BIOLOGICAL EFFECTIVENESS OF *BACILLUS* SPP. AND *TRICHODERMA* SPP. ON APPLE SCAB (*VENTURIA INAEQUALIS) IN VITRO* AND UNDER FIELD CONDITIONS

Maria del Carmen Meza Jimenez
Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología **México**
carmen_16julio@live.com

Francisco Daniel Hernández Castillo Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología **México** fdanielhc@hotmail.com
(Corresponding Author)

Elan Iñaky Laredo Alcalá
GreenCorp Biorganiks de México, S.A. DE C.V. **México**
elan_laredo@hotmail.com

Gabriel Gallegos Morales
Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología **México**
gabgalmor@yahoo.com.mx

Roberto Arredondo Valdés
Universidad la Salle, Saltillo **México** robqfb@gmail.com

Francisco Castillo Reyes
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias **México**
reyes.francisco@inifap.gob.mx

ABSTRACT

Venturia inaequalis causes great economic losses until near 100% in apple production industry in worldwide, generally its control is based in agrochemicals using, but irrational applications had causing resistance problems, so the use of biocontrol agents are an alternative for disease management. In the present research, the antifungal activity of *Bacillus* spp. and *Trichoderma* spp. for the control of apple scab *in vitro* and *in vivo* conditions were evaluated. The *V. inaequalis* strain was isolated from fruits and apple tree leaves with presence of disease symptoms. *Trichoderma* spp. and *Bacillus* spp. against *V. inaequalis* in duals cultures were evaluated; the antagonism by *Trichoderma* spp. was classified according to the Bell scale, while for *Bacillus* spp. the radial growth inhibition percentage was determined. On the other hand, secondary metabolites from *Trichoderma* spp. and *Bacillus* spp. against *V. inaequalis* was evaluated by means the microplate technique to determine the inhibition percentage. *In vivo* conditions, five formulated from *Bacillus* spp. and *Trichoderma* spp. under a randomized block experimental design and four repetitions was evaluated. The incidence and severity in fruits and leaves were variables evaluated. The results in dual cultures technique, showed maximum antagonistic effect by *Trichoderma* strains, and according to the Bell scale were identify in class 1. Regarding, the inhibition of *V. inaequalis* mycelia growth by the *Bacillus* strains ranged 33.4 to 41.3% for *B. subtilis* and *B. licheniformis*, respectively. The secondary metabolites from *T. yunnanense*, *T. harzianum* and *B. licheniformis* showed an inhibition percentage in 100% against *V. inaequalis*. The results from *in vivo* conditions showed decreased in incidence in 53.4% and severity in 63.7% in fruits, by using formulated *Bacillus* spp. at doses of $2Lh^{-1}$, meanwhile, foliage the incidence decreased in 66.7 by formulated *Trichoderma* spp. at doses of $2Lh^{-1}$, but in severity, all treatments has behaved similarly.

Keywords: Antagonism, biocontrol agents, Secondary Metabolites, plant disease.

INTRODUCTION

The apple scab is caused by the fungus *Venturia inaequalis* (Cook) Wint. (Anamorfo *Spilocaea pomi* Fr.), is the most important disease of this fruit at worldwide level, causing great economic losses can be until from 100% of the production, by affecting commercial

quality of fruits (Doolotkeldieva, 2017; Gopaljee et al., 2009). This fungus survives in the leaf litter the end of the crop's vegetative cycle, developing pseudotheces that produce ascospores, which are released with rain and responsible for the primary infection, these require temperatures between 15° and 20°C in periods of continuous humidification from 8 to 12 hours for its germination on plant material, and a few days later, *V. inaequalis* it propagates asexually producing several generations. *V. inaequqlis* is characterized by presence of short conidiophores and producing only conidia at its end, the symptoms are manifesting in leaves and fruits as round spots, at first slightly translucent, then turn olive green and then darken. (Beata, 2015; Lucero, 2003). Generally, its control is based on the use of agrochemicals; which significantly increases the production costs together with the negative impact it causes on the environment and the health of the human (Carisse et al., 2000, Sutton et al., 2000, Misba et al., 2017). Therefore, there is a need to search for alternatives to reduce the use of these products. Biological control has been investigated for several years to control this pathogen, it has been proven by the use of antagonistic microorganisms such as *Athelia bombacina* and *Chaetomium globosum*, which showed a high potential to reduce the formation of ascospores in the leaf litter (Jamar et al.., 2007; Miedtke et al., 1990). In addition, Doolotkeldieva et al. (2017) evaluated the effect of *Trichoderma viride* for the reduction of conidia of *V. inaequalis* in apple tree seedlings, the results showed significantly decreasing the percentage of conidia. From the above mentioned is necessary the search on antagonistic microorganisms that can control the damages caused by *V. inaequalis*. The objective of this work were to determine the antifungal activity of *Bacillus* spp. and *Trichoderma* spp. against *V. inaequalis* under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Obtaining of pathogen and biocontrol microorganisms

The strain of *V. inaequalis* was isolated from fruits and leaves samples of apple tree, with presence of characteristic symptoms of the disease, obtained from commercial orchards located in the Cañon de los Lirios, Arteaga, Coahuila. After mycelial growth, it was purified by the monosporic culture technique using serial dilutions in culture media malt-agar (1:1.5%) and 1000 ppm benzimidazol-2-ylcarbamate, incubated at a temperature of 20 °C for 20 days and was subsequently identified at species level with taxonomic keys from Sivanesan, 1974 and Bowen, 2010. The strains of *Trichoderma yunnanense*, *Trichoderma lignorum*, *Trichoderma asperellum* and *Trichoderma harzianum* were isolated and molecularly identified in a previous work by Hernández et al. (2011). The *Bacillus subtilis* and *Bacillus licheniformis* isolated were provided from collections of the Department of Agricultural Parasitology of the Universidad Autónoma Agraria Antonio Narro.

In vitro antagonistic activity of *Trichoderma* spp. against pathogenic *Venturia inaequalis*

Preliminary screening of *Trichoderma* antagonism against pathogenic isolate *V. inaequalis* was carried out using the dual culture method as described the methodology of Cherif and Benhamou (1990). In Petri dishes with PDA (Agar Potato Dextrose), a 5 mm disc with active mycelium and 10-day-old of phytopathogen was placed on one side, and a 5 mm disc with *Trichoderma* spp. active mycelium was placed on the other side. The plates inoculated only with the pathogen culture served as control. Both plates inoculated were incubated at 20 °C and were observed every 24 h in order to quantify the contact time (contact day) between the antagonist and the phytopathogen; the radial growth of both colonies (cm) and the diameter of intersection and/or overlap were measured (cm). The antagonism was classified according to the scale proposed by Bell *et al*. (1982). A completely randomized design was used with five treatments (four *Trichoderma* strains and control) and four repetitions.

In vitro antagonistic activity of _Bacillus_ spp. against pathogenic _Venturia inaequalis_

Preliminary screening of bacterial antagonism against the pathogen isolate *V. inaequalis* was carried out using the dual culture method according to the methodology of Ríos (2016). PDA disc (5mm) with active mycelium of the phytopathogen were placed in the center of a Petri dish with PDA, in the same plate, at a distance of 1.5 cm in the four cardinal points, a loopful of antagonistic bacterial isolates was placed. Plates inoculated only with the pathogen culture served as controls. In order to quantify the antagonistic potential of bacterial strains, the size of growth inhibition zones was measured after 6 days of incubation at 20 ˚C and the percent of radial growth inhibition (PICR) was calculated using the formula proposed by Suárez et al. (2008). A completely randomized design was used with three treatments and four repetitions.

$$PICR = \left(\frac{R_1 - R_2}{R_1}\right) * 100$$

Where R1 represents the pathogen radial growth (diameter) in control plate, and R2 represents the colony diameter in treated plate.

Production of secondary metabolites of _Bacillus_ spp. and _Trichoderma_ spp

The metabolites of *Bacillus* were producing in culture media with the following composition: infusion of 350 g of potato by one liter of water, 3.5 g of yeast extract, 3.5 g of corn molasses, 15 g of Dextrose and 0.1 g of calcium chloride. After the culture media was inoculated with a loopful of *Bacillus* spp., and was incubated at 28 °C with agitation at 120 rpm for 72 hours, then, was centrifuged at 10,000 rpm for 10 min. and it was filtered on a nitrocellulose membrane (0.25 µm) (Ragazzo, 2011).

The metabolites of *Trichoderma* was producing in culture media with the following composition: infusion of 350 g of potato by one liter of water, 3.5 g of yeast extract, 3.5 g of corn molasses, 15 g of Dextrose and 0.1 g of calcium chloride. After the culture media was inoculated with 50 µl of each *Trichoderma* strain at a concentration of $1x10^8$ was incubated at 28 °C, with shaking at 120 rpm for 72 h, then was centrifuged at 10,000 rpm for 10 min. and it was filtered on a nitrocellulose membrane (0.25 µm) (Stefanova et al., 1999).

***In vitro* antifungal activity of the secondary metabolites of _Bacillus_ spp. and _Trichoderma_ spp. against _Venturia inaequalis_ evaluated by means of the microplate technique**

The antifungal activity of secondary metabolites by the plate microdilution technique proposed by Masoko et al. (2015) was used. The treatments such as filtrates obtained from the fermentation of *Bacillus* spp. and *Trichoderma* spp. were evaluated. In 96-well Elisa plates, 100 µl of liquid malt extract media was placed in all plate. From plate row three, 100 µl of solvent (fermented medium) were mixed with liquid malt extract media placed initially where 100 µl was discarded. Later in the in row four, of each *Bacillus* and/or *Trichoderma* filtrates were placed in plate as serial dilutions (50, 25, 12.5, 6.25, 3.1, 1.5, 0.75, 0.37 and 0.18 percent). Finally, 10 µl of *V. inaequalis* spore solution at a concentration of $1x10^6$ was placed from 2-12 row. Then microplates were covered with a self-adhering plastic film and incubated at 20 °C for 7 days. Finally, the microplates were evaluated at an absorbance of 490 nm in a spectrophotometer (Thermo Scientific Multiskan Go) controlled with the Thermo Scientific SkanIt software. The formulas proposed by Moreno et al. (2011) was used for to determine the inhibition percentage. The four repetitions per treatment were used.

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

$$\%\ inhibition = (100 - \%\ of\ growth)$$

Where, A represents Treatment Absorbance, B represents Negative Control Absorbance and C represents Positive Control Absorbance

In vitro antifungal activity from *Bacillus* spp. and *Trichoderma* spp. against *Venturia inaequalis*

The trial was conducted in an apple commercial orchard, situated in the Lirios Canyon in Arteaga, Coahuila, Mexico, located at the geographic coordinates 25° 27' 0" North and 100° 50' 56" West. Under randomized blocks design, with five treatments and four repetitions in trees of Golden Delicious variety was evaluated (Table 1). Four applications were realized; on July 17 and 31 and August 14 and 28, according to weather conditions. The variables evaluated were incidence and severity in leaf and fruit, so a representative sample from 40 leaves and 40 fruits per tree were selected from four terminal branches per tree with orientation N, S, E and O.

Table 1. Treatments and doses applied for the control of *Venturia inaequalis* in field.

Treatments	Formulates	Doses
1	*Bacillus* spp. *	1L/ ha
2	*Bacillus* spp.	2 L/ ha
3	*Trichoderma* spp. **	1L/ ha
4	*Trichoderma* spp.	2 L/ ha
5	Control	----

* *Bacillus* spp.: Mixture of microbial broth from the fermentations and cells of *B. subtilis* and *B. licheniformis* ($1x10^8$ CFU).
** *Trichoderma* spp. Mixture of microbial broth from the fermentations and cells of *T. lignorum*, *T. asperellum*, *T. harziamun* and *T. yunnanense* ($1x10^8$ CFU).

Statistical analysis. Data of each experiment were analyzed in order to detect differences among treatments and means were compared using the Duncan ($P \leq 0.0001$) multiple range test. Percentage data were transformed before the statistical analysis (ANOVA).

RESULTS

Obtaining of pathogen and biocontrol microorganisms

A strain of *V. inaequalis* (Anamorph *Spilocaea pomi*) was obtained with the following morphological characteristics: macroscopically, a clear mycelium and as it matured the mycelium changed to a greenish-brown color (after three days). Microscopically, in its Anamorph state, was observed several short and erect conidiophores with concentric rings in the apical part, in addition, conidia with one or two cells (Fig. 1). These characteristics agree with the description mentioned by Sivanesan, 1974 and Bowen, 2010. According descriptive characterization of pathogenic strains, so it can be deduced the strain belonging to the genus and species *Venturia inaequalis*.

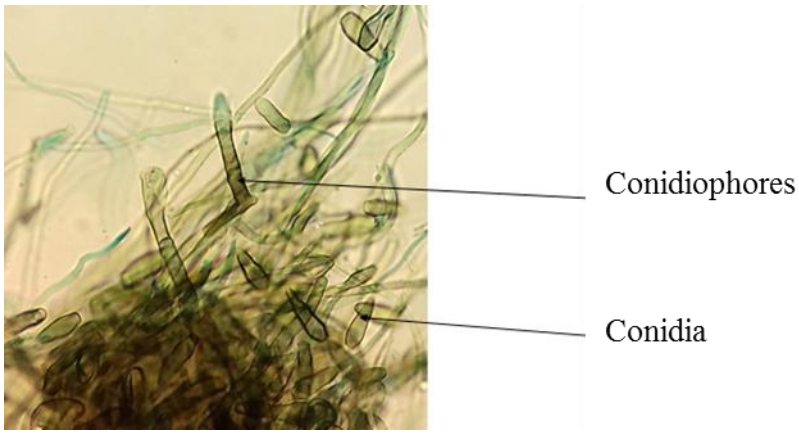

Figure 1. Morphological characteristics of *Venturia inaequalis* (Anamorph *Spilocaea pomi*)

In vitro antagonistic activity of *Trichoderma* spp. against pathogenic *Venturia inaequalis*

The overgrowth of *Trichoderma* strains over *V. inaequalis* (overlap) varied from 6 to 5.8 cm (Table 2). The analysis of variance showed that there is not significant difference (≤ 0.0001) among the treatments. The four *Trichoderma* strains showed the maximum antagonistic effect at two days, according to Bell et al. (1982), and these strains were classified as rank 1of this scale according to the Duncan means comparison procedure (Fig. 2).

Table 2. Overlap of the mycelial growth of *Trichoderma* spp. on *Venturia inaequalis* mycelia growth, days to first contact among microorganisms and class of antagonism.

Strains	Overlap (cm)	Days to contact	Antagonism Class*
T. yunnanense	6.0 ± 0.05 a	2	1
T. lignorum	5.9 ± 0.11 a	2	1
T. asperellum	5.8 ± 0.15 a	2	1
T. harzianum	6.8 ± 0.10 a	2	1

Treatments with the same letter are statistically equal to each other (P <0.05). * Class of antagonism according to Bell et al. (1982).

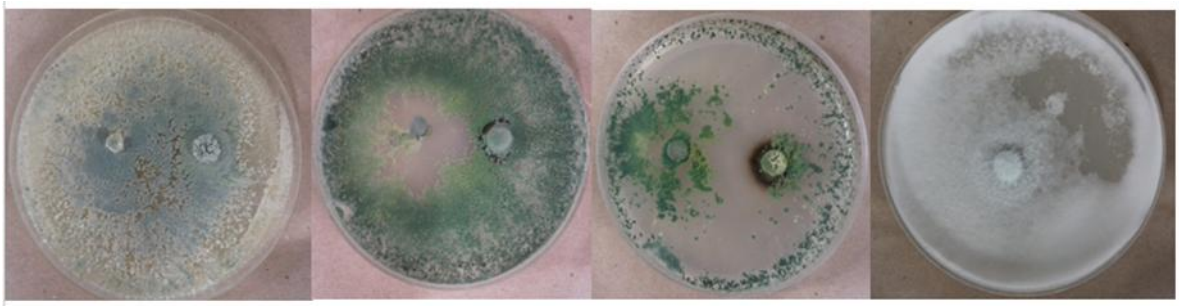

Figure 2. Antagonistic effect from *T. yunnanense, T. lignorum T. asperellum* and *T. harzianum* against *Venturia inaequalis*

In vitro antagonistic activity of *Bacillus* spp. against pathogenic *Venturia inaequalis*

The inhibition of *V. inaequalis* mycelia growth by the *Bacillus* strains ranged 33.4 to 41.3% for B. *subtilis* and *B. licheniformis*, respectively; on the sixth day of the trial (Fig. 3).

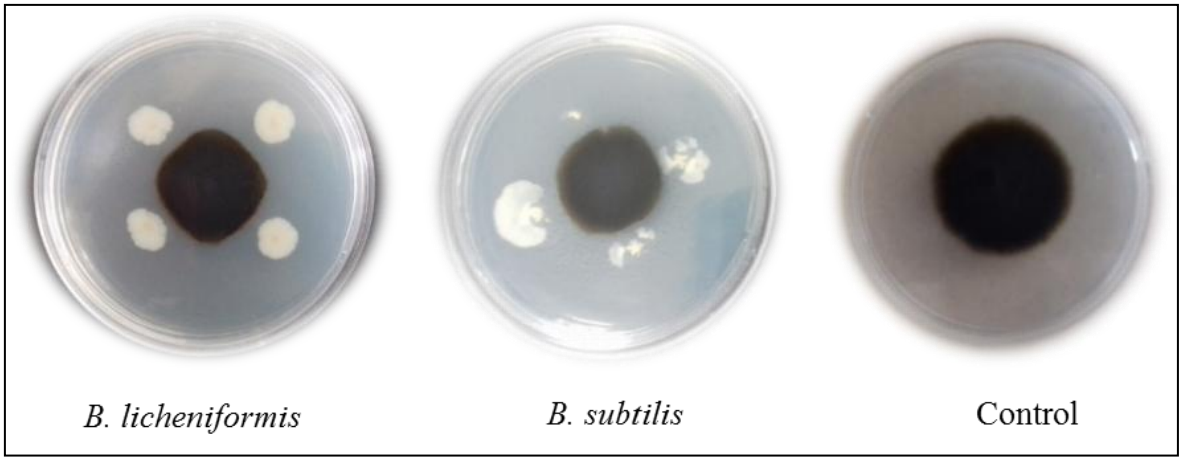

Figure 3. Antagonistic effect of *Bacillus* spp. against *Venturia inaequalis*

***In vitro* antifungal activity of the secondary metabolites of *Bacillus* spp. and *Trichoderma* spp. against *Venturia inaequalis* evaluated by means of the microplate technique**

The secondary metabolites obtained from *B. licheniformis* at a concentration of 50 and 25% showed an inhibiting effect in 100% against *V. inaequalis*, while the metabolites obtained from *B. subtilis* at a concentration of 50% showed an inhibiting effect near to 78% of the development of this pathogen (Fig. 4).

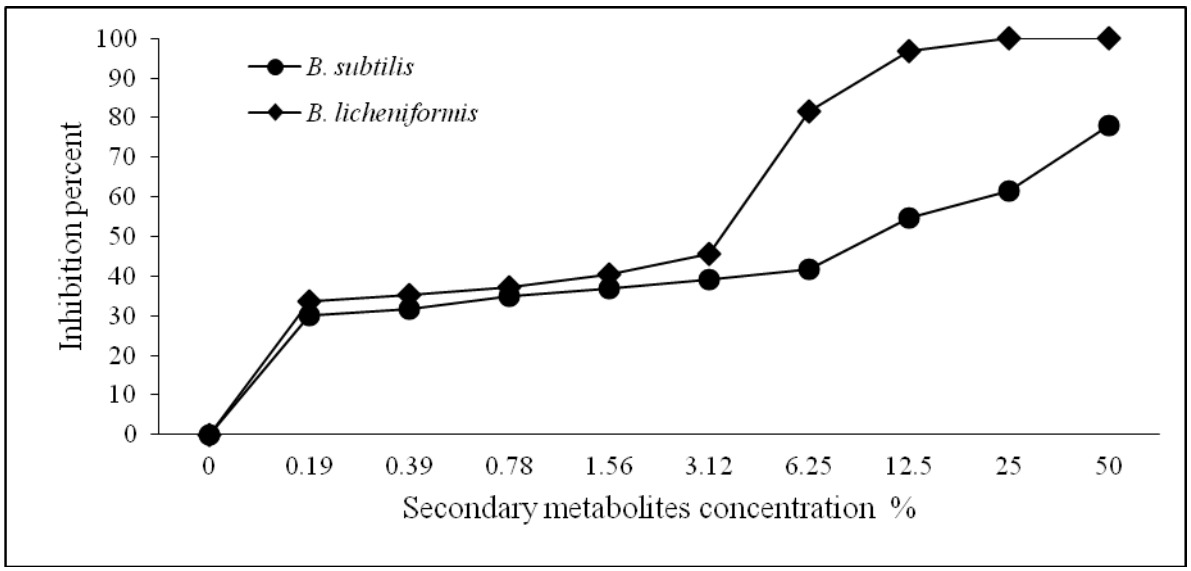

Figure 4. Percentage of inhibition of secondary metabolites obtained from *Bacillus* spp. against *Venturia inaequalis*

On the other hand, the secondary metabolites obtained from *T. yunnanense* and *T. harzianum* at a concentration of 50 and 25% showed an inhibiting total effect (100%) of mycelia growth of *V. inaequalis*, while the metabolites obtained of *T. asperellum* and *T. lignorum* at a concentration of 50% showed an inhibiting effect from 90 and 84%, respectively (Fig. 5).

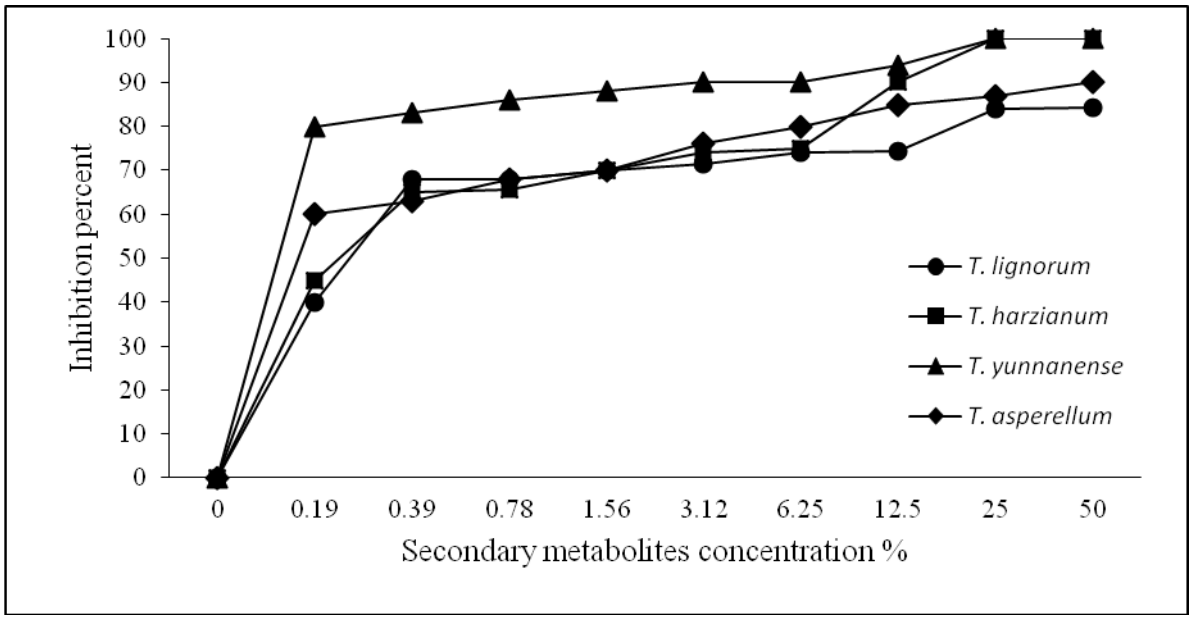

Figure 5. Percentage of inhibition of secondary metabolites obtained from *Trichoderma* spp. against *Venturia inaequalis*

In vivo antifungal activity from *Bacillus* spp. and *Trichoderma* spp. against incidence and severity of *Venturia inaequalis* under field conditions

Incidence and severity in fruit. The statistical analysis of bioassay in field, showed highly significant difference among treatments (P≤0.5) (Table 3). The incidence varied from 5.6 to 19.3% for the *Trichoderma* spp. 2L ha^{-1} and control, respectively, after 15 days of the first application. Sixty days after starting the applications, the incidence was expressed in a range of 42.5% to 91.2% for the *Bacillus* spp. 2L ha^{-1} and control, respectively. According to the severity, highly significant differences between treatments were also observed (P≤0.5). Observing a range of severity between 1.8 and 2.6 of lesions per fruit by treatment *Trichoderma* spp. 2L ha^{-1} and control, respectively, after 15 days of application initiation. After 60 days of application beginning, a range of number of lesions per fruit (severity) from 5.3 to 14.5 was observed, corresponding to *Bacillus* spp. 2L ha^{-1} and control, respectively (Table 4). The treatment with best antagonism effect under field conditions, was *Bacillus* spp. 2L ha^{-1} who expressed a 42.5% of incidence and 5 lesions per fruit in contrast to the control, which showed 91.2% incidence and 14.5 lesions per fruit.

Table 3. Incidence in apple fruits by *Venturia inaequalis*

Treatments	Incidence (%) in fruit			
	15 days	30 days	45 days	60 days
Bacillus spp. 1L/ha	18.12 ± 2.4 a	22.50 ± 2.0 b	23.25 ± 1.8 b	51.87 ± 5.5 b
Bacillus spp. 2L/ha	6.25 ± 5.2 b	18.12 ± 5.5 b	18.63 ± 5.3 b	42.50 ± 6.5 b
Trichoderma spp.1L/ha	15.00 ± 3.5 a	23.75 ± 3.2 b	25.00 ± 3.5 b	55.00 ± 5.4 b
Trichoderma spp.2L/ha	5.62 ± 4.7 b	10.62 ± 2.3 b	11.00 ± 2.7 b	46.62 ± 5.2 b
Control	19.37 ± 4.7 a	56.88 ± 4.7 a	60.00 ± 4.1 a	91.25 ± 4.3 a

Treatments with the same letter are statistically equal to each other (P <0.05).

Table 4. Severity in apple fruits by *Venturia inaequalis*

Treatments	Severity (Lesions) in fruit			
	15 days	30 days	45 days	60 days
Bacillus spp. 1L /ha	1.82 ± 0.6 ab	3.87 ± 0.4 ab	4.52 ± 0.8 a	8.02 ± 0.7 b
Bacillus spp. 2L /ha	1.07 ± 0.8 b	1.85 ± 0.8 ab	1.90 ± 0.7 b	5.30 ±0.5 b
Trichoderma spp.1L/ha	1.77 ± 0.5 a b	2.47 ± 0.4 ab	3.47 ± 0.6 ab	7.62 ± 0.2 b
Trichoderma spp.2L/ha	1.00 ± 0.0 b	1.35 ± 0.3 b	1.72 ± 0.6 b	6.32 ± 0.7 b
Control	2.65 ± 0.5 a	4.17 ± 0.5 a	4.77 ± 0.2 a	14.57± 0.3 a

Treatments with the same letter are statistically equal to each other (P <0.05).

Incidence and severity in apple leaves. The field experiment was carried out to test biocontrol agents for control *V. inaequalis* in commercial apple cultivar, the statistical analysis showed highly significant differences between treatments (P≤0.5). The incidence in foliage treated with *Trichoderma* spp. 2L ha^{-1} was more low in first evaluation (after 15 days first application) and until harvest. This treatment expressed 10.6% incidence and 2 lesions per leaf, in contrast to the control which showed 31.8% and 3 lesions per leaf (Table 5). On the other hand, severity did not show significant differences among treatments (Table 6).

Treatments	Incidence (%) in leaves			
	15 days	30 days	45 days	60 days
Bacillus spp. 1L/ha	8.12 ± 6.9 ab	11.25 ± 1.2 bc	13.12 ± 2.1 bc	22.50 ± 4.8 b
Bacillus spp. 2L/ha	6.25 ± 2.1 ab	11.25 ± 2.5 bc	11.87 ± 2.4 bc	17.50 ± 4.6 b
Trichoderma spp.1L/ha	8.13 ± 3.8 ab	13.12 ± 5.5 bc	13.75 ± 4.7 bc	20.62 ± 2.4 b
Trichoderma spp.2L/ha	2.50 ± 1.7 b	6.25 ± 1.4 c	6.25 ± 1.4 c	10.62 ± 1.3 c
Control	18.12 ± 4.1 a	21.87 ± 3.1 a	23.75 ± 4.3 a	31.87 ± 3.8 a

Table 5. Incidence in apple leaves by *Venturia inaequalis*
Treatments with the same letter are statistically equal to each other (P <0.05)

Treatments	Severity (Lesions) in leaves			
	15 days	30 days	45 days	60 days
Bacillus spp. 1L/ha	1.07 ± 0.2 a	1.60 ± 0.3 a	1.80 ± 0.3 a	3.00 ± 0.7 a
Bacillus spp. 2L/ha	1.30 ± 0.3 a	1.37 ± 0.5 a	1.77 ± 0.3 a	2.00 ± 0.2 a
Trichoderma spp. 1L/ha	1.20 ± 0.9 a	1.47 ± 0.6 a	1.85 ± 0.7 a	2.60 ± 0.6 a
Trichoderma spp. 2L/ha	0.75 ± 0.5 a	1.27 ± 0.2 a	1.40 ± 0.4 a	2.10 ± 0.3 a
Control	1.52 ± 0.5 a	1.72 ± 0.2 a	1.90 ± 0.3 a	3.30 ± 0.7 a

Table 6. Severity in apple leaves by *Venturia inaequalis*
Treatments with the same letter are statistically equal to each other (P <0.05)

DISCUSSION

Both antagonistic agents showed inhibiting effects against *V. inaequalis*, *in vitro* and *in vivo* conditions. The strains of *Trichoderma* showed antagonistic and hyperparasitic activity on the mycelial growth of *V. inaequalis*, on the other hand, it was observed that the contact time was two days, similar to results obtained by Osorio et al. (2016) in your antagonistic test of *Trichoderma* spp. over *Rhizoctonia solani*. This effect is possibly attributed to the production of various lytic enzymes, such as glucanases, chitinases, proteases among others, which helps cell wall degradation of the pathogenic fungus (Gonzales et al., 2012; Guédez, 2009). The confrontation results of *Bacillus* spp. against *V. inaequalis*, on the sixth day after of the test was conducted, a positive antagonistic effect was observed by these bacteria. This effect could be attributed to the production of lytic enzymes such as chitinases, hydrolases and proteases, as well as the production of bioactive antibiotics that allows the cytoplasmic

membrane interruption of the pathogenic fungus and this decreasing its growth (Yesid, 2012; Tejera, 2011).

In the test of the antifungal activity with secondary metabolites of *Bacillus* spp. against *V. inaequalis* positive results were presented, in range of 80 to 100% of inhibition, this effect is possibly due to the production of lipopeptides such as inturinas, surfactinas and fengicinas, which, are responsible for interacting with the molecules and inhibiting the growth of the conidia of the fungus (Baysal, 2013; Wu et al., 2014; Chung et al., 2015). On the other hand, the effect of the secondary metabolites produced by *Trichoderma* spp. inhibited in greater proportion to *V. inaequalis* with 84%, 90% and 100%, this is possibly attributed to the concentration of metabolites in the supernatant such as gliotoxin, viridin, pacibasin, trichodermin, furanone, 6 penthyl α-pyrone, almenticines and others; acting in the disintegration of the cytoplasm, inhibition of conidia germination and growth of germinative tubes of the pathogenic fungus (Hernández et al., 2011, Michel, 2004).

Finally, in the in vivo evaluation the applications of *Trichoderma* spp. and *Bacillus* spp. decreased the incidence and severity of the scab apple disease. This also coincides with reporters by Doolotkeldieva et al. (2017), which evaluated the application of *Trichoderma viride* over *V. inaequalis* in apple tree seedlings. They observed that in 35 days the disease was stopped in the leaves. These results are possibly attributed to the mechanisms of action mentioned in *in vitro* conditions by these biological agents which cause structural changes at the cellular level, disintegration of the cytoplasm and cell lysis of the pathogenic fungus (Harman, et al., 2004; Hernández, 2011). On the other hand, these microorganisms induce systemic resistance in the leaves, forming a protective film as a defense (Compant et al., 2005, Rudrappa et al., 2010, Ongena et al., 2007, Santoyo et al., 2012).

CONCLUSION

The strains of *Trichoderma* and *Bacillus* were able to inhibit the growth of *V. inaequalis* under *in vitro* conditions.

The secondary metabolites of *Trichoderma* spp. presented antifungal activity against *V. inaequalis*, being the strains *T. yunnanense* and *T. harzianum* more effectives for inhibition of mycelia growth. Similarly, the secondary metabolites of *Bacillus* spp. presented antifungal activity over *V. inaequalis*, being *B. licheniformis* the strain that showed the highest percentage of inhibition.

The formulations of *Bacillus* spp. and *Trichoderma* spp. applied, are effective for to managed and reduce the damages caused by *V. inaequalis* under field conditions in the Cañon de los Lirios, Municipality of Arteaga, Coahuila, Mexico.

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