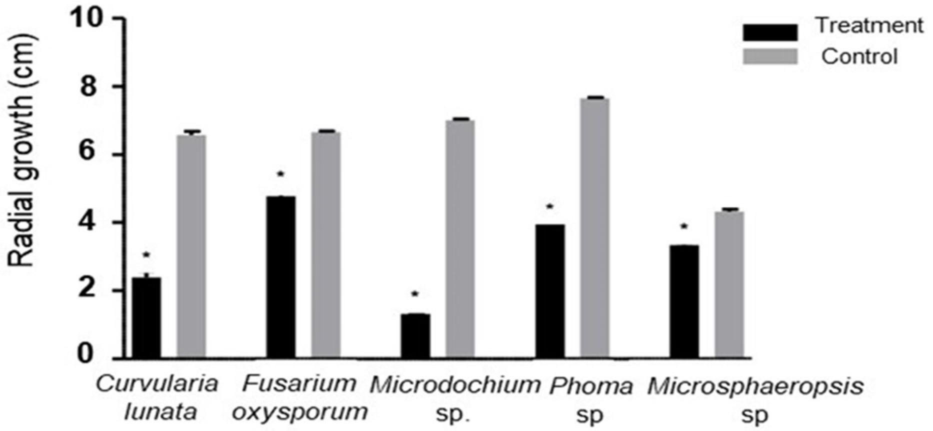

Figure. 2. Radial growth (mean ± 1EE) of the fungi in the absence and the presence of *B. licheniformis* M2-7. The asterisk indicates significant differences

Tar spot complex is an emerging disease in the coldest regions, such as the high-elevation plateaus of central Mexico at more than 2600 meters above sea level or the United States, where temperatures are below 0ºC (McCoy et al., 2018; Ruhl et al., 2016). The tar spot complex is a phytosanitary problem of the maize crop, which has destroyed and limited the production yield in Central and South American countries in the last 20 years (Ríos et al., 2017). Biological pest and disease control has proven to be efficient and more friendly to the environment and other non-target organisms (Chen et al., 2014). Different strains of *Bacillus licheniformis* have been reported as producers of different antifungal compounds, such as β-1,3-glucanase (Tendulkar et al., 2007), biosurfactants such as surfactin (Tucuch et al., 2018), one of the most powerful surfactants and chitinase (Tendulkar et al., 2007). In this work, *Bacillus licheniformis* M2-7 showed an effect *in vitro* on the growth of phytopathogenic fungi *Phoma, Curvularia, Microspheropsis, Fusarium* and *Microdochium*. The results match Tendulkar et al., (2007), where *B. licheniformis* BC98 induced abnormal growth in *Magnaporthe grisea*, showing a fungicidal effect Similarly, Won et al. (2019b) found that the strain *B. licheniformis* MH48 can inhibit foliar pathogens of *Camellia oleifera* (*Glomerella cingulata, Pestalotia diospyri, Pestalotiopsis karstenii, Botrytis cinerea*), by lytic enzymes that cause deformation, rupture and degradation in the hyphae of plant pathogens.

The inhibition percentages of up to 81.1 % obtained in this work differ from those reported by Tucuch et al. (2018), where *B. licheniformis* metabolites inhibited 80-100% to *F. oxysporum*. Castañeda and Sanchez (2016) reported a percentage inhibition of 66.2 % for *Fusarium* sp. Won et al. (2019a), in an experiment with *Bacillus licheniformis* MH48, reported lower percentages of inhibition than those reported in this work since the highest inhibition rate was 50.5 % against *Pestalotiopsis karstenii* . The difference in inhibition percentages may be due to the different responses of the fungi to the antagonistic organisms.

Bacillus licheniformis causes structural damage to *Phoma, Curvularia, Microspheropsis, Microdochium* and *Fusarium*.

In the microscopic observation of the hyphae of the fungi in the absence of the bacteria, they are complete, without deformation or lysis. In the case of *Fusarium*, many spores were distinguished (Figure 3). In comparison, the fungi against *B. licheniformis* M2-7 showed a decrease in mycelium, abnormal growth, damaged liver cells and no or minimal production of spores (Figure 3).

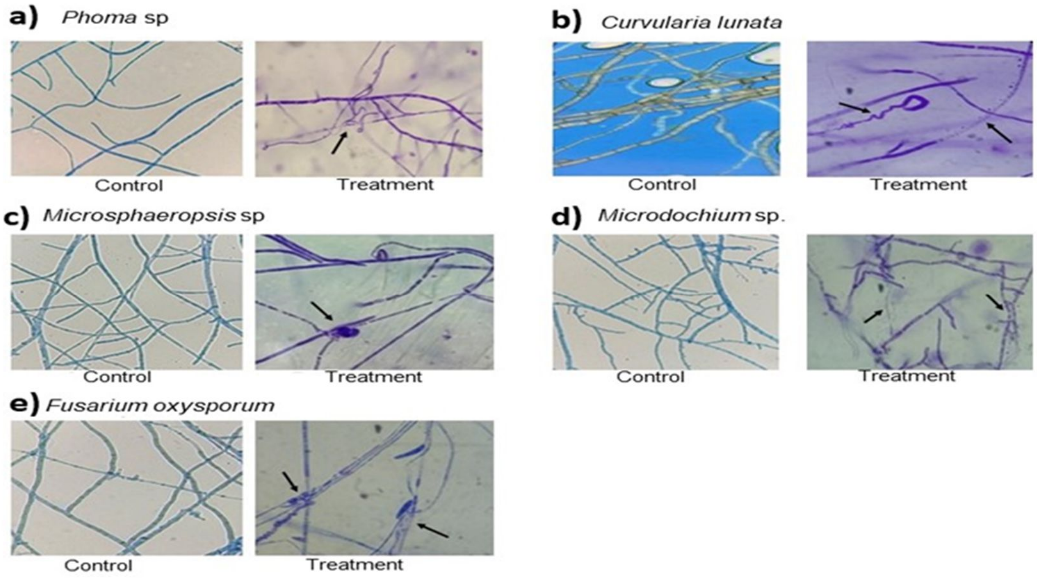

Figure. 3. Microscopic observations of the morphology of the hyphae of (a) *Phom asp*., (b) *Curvularia lunata*, (c) *Microsphaeropsis* sp., (d) *Microdochium* sp. and (e) *Fusarium oxysporum*. in the absence (control) and in the presence of *B. licheniformis* M2-7 (treatment), where deformity, swelling and rupture of the hyphae can be observed

The finding that the strain *Bacillus licheniformis* M2-7 inhibited the growth of the fungi evaluated in this work prompted us to investigate the effect on the growth of the hyphae structure, observing that the presence of the bacteria caused the cellular change in hyphae morphology, such as swelling, distortion, and cytoplasm aggregation (Figure 3). The enzymes chitinase and β-1,3-glucanase are capable of lysing fungal cell walls and are responsible for the suppression of fungal growth (Castañeda and Sanchez, 2016).

It is known that within the causal agents of the disease, tar spot is found in *Curvularia* and *Microdochium*; having obtained antagonistic activity with *Bacillus licheniformis* M2-7 allows considering this bacterium as an alternative for the biological control of the tar spot complex disease. The results of this work will serve to have new alternatives to chemical fungicides in the fight against the tar spot Complex disease and other fungal diseases in corn; with this, we help protect native corn that is the most affected by this disease and decrease the contamination by the excessive use of chemical fungicides.

Conclusions

The strain M2-7 has great potential and can be used in specific formulations to protect against phytopathogenic fungi tar spot complex disease and other fungal diseases in corn.

Acknowledgments

This work was supported by grants 858637 from CONACyT and SAGARPA 18-PFA-IIDTTT-000089-L000-DF. Rosibel Bahena Oregon thanks CONACyT for M.Sc. scholarships.

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