ANTIFUNGAL ACTIVITY OF METABOLITES FROM *Bacillus* spp. AGAINST *Fusarium oxysporum* USING MICRO DILUTION IN PLATE METHOD

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ABSTRACT

Fusarium oxysporum is a plant pathogen that causes great losses in tomato crops because affect the vascular system of plants; at present the principal control of this phytopathogen is the chemical control, nevertheless the indiscriminate use of those products have caused resistance and environmental problems. For this reason the use of antagonistic microorganisms like bacteria of the genus *Bacillus* have arised as an alternative sustainable and friendly to the environment. For this reason this research determined the antifungal activity and the minimum inhibitory concentration to 50% (IC_{50}) of crude extracts with metabolites from six strains of bacteria of the *Bacillus* genus (B-AN1, B-AN2, B-AN3, B-AN4, B-AN5 y B-AN6) against *F. oxysporum*, by the micro dilution plate method. The *Bacillus* strains were grown in potato dextrose broth enriched with yeast extract and malt extract, to get crude extracts with metabolites; the concentrations studied were 50.00 %, 25.00 %, 12.50 %, 6.25 %, 3.13 %, 1.56 %, 0.78 %, 0.39 % and 0.20 %.The results showed the strains B-AN3 and B-AN4 corresponding to *Bacillus licheniformis* and *Bacillus subtilis* are which presented the highest percent of inhibition with 100 % to 3.13 % of microbial extract; for the IC_{50}, the lowest IC_{50} was by the strain B-AN4 with 0.01 %. It concludes that the strain B-AN4 (*B. subtilis*) in this work was the best for control *F. oxysporum* with the lowest IC_{50} of all; also this work concludes that the micro dilution in plate method is a fast and effective method for studies of substances with antimicrobial activity.

Keywords: *Fusarium oxysporum*, *Bacillus*, antagonistic bacteria, IC_{50}, micro dilution, bio control, antifungal activity.

INTRODUCTION

In Mexico tomato crop registered an increase on production from the year 2005 at 2015 with 246,246.34 t to 3,098,329.41 t (SAGARPA, 2017). Nevertheless the tomato crop is affected by several fungus diseases as gray mold (*Botritys cinerea*), early blight (*Alternaria solani*) and vascular wilt caused by *F. oxysporum* (Villasanti & Pantoja, 2013). Actually *F.*

oxysporum represents an important limiting on systems of production, because the disease cause yield losses until 60 % penetrating the roots colonization the vascular system of the plants. The typical symptoms of vascular wilt are chlorosis in plant leaves, although occasionally it is present just in the half plant; vascular wilt of stems and petioles of big leaves; and atrophied radicular system (Turlier et al., 1994; González et al., 2012). Currently for *F. oxysporum* control the farmers use chemical products, nevertheless the indiscriminate use of these products has been produced a negative impact on environment; also has occasioned resistance in pathogens as the reported to the fungicides trifloxystrobin, carbendazim and some azoles and an increase in production costs (Ramírez & Jacobo, 2002; Bautista-Baños, 2006; Chen et al., 2007; Ascencio-Álvarez et al., 2008; Liu et al., 2011; Dubos et al., 2013). In addition, the planted surface in Mexico under protected agriculture conditions have been augmenting; in the year 2005 enhanced from 395 ha to 13 743 ha in the year 2015 (FIRA, 2015). For this reason exist a necessity to develop alternative methods for the control of diseases in plants (Jeong et al., 2017).

In this sense the use of biological control and natural products represent an alternative environmentally safety and sustainable, and in some cases is the only option for protection of plants against pathogens (Baker, 1987; Cook, 1993; Wafaa & Haggag, 2002). The genus *Bacillus*, result an important option for control of phytopathogen organisms because *Bacillus* produce a vast variety of secondary metabolites with antimicrobial potential and also form resistant spores to adverse environmental conditions that allow easy formulation of feasible long-term commercial products, cyclic amphiphilic lipopeptides probably represent the most common class of compounds produced by *Bacillus*, these cyclic lipopeptides are classified into three different families depending on the amino acid sequence (Osouli & Afsharmanesh, 2016); surfactins that induces resistance and contribute to formation of protecting biofilms; Iturins as mycosubtilins and bacillomycin which present a strong fungitoxic activity due to their ability to permeate membranes; and fengycins that can interact with the layers of lipids and also can alter the structure of the membranes and permeabilize it; finally also enzymes like lipases, proteases and β- glucanases are produced by these microorganisms. All these compounds may be used for the control of phytopathogen fungus; some species of *Bacillus* that produce this kind of metabolites are *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* (Reinoso-Pozo et al., 2006; Tejera-Hernandez et al., 2011; Yesid & Ligia, 2012; Astorga-Quiros et al., 2013; Pila, 2016; Vieira et al., 2017).

Different researches have showed the antifungal effect of extracts from *B. subtilis* against phytopathogen fungus; such as *B. cinerea* where the extract affect the growth and morphology of the fungus, also a reduction of 79 % of the disease incidence in tomato fruits have been observed (Kilani-Feki et al., 2017); Zouari et al. (2016) indicate that metabolites from *B. amyloliquefaciens* inhibit the growth of *F. oxysporum*, *Pythium aphanidermatum*, *Botrytis cinerea*, *Alternaria alternata* and *Aspergillus niger*.

The most used method for determinate the antifungal activity of metabolites from *Bacillus* in laboratory have been realized with the diffusion in agar while the method of micro dilution in plate has not been used; this method use the 2,3,5- triphenyltetrazolium chloride (TTC) due to its high sensibility allow detect inhibition of microorganisms with very small amounts of antimicrobial products, also the red coloration is a visual indicator of the antimicrobial activity from the treatments (Gabrielson et al., 2002). For the previous reasons the objective of this research was determinate the antifungal activity of microbial extracts with metabolites from six strains of *Bacillus* against *F. oxysporum* using the micro dilution in plate method.

MATHERIALS AND METHODS

Obtaining of bio control microorganisms and *F. oxysporum* strain

The *Bacillus* strains were provided by the Phytopathology Laboratory, these strains were identified previously for several works carried out as B-AN1 (*B. subtilis*), B-AN2 (*B. licheniformis*), B-AN3 (*B. licheniformis*), B-AN4 (*B. subtilis*), B-AN5 (*B. subtilis*) y B-AN6 (*B. subtilis*). The *F. oxysporum* strain isolated from tomato plants was provided from microbiological collection of the Mycology and Biotechnology Laboratory, the *F. oxysporum* was identified with specific primers ITS1-ITS4 with the access key in GenBank: KU533843.1. Both laboratories belong to the Department of Parasitology from the Universidad Autonoma Agraria Antonio Narro located in Saltillo, Coahuila, Mexico.

Secondary metabolites production from liquid fermentation of *Bacillus*

The liquid fermentation was obtained using potato dextrose broth (350 g of potato in a water liter, later broth was filtered and finally 3.5 g of yeast extract, 3.5 g of malt extract, 15 g of sugar and 0.1 g of calcium chloride were added). Six flasks were prepared with 50 mL of potato dextrose broth enriched with yeast and malt, each flask were inoculated from the Petri dishes; later the flasks were placed in agitation during four days to 150 rpm to 28 °C; at last the bacterial broth was centrifuged to 5000 rpm during 10 min, the supernatant was filtered using filter of 0.2 µm.

In vitro tests of the Antifungal Activity of *Bacillus* Secondary Metabolites on *F. oxysporum* by micro dilution in plate method

For *in vitro* tests, were adapted the techniques proposed by Masoko et al. (2005), Gabrielson et al. (2002) and Eloff (1998). For this experiment polystyrene micro plates of 96 wells were used; in all wells, 100 µl of Sabouraud liquid medium was placed; the column one was the negative control, the column two consisted of the positive control and the column three was a control which consisted of the *Bacillus* fermentation medium. From column four, 100 µl of the fermented extract of the strains was placed which was mixed with a pipette with the 100 µl of the liquid medium and then 100 µl of the mixture was transferred to the next column so on and so forth discarding the last 100 µl from the column 12, in order to get serial micro dilutions to 50.00 % of the microbial extract. Once the micro dilutions were carried out, growth developer 2,3,5-triphenyltetrazolium chloride 0.01% was added in the whole plate, due its the lowest concentration reported in the literature, in as much as an excess of this indicator can interfere with the growth of the pathogen or react with reagents from the medium; This indicator measures the respiratory activity associated with electron transport chains and when reduced, it precipitates forming a complex intense red color; Its use is due to its high sensitivity to detect inhibition of microorganisms with very low amounts of antimicrobial products, besides that the red coloration is a visual indicator of the antimicrobial activity of the treatments (Gabrielson et al., 2002). Finally starting by the column two were added 10 µl of a spore solution of *F. oxysporum* at a concentration of $1x10^8$ in all wells, keeping all the wells a volume of 150 µl are in total. Each microplate was considered a replicate, and the test was realized by triplicate. The microplates were incubated at 28 °C for 48 h and a lecture of absorbance was realized at 490 nm in a spectrophotometer (Thermo Scienctific Multiskan Go, model 51119200). Staying the six microbial extracts in the rows A to F. To calculate the inhibition percentage, the Moreno-Limon et al. (2011) formulas were adapted:

$$\%\ growth = \frac{A - B}{C}(100)$$

A=Treatment absorbance B=Negative witness absorbance C=Positive witness absorbance

$$\%\ inhibition = 100 - \%\ growth$$

Statistical Analysis

A Probit analysis was performed to determinate the inhibitory concentration to 50% (IC_{50}) of each *Bacillus* strain. With the results obtained, a variance analysis was performed with the inhibitory concentrations, in the analysis the six treatments were evaluated with three repetition and a Tukey's range tests was performed ($p<0.05$).

RESULTS AND DISCUSSION

Antifungal activity of Secondary metabolites from *Bacillus* against *F. oxysporum* using micro dilution in plate method

The six *Bacillus* strains used presented antifungal activity against *F. oxysporum*. The strains B-AN2, B-AN3 and B-AN4 showed the highest inhibition percentages (80 %) in the lowest concentration of metabolites of 0.20 %; nevertheless, starting by the concentration 3.13 % of the extract there was inhibition of 100 % with the strains B-AN3 and B-AN4, while the strain B-AN2 obtained a inhibition of 100 % to concentration of 6. 25 % of microbial extract (Figure 1). At last B-AN5 and B-AN6 strains inhibited 68 % of the fungi development with highest concentration used in this study of 50 %.

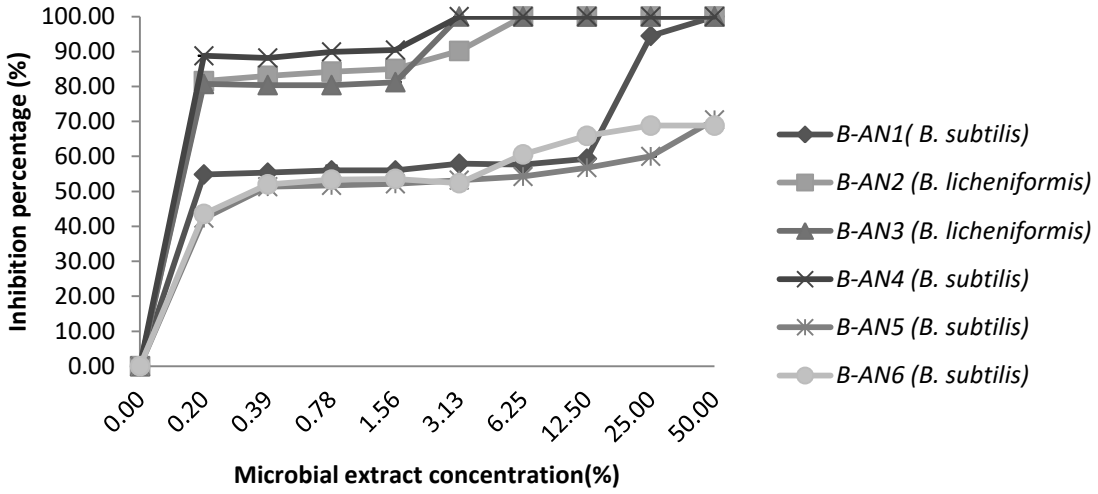

Figure 1. Percentage inhibition of the microbial extracts with *Bacillus* metabolites against *F. oxysporum*.

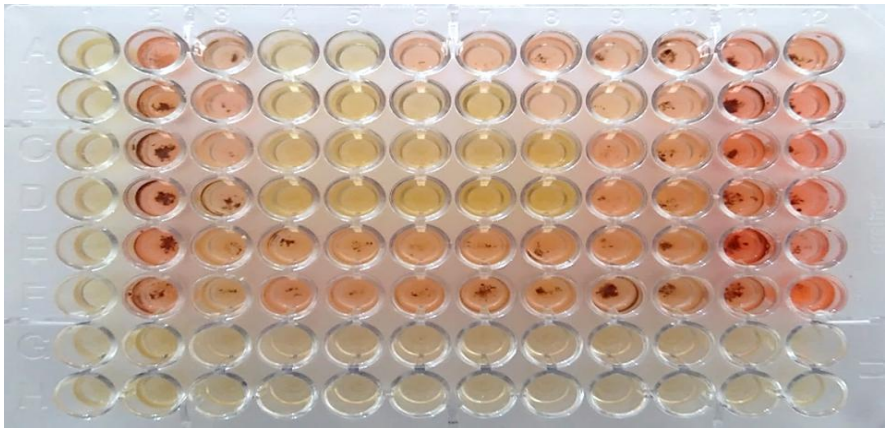

Figure 2. Microplate with treatments to several concentrations and the pathogen elapsed 48 hours after incubation. Row A= B-AN1, B=B-AN2, C=B-AN3, D=B-AN4, E=B-AN5, F= B-AN6; Column 1= Negative witness, Column 2= Positive witness, 3= Growth medium of Bacillus, 5= 50 %, 5= 25 %, 6= 12.50 %, 7=6.25 %, 8= 3.13 %, 9 = 1.56 %, 10= 0.78 %, 11= 0.39 % y 12= 0.20 %. Each microplate was considered a repetition, for this work were made three repetitions. Red coloration indicates pathogen growth.

The variance analysis showed significative difference between the IC_{50} of the metabolites from *B. subtilis* and *B. licheniformis*, being the B-AN4 strain with the lowest IC_{50} of 0.01 % (Table 1). The obtained results showed the effectiveness of metabolites from *B. subtilis* and *B. licheniformis* for inhibit the *F. oxysporum* development, as the IC_{50} of the different extracts oscillated of 0.01 % to 0.86 %, and that the B-AN3 and B-AN4 strains inhibited *F. oxysporum* from the concentration of 3.13 %.

Table 1. Inhibitory concentration to 50 % (IC_{50}) of microbial extracts with metabolites from *Bacillus* strains, for inhibition of *F. oxysporum*.

Cepa	$CI_{50}{}^{x}$
B-AN1(*B. subtilis*)	0.41± 0.17b
B-AN2 (*B. licheniformis*)	0.03±0.06cc
B-AN3 (*B. licheniformis*)	0.05±0.04cc
B-AN4 (*B.subtilis*)	0.01±0.06c
B-AN5 (*B.subtilis*)	0.86±0.04a
B-AN6 (*B.subtilis*)	0.54±0.03bb

xValues with same letter are same (Tukey, p≤0.05); ± Standar deviation

The inhibition percentages obtained in this work was higher than the percentages reported by Ramyabharathi & Raguchander (2014); according with their results the *B. subtilis* metabolites against *F. oxysporum* reached a maximum inhibition of 46.04 %, also the results reported by Sarti and Miyasaky (2013) with *B. subtilis* only presented an inhibition of 50 % against *F. solani*. In the case of *B. licheniformis* the development inhibition of *F. oxysporum* showed in this work was higher than the reports of Jeong et al. (2017) against *Rhizoctonia solani* and *Colleteotrichum gloesporoides* where the inhibition wasn't higher to the 60 %, nevertheless the results of this work coincide with the results reported by Tendulkar et al. (2007) who reported high inhibition in spores *Magnaporthe grisea* with metabolites of *B. licheniformis*. This could be due because the method used in the literature is the method diffusion in agar being a less sensible than the micro dilution in plate method.

It has been shown that *B. subtilis* and *B. licheniformis* metabolites are capable of protect the plants against phytopathogens trough diverse mechanism, in particular trough the synthesis of cyclical lipopetides with antifungal activity (Hossain et al., 2015). So that the antifungal activity showed by the microbial extract in this work maybe be attributed to the presence of lipopetides that *B. subtilis* and *B. licheniformis* produce as part of their metabolism; some of these metabolites are surfactins considered one of the most powerful known biosurfactants, consists of four isomers (surfactin A–D) that exhibit a wide variety of physiological activities like the cell lysis; fengycins that consist of a β-hydroxy fatty acid connected to the N-terminus of a decapeptide that includes four D-amino acids and the non-proteinogenic amino acid L-ornithine; and iturins that are a large family of cyclic heptapeptides with a C14-C17 aliphatic β-amino fatty acid. They have chiral peptide sequences of L- and D- amino acids (LDDLLDL) and are cyclized by the formation of an amide bond between the N-terminal β-amino fatty acid and the C-terminus of the peptide, these group includes iturin (variants A, C, D and E), bacillomycin (D, F, L and Lc) and mycosubtilin (Harwood et al., 2018). The inhibition by the metabolites mentioned could be due the disruption of the cellular wall and the inhibition in the conidia development, also these metabolites may also play different roles in the development and survival of *Bacillus* strains in their natural habitat: increasing bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing, motility and biofilm formation. (Arguelles-Arias et al., 2009; Mora et al., 2011; Falardeau et al., 2013; Wise et al., 2014; Hossain et al., 2015). Other compounds secreted by antagonistic bacteria are enzymes such as hydrolases, chitinase,

chitosanase and other fungal wall degrading enzymes (Baysal et al., 2013; Wu et al., 2014; Kilani-Feki et al., 2017).

Regarding the similar behavior between both species identified in this work at the time of inhibiting the development of *F. oxysporum*, this may be due to the fact that both produce metabolites with antimicrobial activity capable of affecting in various ways the cells of phytopathogenic organisms (Sansinenea & Ortiz, 2011).

CONCLUSIONS

With the micro dilution plate method the antifungal activity of the *B. subtilis* and *B. licheniformis* extracts of this study was determined; the strains B-AN3 and B-AN4 were able to inhibit the development of *F. oxysporum* to 100% from the concentration of 3.1% of the extract and strain B-AN2 to 6.2%.The IC_{50} varied from 0.01 to 0.86 and not differences were observed in antifungal effect of the extracts between the species of *B. subtilis* and *B. licheniformis*, because both species produce metabolites with antimicrobial activity that affect the cellular structures of the fungus in various ways.

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