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Chapter

Biological Efficacy of *Trichoderma* spp. and *Bacillus* spp. in the Management of Plant Diseases

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Abstract

This chapter will cover topics about the microbial antagonists *Trichoderma* spp. and *Bacillus* spp. from the perspective of use as potential biological control agents on plant diseases. Results obtained in the laboratory about from their isolation, microbial strain collections for both genera, taxonomic identification, antifungal activity in in vitro tests, obtained evaluation of the antifungal effect of secondary metabolites from microbial antagonists will be shown. Besides, results obtained from bioassays in the greenhouse and field are used as biopesticides in the control of diseases in fruit trees and vegetables and their effects on the promotion of plant growth and increased crop yield.

Keywords: inhibition, disease, plant pathogen, incidence, severity, antagonist microorganisms

1. Introduction

The agricultural production systems are generally based on technological dependence of high-yield varieties and the use of agrochemicals, causing an imbalance in different agroecosystems. The crops had turned to be susceptible to plague organisms to which before they were not and have proliferated because their natural enemies have eliminated the selection pressure has been favored by the monoculture or by the excessive use of plaguicides, what has conducted to the rupture of resistance of host and resistance toward the pesticides.

Those, as mentioned above, do rethink the actual technological management of the crops, researching less aggressive options with low or without environmental impact. This focus allows the searching of microbial alternatives as biological control agents of diseases, as a viable option to reduce their impact, enhancing the yield and quality of the agricultural products. The economic production losses caused by pests and diseases worldwide are estimated to be 36.5% on average, where 14.1% is caused by diseases, 10.2% by insects, and 12.2% by weeds, without considering the 6–12% of agricultural products postharvest losses. Although it estimated that in developing countries, these could reach up to 50% of production losses, considering only the disease, it estimates that annual losses worldwide can reach about 220 billion dollars. Overall, the diseases from plants can destroy crops before and after harvest or yield partial losses and cause loss of quality in the products harvested.

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For example, the apple scab caused by *Venturia inaequalis* (Cook) Wint. (Anamorph: *Spilocaea did Fr*.) is the most important disease of this fruit at a worldwide level, which can cause significant economic losses until 100% of the production, affecting the commercial quality of fruits [1, 2]. Generally, its control is based on the use of agrochemicals. In vegetables, wilting of chili pepper and tomato crops is one of the main biological limitations in the production of these crops and can be caused by *Phytophthora capsici*, *Rhizoctonia solani*, and *Fusarium oxysporum* [3]; this disease is reported throughout Mexico, estimating losses of up to 80% due to root rot by invading the vascular system of plants. Likewise, chemical control is the most used method for disease management and is common to reduce the inoculum by disinfecting the soil with metam sodium, 2-thiocyanomethyl benzothiozole (TCMTB), metalaxyl, azoxystrobin, and propanocarp fungicide applications to control *P. capsici* [4]. *R. solani* and *Fusarium* spp. are controlled with tebuconazole, carbendazim, thiabendazole, and methyl thiophanate [5]. The use of this control method significantly increases the production costs and the negative impact it causes on the environment and to human health and induces resistance of the pathogens toward the active ingredients. An alternative is the use of biological control by microorganisms antagonistic to fungi and stramenopiles from the soil, which has little or no effect on the environment and human health.

2. Biopesticides market

The worldwide market of biopesticides was of 1213 million dollars in 2010 and 3222 million dollars in 2017; the annual rate increases to 15.8% since 2012 besides 2017. Within this market, bioinsecticides represented 46% in 2011, and biofungicides were of 600.5 million dollars, reaching 1447 million in 2017. The annual rate from 2012 to 2017 grows up at 16.1%. Given that there currently exists a market demand for free products of pesticide waste, huge agrochemical companies are in the market of bioproducts, acquiring biocontrol companies and developing new biotechnological products.

3. Isolation and identification of *Bacillus* spp. and *Trichoderma* spp.

Trichoderma and *Bacillus* are essential genera of antagonistic microorganisms for control of a large number of phytopathogens. *Trichoderma* is a cosmopolitan soil fungus, which is frequently on soil from the plant root system. This fungus is attractive for organic management of diseases because present different action modes against phytopathogens as competition for nutrients, mycoparasitism, and antibiosis by hydrolytic enzymes and metabolites also produce substances that promote plant growth [6, 7]. On the other hand, *Bacillus* spp. is a large and heterogeneous group of Gram-positive, rod-shaped, aerobic and facultative anaerobic, and endospore-forming bacteria; same as *Trichoderma*, *Bacillus* is an alternative of biological control of plant diseases due to its capability to inhibit phytopathogens and growth promotion in plants [8, 9].

Due to the abovementioned and because there is a large number of species from both microorganisms, their isolation and identification for their possible commercial use are necessary; some of the species of *Trichoderma* are *T. virens*, *T. harzianum*, and *T. viride* and of *Bacillus* spp. are reported as antagonists *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, and *B. pumilus* [10, 11]. Thus a correct identification of the species which needs work is necessary.

3.1 Isolation and identification of *Trichoderma* spp.

3.1.1 Isolation and morphological identification of Trichoderma spp.

The first step for correct identification of antagonist microorganisms depends on isolation. In the case of *Trichoderma* spp., they are present in a great variety of agricultural and natural soils. The soil sampling for its isolation is relatively simple; using a shovel at 10–20 cm depth, 500 g of soil is taken and deposited in plastic bags; after, the samples will be moved to a laboratory and placed on storage at 4°C until used. Purification of *Trichoderma* spp. it is essential on investigations and present many ways or techniques; nevertheless, the monosporic culture is suggested by Trichoderma on culture media as potato dextrose agar (PDA) or *Trichoderma* Specific Medium (TSM), and incubated at 28 ± 2°C for 96 h [11–13]. Once the monosporic culture is obtained, the identification of *Trichoderma* species can be realized using taxonomic keys through its morphological features or with molecular biology, extracting DNA and utilizing general or specific primers. In case of the use of taxonomic keys, structures as width and length of phialide, length and width of conidia, and presence of chlamydospores will be observed [7, 14].

3.1.2 Molecular identification

This kind of identification has gained acceptance because it presents more precision and reliability among several strains of *Trichoderma.* The phylogeny of this genus has been based in the sequence analysis of the internal transcribed spacers of ribosomal DNA using the universal primers ITS1 and ITS4 with a subsequent sequencing and analysis through databases [15] but also can be identified through specific primers which are a powerful tool that allows to identify a specific species of *Trichoderma* [16] (**Table 1**). In Mexico, diverse species of *Trichoderma* have been isolated and identified [6]; they identified *T. atroviride*, *T. asperellum*, *T. citrinoviride*, *T. ghanense*, *T. harzianum*, *T. inhamatum*, *T. longibrachiatum*, and *T. yunnanense* (**Figure 1**) from samples taken from several agricultural regions. In a similar research, Osorio et al. [23] identified the species as *T. asperellum*, *T. rossicum*, and *T. hamatum* from different localities of Mexican Northeast region.

Species specificity	Primer sequences (5′–3′)	Product size (bp)	References
T. harzianum	HAR-1.6F: GTACCTCGCGAATGCATCTA HAR-1.6R: GGCTATGACCATGATTACGC	1600	[17]
T. asperellum	T2AF: CTCTGCCGTTGACTGTGAACG T2AR: CGATAGTGGGGTTGCCGTCAA	507	[18]
T. virens	TvCTT56f: CTTGATGACAAGCCAAAAGG TvCTT56r: GAAGAGAGGACATAGGGTCTGG	289	[19]
T. atroviride	Q01_4F: GCACACCAACTGCTGGAGCTT Q01_4R: CACGCTGACAATGACCGACAC	1017	[20]
T. aggressivum	Th-F: CGGTGACATCTGAAAAGTCGTG Th-R: TGTCACCCGTTCGGATCATCCG	444	[21]
T. pleuroti	FPforw1: CACATTCAATTGTGCCCGACGA PSrev1: GCGACACAGAGCACGTTGAATC	218	[22]

Table 1.
Examples of species-specific primers for Trichoderma *spp.*

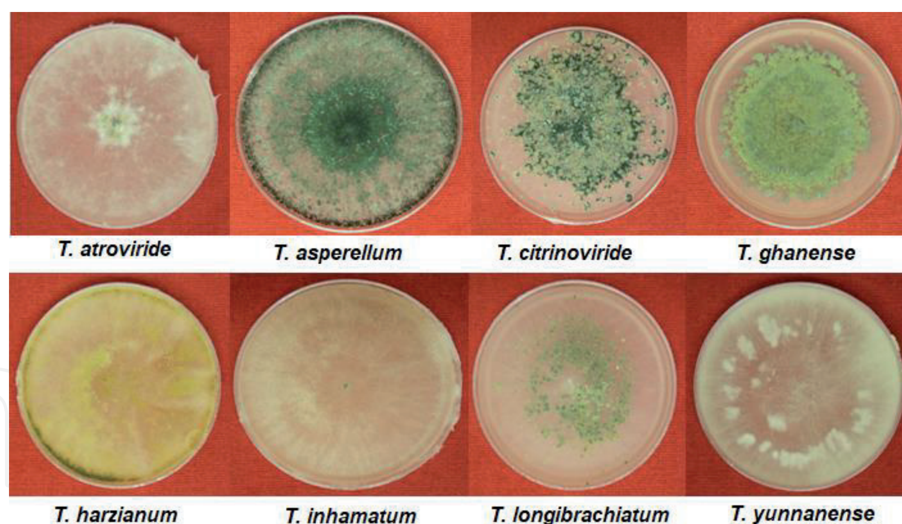

Figure 1.
Morphologic characteristics of different Trichoderma *spp. isolated from samples of different agricultural systems of Mexico.*

3.2 Isolation and identification of *Bacillus* spp.

Bacillus spp. is a genus present in the soil of a considerable amount of crops and naturally is on the rhizosphere; due to this, the traditional tools for determining the soil bacterial community and diversity are used [24]. The first step is to make the collecting of the rhizosphere soil, take 10 g of soil with a sterile spoon, and store the sample at 4°C. Heat or pasteurization treatments are the most commonly used techniques to select spores due to this, the sample is diluted in 90 mL of sterile normal saline and heated at 80°C for 10 min to eliminate vegetative cells; once heated, the sample is serially diluted (10^{-1}–10^{-4}) and placed on 1 mL nutrient agar (NA) medium with cycloheximide (100 mg mL^{-1}) to prevent fungal growth or carboxymethylcellulose (CMC) agar, and it is incubated at 37°C for 24 h [25, 26].

However, the treatment with heat can be different depending on the species because endospores of some strains are more resistant to heat than others [24]. Due to this, the drying treatment is considered more gentle; this method consists of placing the samples on a dryer at 70°C for 1 h [25]. The considerable variety of physiology of *Bacillus* spp. requires elaborate biochemical and morphological tests for species identification; as colony growth in artificial media, form cell unit, presence, number and orientation of flagella, Gram stain, spore form-position and specific environmental conditions of growth and finally the specific use of carbon sources gave its metabolic diversity [27].

3.2.1 Molecular identification of Bacillus spp.

Several molecular approaches are currently utilized for the identification of microorganisms; in this sense, the use of polymerase chain reaction (PCR) in combination with 16S rRNA is a tool frequently used for identification of *Bacillus* spp. from various environments including soil. Using the 16S rRNA sequence, five groups within biological control of root pathogens by plant growth-promoting *Bacillus* spp., the genus *Bacillus* spp., where group 1 comprises species *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, and *B. licheniformis*, have been identified [24, 25].

Bacillus spp. can identify through specific primers (**Table 2**). Such as *Trichoderma* spp., the Mexican agricultural systems are an excellent source to obtain *Bacillus* spp., such as mentioned by Guillén-Cruz et al. [9] and Hernandez-Castillo et al. [32] whom identified several *Bacillus* spp. species as *B. amyloliquefaciens*,

Species specificity	Primer sequences (5′–3′)	Product size (bp)	References
B. subtilis	p-gyrA-f: CAGTCAGGAAATGCGTACGTCCTT p-gyrA-r: CAAGGTAATGCTCCAGGCATTGCT	741	[28]
B. amyloliquefaciens	trpE(G) F: TTTGAATCCGAGCCCTTATG trpE(G) R: ACATACATTTCGGGGGATGA	78	[29]
B. pumilus	GC-U968(F): GCAACGCGAAGAACCTTAC L1401(R): GCGTGTGTACAAGACCC	490	[30]
B. licheniformis	BL8AF: TCACAACCCGTTGACGACAA BL8AR: CGTGTCCGAGTGTGCGTTATAT	247	[31]

Table 2.
Examples of species-specific primers for Bacillus *spp.*

B. pumilus, *B. licheniformis*, and *B. subtilis* from samples coming from several regions of the center and northern of Mexico.

4. Antifungal activity in vitro of *Trichoderma* spp. and *Bacillus* spp.

The antifungal activity of *Trichoderma* species has been evaluated in in vitro studies against soilborne and foliar fungi, and there have been acceptable results. The antifungal activity can be determined such a direct manner as indirect manner. In the case of a direct manner, the most used technique is the dual culture where the inhibition percentage, Bell scale, and the days to contact are evaluated to determine the antagonistic activity of *Trichoderma* species. Dual culture consists of Petri dishes with PDA where a disk (5 mm in diameter) with mycelium of the plant pathogen is placed and, on the other side of the Petri dish equidistantly, a disk of mycelium of the same diameter of *Trichoderma* strains under study is placed. The plates inoculated are incubated at 27 ± 1°C until the growth of control treatment (with only plant pathogen disk) covered the Petri dish. The effect of *Trichoderma* strains on plant pathogens is determined by the percentage of mycelial growth inhibition. The days of contact between plant pathogen antagonistic and antagonistic ability of *Trichoderma* isolates according to the methodology proposed by Bell et al. [33] are also determined. Bell et al. [33] classified the antagonism produced by *Trichoderma* as follows: Class I, *Trichoderma* overgrows completely to pathogen and covers the whole surface of the medium; Class II, *Trichoderma* overgrows two-thirds of the surface of the medium; Class III, *Trichoderma* and pathogen colonized each half of the surface, and nobody seems to dominate the other; Class IV, the pathogen colonizes the two-third parts of the media surface and resists invasion by *Trichoderma*; and Class V, the plant pathogen overgrows completely to *Trichoderma* and covers an area total culture media [6]. In case of the desire to determine the antifungal activity of an indirect manner, the volatile compounds are an option; this method is realized as follows. In the center of a Petri dish having only PDA medium, a disk of 5 mm in diameter with active mycelia of the plant pathogen is placed, and the top of the dish is replaced with another Petri dish in which a disk with mycelia of *Trichoderma* strain is placed; in this case, the lid is pierced with a punch (10 mm in diameter), and the Petri dishes are joined and sealed with parafilm paper and incubated at 26 ± 1°C until each pathogen control covered the Petri dish. The effect of volatile compounds is measured considering the diameter of pathogen colonies and expressed as percentage of mycelial growth inhibition [6].

Several research were carried out to determine the antifungal activity of *Trichoderma* spp. strains, due to its potential as biocontroller of plant pathogens,

as reported by Hernandez et al. [6] who evaluated several strains of *Trichoderma* spp. against *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* through dual culture and observed rate of inhibition of 45–63.8% and 50.9–81.5 for *S. sclerotiorum* with *T. ghanense* and *T. longibrachiatum*, respectively, and 81.5 and 81.2% of *S. cepivorum* with *T. inhamatum* and *T. asperellum*, respectively. For the Bell scale and contact days for both phytopathogenic fungi, the mean was of 2 days and scale of I, II, and III with all the *Trichoderma* spp. strains. Some research about the inhibition of secondary metabolites, precisely the volatile compounds, present inhibition of *S. sclerotiorum* and *S. cepivorum* against *T. longibrachiatum* with 28.1 and 73.8%, respectively, followed by *T. harzianum* with 12.5 and 62.5%.

Some studies by Osorio et al. [23] mentioned a Trichoderma spp. as controller. They reported the overgrowth of *Trichoderma* spp. strains over *Phytophthora capsici*; in total 13 *Trichoderma* spp. strains showed level 1 according to the Bell scale. This effect can be attributed to enzyme production (β-1, 3-glucanase, chitinase, protease, and cellulose) by these *Trichoderma* spp. strains. As too volatile compounds produced by the *Trichoderma* spp. strains, they reported inhibition of *P. capsici* mycelial growth ranged between 4.3 and 48.8, the major effect observed with the *T. asperellum* and the least with *Trichoderma* sp. strain. Some studies mentioned that *Trichoderma* spp. produces volatile compounds, carbon dioxide, oxygen, ethylene, and 6-pentyl-α-pyrone (6PP) which cause adverse effect in the development of phytopathogenic fungus. Furthermore, *Trichoderma* spp. can inhibit the growth of foliar fungus as *Colletotrichum* spp., such as mentioned by Tucuch et al. [34] who determined the antifungal activity *Trichoderma* spp. strains and reported antagonism level 1 in Bell scale by *T. asperellum*, while for volatile compounds, the species *T. lignorum* affected the fungus inhibiting its developments in 24.02% (**Figure 2**).

Usually, *Trichoderma* spp. inhibit several phytopathogenic fungi due to their capacity to produce enzymes, volatile compounds and compete for nutrients against phytopathogens. Studies realized by Samaniego-Fernández et al. [35] and Kumar et al. [7] showed the antagonistic capacity of *Trichoderma* spp.; in the first case, the species *T. harzianum* and *T. viride* are controlled to *S. rolfsii* y *Fusarium* spp.; in the second study, several *Trichoderma* spp. showed mycelial growth inhibition of *S. rolfsii* more than 50%.

Likewise, then *Trichoderma* spp. strains, the antifungal activity of *Bacillus* spp., can be tested by dual culture; nevertheless, the method is different than with *Trichoderma* spp. PDA disk (5 mm) with active mycelium of the phytopathogen is placed in the center of a Petri dish with PDA; on the same plate, at a distance of 1.5 cm in the four cardinal points, a loopful of antagonistic bacterial isolates is placed. Plates inoculated with the pathogen culture serve as controls. In order to quantify the antagonistic potential of bacterial strains, the size of growth inhibition zones measured after 6 days of incubation at 25–28°C and the percent of radial growth inhibition (PICR) are calculated [36]. In this sense, study also showed the capacity of *Bacillus* spp. to inhibit the growth of phytopathogenic fungi. Thereby

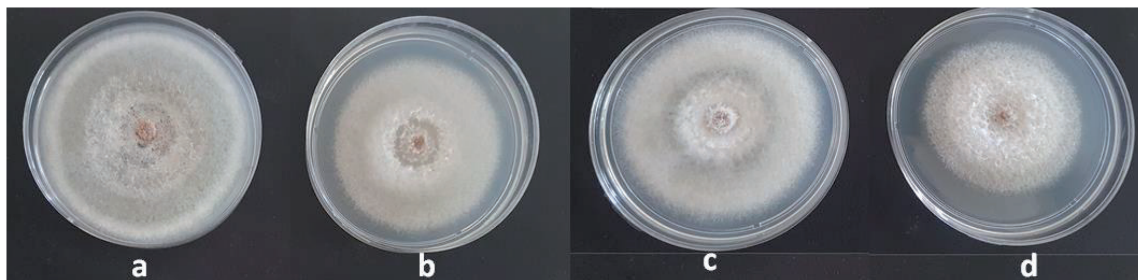

Figure 2.
Inhibition of Colletotrichum *spp. by volatile compounds produced by different* Trichoderma *spp. strains (a) control, (b)* T. asperellum, *(c)* T. yunnanense, *and (d)* T. lignorum.

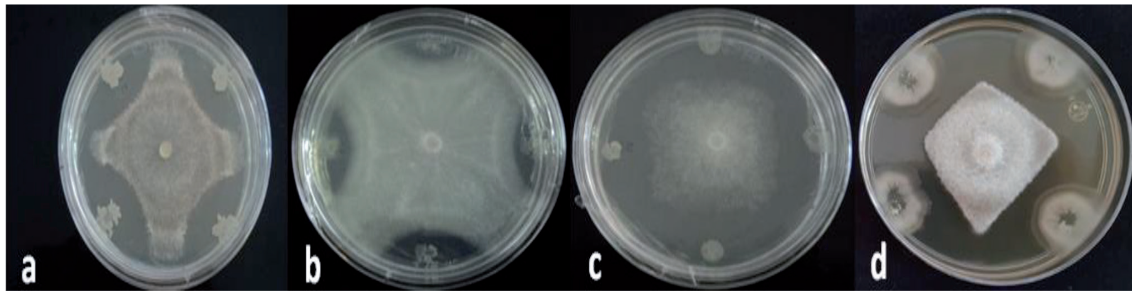

Figure 3.
Antagonistic effect of Bacillus *spp. strains against different phytopathogenic fungus (a)* Rhizoctonia solani*, (b)* Fusarium oxysporum*, (c)* Phytophthora capsici*, and (d)* Colletotrichum *spp.*

Jimenez et al. [36] reported the inhibition of *Venturia inaequalis* mycelia by *B. subtilis* and *B. licheniformis* ranged 33.4–41.3%, respectively, and Tucuch et al. [34] observed 50% of inhibition from *B. subtilis* against *Colletotrichum* spp. (**Figure 3**).

5. Obtaining secondary metabolites of *Trichoderma* spp. and *Bacillus* spp. and their antifungal activity in vitro (PDA methods and microplate dilution)

Generally, the production of the secondary metabolites from biological agents such as *Trichoderma* spp. and *Bacillus* spp. is carried out using liquid media by a fermentation process in a reactor, which can be of different types from a simple bottle to until an automated reactor, where the temperature and shaking rate are the key variables for the emission of secondary metabolites. The liquid medium is integrated by several components such as carbon sources and mineral salts, where the biological microorganism is inoculated [37]. The secondary metabolites obtained from the fermentation of *Bacillus* spp. and *Trichoderma* spp. are filtered with nitrocellulose membrane of 0.22 um; after the recovery of these metabolites, it is necessary to perform a screening to determine their ability to inhibit phytopathogenic microorganisms. There are many methods to determine the antifungal activity from secondary metabolites of antagonistic microorganisms, the most common is the poisoned medium, adding the substance to evaluate in the culture medium before solidification, which consists in adding 200 μl of the secondary metabolites on PDA medium in the center of Petri dish and 5 mm mycelium disk of the phytopathogen, then Petri dishes are incubated at 28 ± 1°C until the control treatment covers the petri dish, after that the percentage inhibition is determined [23].

However, our workgroup has standardized the method in microdilution on plate, which consists in an adaptation of the technique proposed by Masoko et al. [38]; polystyrene microplates of 96 wells are used; in all wells, 100 μl of liquid medium is placed; column 1 is the negative control, column 2 consists of the positive control, and column 3 is a control which consists of the fermentation medium. Starting in column 4, 100 μl of the secondary metabolites from strains is mixed in a pipette with 100 μl of the liquid medium, and then 100 μl of the mixture is transferred to the next column, discarding the last 100 μl from column 12, to get serial microdilutions to 50.00% of the secondary metabolites. Once the microdilutions are carried out, the growth developer 2,3,5-triphenyltetrazolium chloride is added in the whole plate; the concentration of the growth developer is the lowest reported in the literature, 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 deficient amounts of antimicrobial products; besides that, the red coloration is a visual indicator of the antimicrobial activity of the treatment. Finally, starting in column 2, 10 μl of a spore solution of the fungus at a concentration of 1×10^8 in all wells is added, keeping all the wells a volume of 150 μl in total; each microplate is considered a replicate. The microplates are incubated in agreement with the necessary conditions of the fungus on absorbance realized at 490 nm in a spectrophotometer. The secondary metabolites from the different strains placed in the rows A to F. To calculate the growth and inhibition percentage, the following formulas used: % Growth = (A − B/C)(100); where A is treatment absorbance, B is negative control absorbance, C is positive control absorbance, and % Inhibition = 100 – % Growth.

In general, the selection principle of strains is the determination of their antagonistic capacity; the method of microdilution in plate is to some extent interesting since it allows determined quickly and efficiently in time and costs its capacity of antifungal inhibition. Several studies demonstrate the effectiveness of secondary metabolites in the control of phytopathogenic fungi with PDA medium; Osorio et al. [23] mentioned that the inhibiting effect by *T. asperellum* and *T. hamatum* against *P. capsici* ranged to 15–20%; this inhibition attributed to the concentration of metabolites like glycotoxins, viridine, trichodermin, furanone, and 6-pentyl-α-pyrone (**Figure 4**).

Likewise, the PDA method or the microdilution in plate method can be an excellent technique to evaluate substance with antifungal activity as the secondary metabolites; in this sense, Jimenez et al. [36] observed that secondary metabolites obtained from *T. yunnanense* and T. *harzianum* at a concentration of 50 and 25% showed an inhibiting total effect of 100% of mycelial growth of *V. inaequalis*, while the metabolites obtained from *T. asperellum* and *T. lignorum* at a concentration of 50% showed an inhibiting effect from 90 to 84%, respectively (**Figure 5**). On the other hand, Jimenez et al. [36] reported that 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 (**Figure 5**).

In another study, Tucuch-Pérez et al. [39] reported six *Bacillus* spp. strains with antifungal activity against *F. oxysporum*; in this case, the species *B. licheniformis* and *B. subtilis* showed the highest inhibition percentages ranged from 80 to 100%, being the lowest inhibition percentage registered of 50% (**Figure 6**).

The results generated by the microplate dilution method are consistent with the results obtained from indirect test by confrontation or dual test, such as shown in the following results against fungi isolated from crops as melon, pepper, and others, which are produced in different zones from Mexico. In melon crop, the root and stem rot disease is a big problem; in this context, Espinoza-Ahumada et al. [40] studied the in vitro antagonist effects of *Trichoderma* spp. and found that *T. asperellum* have excellent biological activity against *Fusarium* strains, isolated from melon,

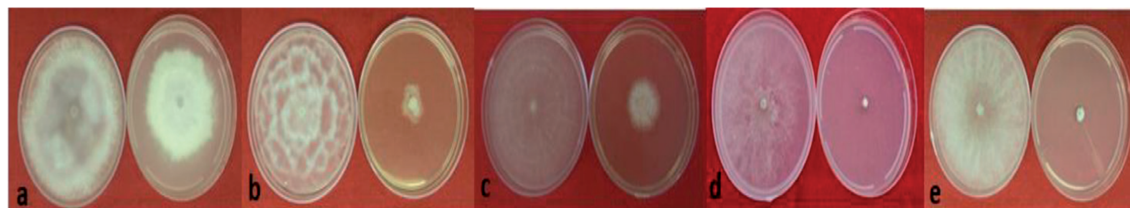

Figure 4.
Antifungal activity of secondary metabolites from different Trichoderma *spp. strains against phytopathogenic fungi; (a)* T. asperellum *vs.* Fusarium oxysporum, *(b)* T. yunannense *vs.* Phytophthora capsici, *(c)* T. longibrachiatum *vs.* Rhizoctonia solani, *(d)* T. asperellum *vs.* Sclerotinia sclerotiorum *and (e)* T. asperellum *vs.* Sclerotium cepivorum.

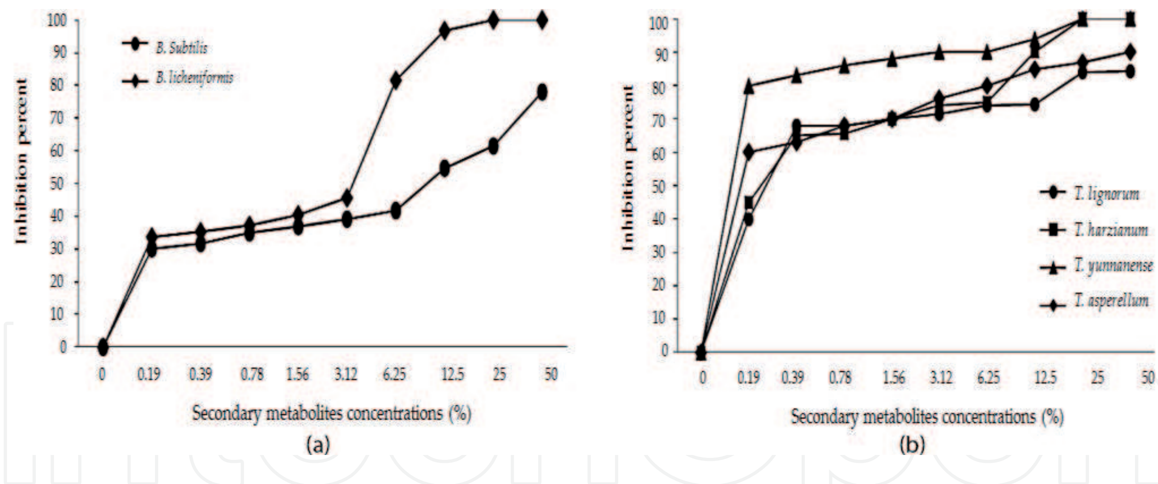

Figure 5.
Percentage of inhibition of secondary metabolites obtained from Bacillus *spp. (a) and* Trichoderma *spp. (b) against* Venturia inaequalis.

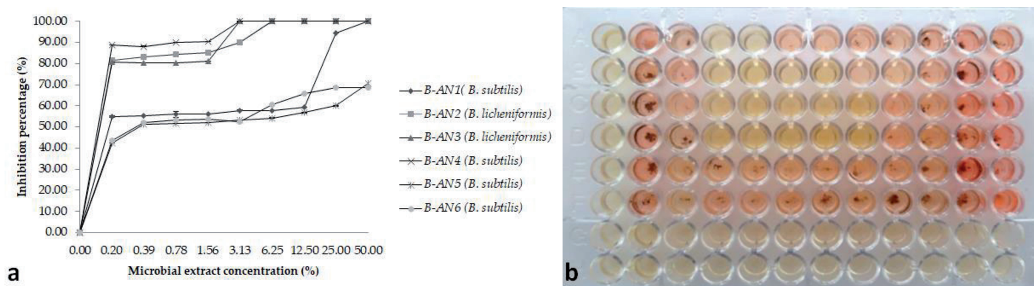

Figure 6.
(a) Percentage inhibition of microbial extracts from Bacillus *spp. metabolite dilutions against* F. oxysporum. *(b) Microplate with treatments to several concentrations, and the pathogen elapsed 48 h after incubation. Row A= B-AN1, B = B-AN2, C = B-AN3, D = B-AN4, E = B-AN5, F = B-AN6; column 1 = negative witness, column 2 = positive witness, 3 = growth medium of* Bacillus *spp., 5 = 50%, 5 = 25%, 6 = 12.50%, 7 = 6.25%, 8 = 3.13%, 9 = 1.56%, 10 = 0.78%, 11 = 0.39%, and 12 = 0.20%.*

shown in **Table 3**. In general, these authors report that the inhibition of *Fusarium* spp. is higher when *Trichoderma* spp. are used (62.4–54.8%), in contrast when *Bacillus* spp. (44.5–36.9%) is used (**Figure 7**).

In a work carried out by Francisco et al. [41], where the behavior of *Bacillus* spp. against *Fusarium* species was studied, it showed low inhibition values. However, they report that the species *B. pumilus* and *B. liquefaciens* can be used effectively against many *Fusarium* species. On the other hand, higher effectiveness of *Bacillus*

Antagonistic agent	***Fusarium* strain**					
	FRR-1	**FRG-2**	**FAF-3**	**FRE-4**	**FCA-5**	**FHA-6**
B. liquefaciens	$46.9^{A,ab}$	$38.4^{B,abc}$	$31.4^{BC,bc}$	$34.8^{CD,bc}$	$28.2^{CD,c}$	$41.6^{DE,ab}$
B. amyloliquefaciens	$51.4^{AB,ab}$	$38.9^{B,c}$	$37.8^{B,c}$	$42.9^{BC,bc}$	$41.2^{BC,bc}$	$55.2^{C,a}$
B. subtilis	$45.7^{B,a}$	$38.6^{B,a}$	$37.2^{B,a}$	$42.4^{B,ca}$	$34.6^{C,a}$	$46.4^{D,a}$
T. asperellum	$57.2^{A,b}$	$61.5^{A,ab}$	$64.3^{A,ab}$	$62.3^{A,ab}$	$54.9^{A,b}$	$74.1^{A,a}$
T. harzianum	$54.9^{AB,b}$	$55.4^{A,b}$	$56.1^{A,ab}$	$60.5^{A,ab}$	$57.9^{A,b}$	$64.7^{B,a}$
T. viride	$49.8^{A,ab}$	$58.3^{A,a}$	$54.9^{A,a}$	$51.1^{A,ab}$	$50.3^{BC,a}$	$64.7^{B,a}$

High letters indicate comparison between columns; low letters indicate comparison between rows. Percentages of inhibition with different letters are significantly different (p ≤ 0.5).

Table 3.
Percentage of antagonism of different biological agents against Fusarium *spp. strains.*

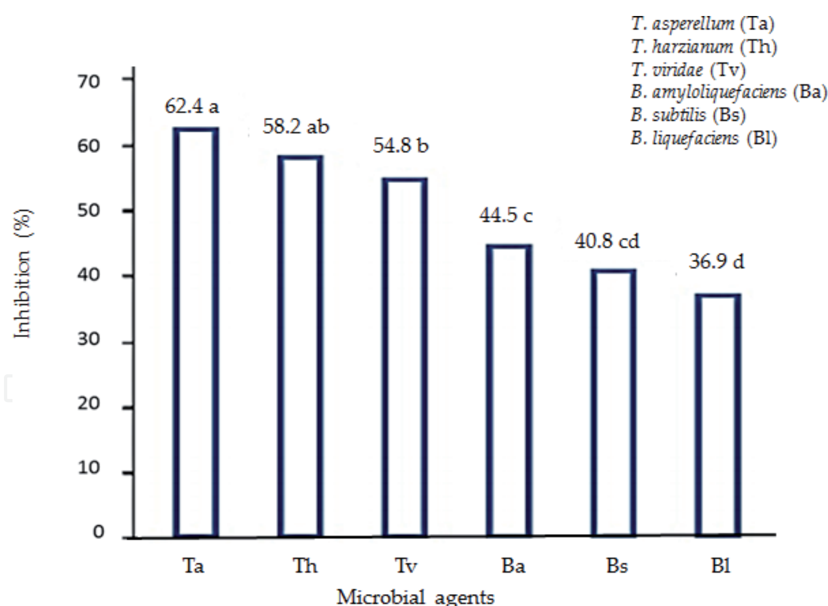

Figure 7.
Microbial agents antagonist to Fusarium oxysporum *(FAF-3, FRE-4, FHA-6) and* Fusarium solani *(FRR-1, FRG-2, FCA-5).*

spp. was observed when applied in the early stage of growth [42], showing that *B. cereus* was most effective against Fusarium dry rot when applied as young cultures (24 h), however *B. thuringiensis* strains was most effective when applied as older cultures (48–72 h). Nevertheless, different studies revealed that *B. pumilus* produced different antifungal compounds as "iturin" which inhibits the growth of *Aspergillus* sp. and their production of aflatoxins [30]. Osorio et al. [23] found an inhibition ranged between 4.3 and 48.8% of *P. capsici* mycelial growth induced by the volatile compounds produced by *Trichoderma* spp. strains. The Tukey test indicated that 21 *Trichoderma* spp. strains showed the highest percentage inhibition. *T. asperellum* (T25) strain present the best result for activity inhibition, strain (T9) being the one with the least inhibition activity. It observed that the 31 *Trichoderma* spp. strains were able to produce volatile compounds with inhibitory properties against *P. capsici*.

6. Antifungal activity bioassay under greenhouse conditions

From different research projects under greenhouse conditions, we have found satisfactory results both in the disease suppression and in the promotion of growth and quality in crops. Hernández-Castillo et al. [24], made an experiment under greenhouse conditions using silty clay soil from an experimental batch previously plot with chile crop and were symptoms of wilting incidence were express. The experiment included three bacterial strains of the genus *Bacillus* spp. (B1, B3, and B13), a chemical control (thiabendazole, T), and control (TA) without fungicide. Before the application of the suspension, an initial colony-forming units (CFU) count of the pathogen involved by the dilution method is performed. The application of spores of the bacterial strains is performed at the time of the transplant. The seedlings are immersed in a spore suspension at a concentration of 10^8 (CFU/mL for 15 min). Subsequently, at 20 and 40 days after transplantation, the same spore suspension was applied to the stem base. In the final evaluation of each treatment (10 adult plants), plant height, fresh fruit weight per cut, root length, dry root weight, and incidence and severity of wilt are measured. The determination of the severity of the disease was with the scale reported by Copes and Stevenson [43]. As a result of this work, a very low wilt incidence found for plants

is inoculated with biological strains (B13 and B3) with an incidence of less than 10%, while values of 60 and 40% for TA and T, respectively, were observed (**Figure 8A**). Likewise, the wilting showed a reduction in severity in those treatments where three bacterial strains were applied (**Figure 8B**), in contrast to the control treatments where the severity of the damage was more considerable.

In **Figure 9**, we can see that the harmful microbiological population rate also reduced with the use of organisms considered as beneficial, according to the final count at the end of the experiment; that could be because antagonistic bacteria are capable of influencing biocontrol mechanisms against phytopathogenic fungi such as antibiosis, siderophores, competition for nutrients, and production of hydrolytic enzymes. Similarly, Ulacio et al. [44] evaluated organic matter and antagonistic microorganisms as management strategies against white rot in garlic cultivation. These authors reported that the fungus *Sclerotium cepivorum* is significantly reduced and there was a lower incidence of the disease in the treatments where the fungus *T. harzianum*, the bacteria *B. firmus*, and vermicompost were combined.

Some microorganisms posess the ability by several ways to reduce the incidence and severity of diseases in crops, and also can participate in the stimulation of plant growth, yield, and crop quality. **Figure 10A** and **B** shows the values related to the promotion of root length (A) and its weight (B), where this effect is clearly observed. In **Figure 10C** and **D**, it was observed that *Bacillus* spp. strains increase the height of the plant by 28% compared to treatment T, and 34.5% concerning the TA. These results coincide with previous work where the biological effectiveness

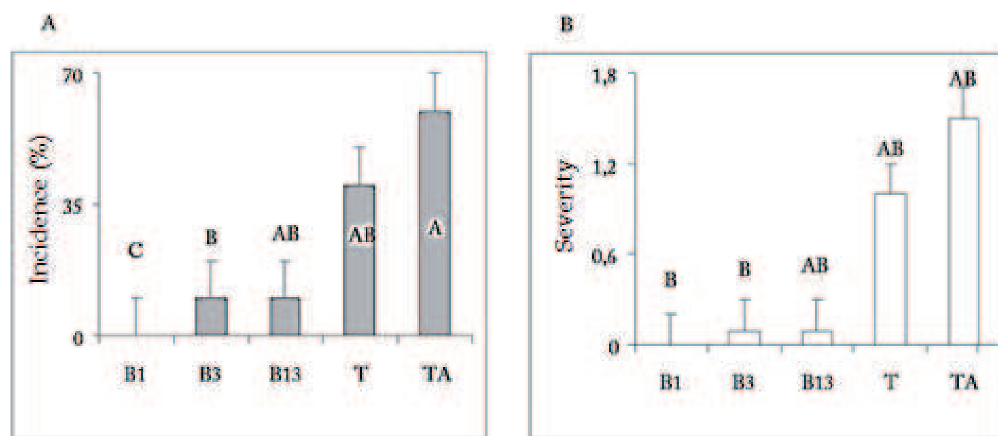

Figure 8.
Incidence (A) and severity (B) in plant traits with Bacillus *spp. strains (B1, B2, B3) in contrast with control (TA) and chemical control (T = thiabendazole).*

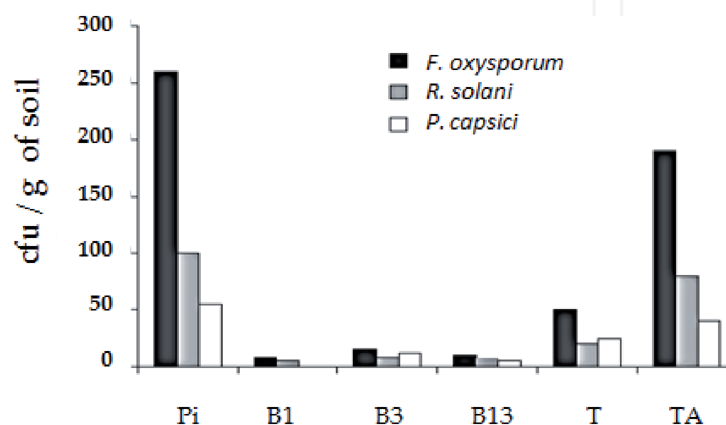

Figure 9.
Colony-forming units from initial (Pi) and final populations of phytopathogenic soil fungi after applying Bacillus *spp. strains (B1, B2, B3) against chemical (T = thiabendazole) and control (TA).*

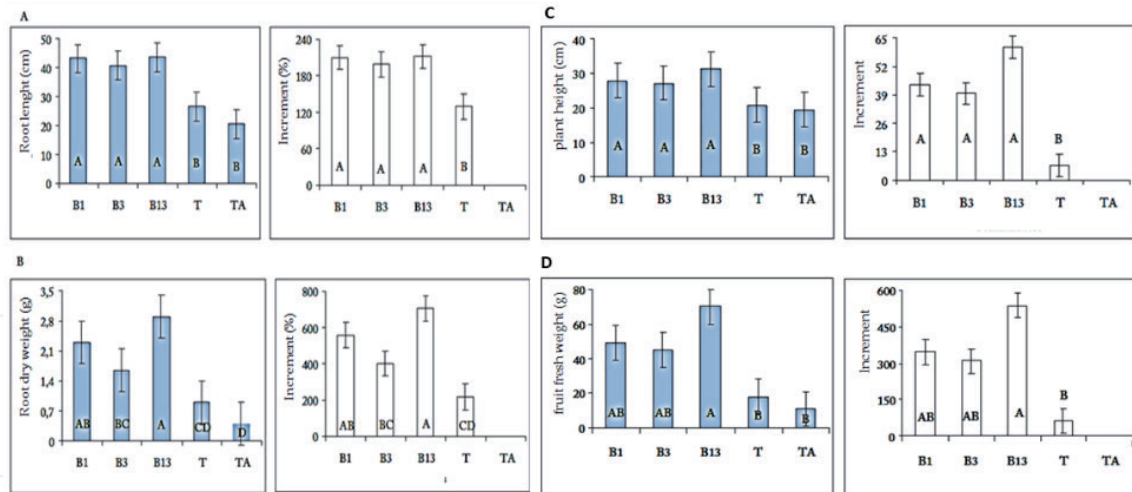

Figure 10.
Root length (A), dry rot weight (B), height (C), fresh fruit weight (D), and increments in chile pepper plant by effect Bacillus *strains (B1, B3, B13) against chemical (T = thiabendazole) and control (TA). Different letters with bars indicate significant differences among treatments ($p \leq 0.05$).*

of 57 strains of the genus *Bacillus* spp. isolated from the rhizosphere of commercial sowing chile plants in Northeast Mexico was analyzed, which showed an apparent antagonistic effect against *P. capsici*, *F. oxysporum*, and *R. solani* fungi. The plants inoculated with *Bacillus* spp. strains significantly increased height and dry weight in 191 and 60.2%, respectively [12]. The application of native *Bacillus* spp. strains shows a clear tendency to produce more biomass compared to chemical (T) and control (TA) treatments.

Likewise, del Ángel et al. [45] found a decrease in the incidence and severity of the disease caused by *Rhizoctonia solani* and *Fusarium oxysporum* with formulated endophytic bacteria, which induce a positive effect on the promotion of growth in the bean crop, increasing height and stem diameter in the treatments. Those formulated with bacteria in the absence of the phytopathogen stood out for their stimulating effect on the growth of the plants under study. This stimulating growth effect is observed in

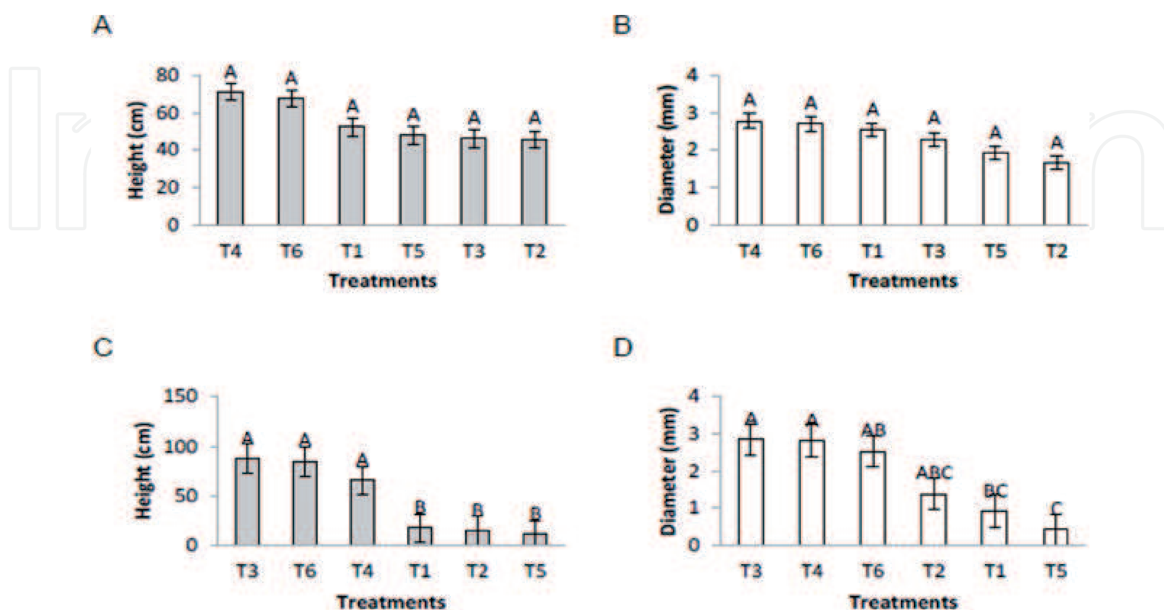

Figure 11.
Effect of endophytic bacteria on plant height and stem diameter in bean crop under greenhouse condition. Fusarium solani*: height (A), diameter (B), and* Rhizoctonia solani*: height (C), diameter (D). Means with the same letter are not significantly different according to the Tukey test ($p \leq 0.05$). Error bars are a standard error of the mean.*

plants treated with those formulated and inoculated at the same time with pathogens. It is essential to mention that the plants grew under no chemical treatment. Therefore, they did not receive fertilization by any chemical source (**Figure 11**).

7. Bioassays of antifungal activity under field conditions

7.1 Fruits results

Jimenez et al. [36] report results obtained on apple fruit and trees under the direct influence of the application of CFU from *Bacillus* spp., and *Trichoderma* spp., as control agents against the incidence and severity of *Venturia inaequalis* under field conditions in commercial apple cultivar. **Table 4** shows the incidence of fungus *Venturia inaequalis* in fruit, and this incidence varied from 5.6 to 6.25 when biological agents (*Trichoderma* spp. and *Bacillus* spp.) were used in maxima doses (2 L ha^{-1}) to 19.3% for the control, respectively, after 15 days of a first application. After 60 days from the start of the applications, the incidence is expressed in a range of 42.5–46.62% for *Bacillus* spp. and *Trichoderma* spp. at doses of 2 L ha^{-1} and for the control observed a 91.2%. The range of severity is observed between 1.8 and 2.6 of lesions per fruit by treatment *Trichoderma* spp. 2 L ha^{-1} and control, respectively, after 15 days of application initiation. After 60 days of treatment application appears first symptoms, so it was evaluated on a range of the number of lesions per fruit (severity) from 5.3 to 14.5 corresponding to *Bacillus* spp., 2 L ha^{-1}, and control, respectively (**Table 4**). The treatment with the best antagonism effect under field conditions was *Bacillus* spp., 2 L ha^{-1}, who expressed 42.5% by incidence and five lesions per fruit in contrast to the control, which showed 91.2% incidence and 14.5 lesions per fruit (**Figure 12**).

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

7.2 Vegetable results

Espinoza-Ahumada et al. [40] aimed to find more environmentally friendly alternatives to the wilting of chile pepper; they evaluated the application of

Treatment	Incidence (%) in fruit		Severity (lesions) in fruit	
	15 days	60 days	15 days	60 days
Bacillus spp. 1 L ha^{-1}	18.12 ± 2.4a	51.87 ± 5.5b	1.82 ± 0.6ab	8.02 ± 0.7b
Bacillus spp. 2 L ha^{-1}	6.25 ± 5.2b	42.50 ± 6.5b	1.07 ± 0.8b	5.30 ± 0.5b
Trichoderma spp. 1 L ha^{-1}	15.00 ± 3.5a	55.00 ± 5.4b	1.77 ± 0.5ab	7.62 ± 0.2b
Trichoderma spp. 2 L ha^{-1}	5.62 ± 4.7b	45.62 ± 5.2b	1.00 ± 0.0b	6.32 ± 0.7b
Control	19.37 ± 4.7a	91.25 ± 4.3a	2.65 ± 0.5a	14.57 ± 0.3a

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

Table 4.
The incidence in apple fruits by Venturia inaequalis.

Figure 12.
Expression of symptoms caused by Venturia inaequalis *in apple trees. (a) Without treatment, (b)* Bacillus *spp. effect, and (c)* Trichoderma *spp. effect.*

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

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

Table 5.
The incidence in apple leaves by Venturia inaequalis.

biological agents for this purpose under field conditions. For this, an experiment is established where different genotypes of chile pepper are evaluated (Serrano, HS-52, Coloso, HS-44, Centauro, Paraíso and Tampiqueño 74 cv.) generated by INIFAP-Mexico. In this experiment, the microbial agents *T. asperellum*, *T. harzianum*, *T. yunnanense* [23, 29], *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* [24] under a mixture of microbial propagative ferment (consortium ferment) are based on *Trichoderma* spp. and *Bacillus* spp. Treatments of bioassay by *Trichoderma* spp. were different: consortium treatment one consists of a *Trichoderma* spp. at $1{\times}10^8$ CFU; treatment two consists of ferment consortium; treatment three consists of a *B. consortium* at $1{\times}10^8$ CFU; treatment four consists of a chemical control by thiabendazole prepared at 60% W/V; and the treatment five consists of an absolute control. A dose of 1 $L.ha^{-1}$ was applied for treatments one, two, and three, while the dose applied for thiabendazole was 0.5 $kg.ha^{-1}$. Field sowing is done with chile seedlings (10 cm), transplanted in 1.5 m double row beds. The application is made to drench with a manual sprinkler at 7, 28, and 49 days after the transplant (DDT). After 85, 105, 125, and 145 DDT, the yield per block (4.5 m^2) is determined and transformed to t ha. To determine yields and improvements of treatments, ten fruits were evaluated, where the weight (g) and size (mm) per fruit were determined. In the first and last harvest, the incidence assessed and transformed into a percentage. The severity is evaluated through the visual scale, where 0 = no visible symptoms; 1 = initial light chlorosis and presence of flowers and fruits; 2 = intermediate, partial wilt, severe chlorosis, and premature ripening of fruits; and 3 = advanced. For total wilt without recovery, the leaves and fruits remain stuck to the stem. The field results observed as the effects of biological agents are shown in **Table 6**. The disease incidence values between HS-52 and Coloso treatments were statistically different ($p \leq 0.05$); in the other varieties, there were no differences between treatments. The treatment based on *Trichoderma* is the biological one that suppresses in higher

Microbial agents	Serrano chile pepper varieties					
	HS-52	Coloso	HS-44	Centauro	Tampiqueño 74	Paraíso
Trichoderma spp.	10.67a	18.17ab	16.84a	19.17a	12.5a	10.00a
Consortium	26.67ab	15.5ab	10.50a	15.33a	19.83a	10.67a
Bacillus spp.	29.17ab	29.67b	20.07a	20.5a	21.83a	19.5a
Thiabendazole	21.00ab	6.83a	19.51a	24.17a	10.33a	16.17a
Control	31.83b	21.33ab	21.51a	23.33a	24.67a	22.33a

Mean values on the same column indicated by different letters are statistically different ($p < 0.05$) according to the LSD test.

Table 6.
Incidence of the disease (%) in serrano chile pepper varieties inoculated with microbial agents in the field.

percentage the incidence of wilting disease in chile pepper crops; in this case, the lowest incidence was in the HS-52 variety which showed a value of 10.67%, while that in the witness it was 31.87%, which represents a decrease of 71% concerning the latter.

Disease evaluation in the presence of treatments of consortium and *Trichoderma* demonstrates the lowest incidence percentage with values between 14.39 and 16.39%, while the control and *Bacillus* spp. were having high levels of the presence of symptoms (24.08 and 23.36%). In the case of severity, it also behaves differently between treatments. **Table 7** shows the values related to the severity of the disease

Treatment	Serrano chile pepper varieties					
	HS-52	Coloso	HS-44	Centauro	Tampiqueño 74	Paraíso
Trichoderma	11.3ab	18.43a	10.8a	6.83a	7.83ab	6.93a
Consortium	8.33a	16.28a	24.45a	12.76a	14.35ab	6.46a
Bacillus spp.	14.4ab	19.16a	15.09a	11.54a	17.6b	16.22ab
Thiabendazole	20.04b	14.07a	17.97a	15.74a	6.56a	18.24b
Control	19.45ab	24.35a	24.07a	13.65a	17.96b	18.43b

Mean values on same column indicated by different letters are statistically different ($p < 0.05$) according to LSD test.

Table 7.
Severity of the disease (%) in serrano pepper with respect to treatments.

Microbial agents	Total yield in chile pepper varieties ($t\ ha^{-1}$)			
	HS-52	Centauro	Paraíso	HS-44
Trichoderma spp.	15.67a	13.22a	8.48b	7.55a
Consortium	10.37ab	11.52ab	10.59a	13.04a
Bacillus spp.	7.26b	8.18ab	5.41b	10.3a
Thiabendazole	10.02ab	8.69ab	7.44b	10.62a
Control	5.98b	5.15b	2.59b	6.94a

Mean values on the same column indicated by different letters are statistically different ($p < 0.05$) according to the LSD test.

Table 8.
Total yield, length, and weight of fruit of the serrano chile pepper crop obtained with the use of microbial agents.

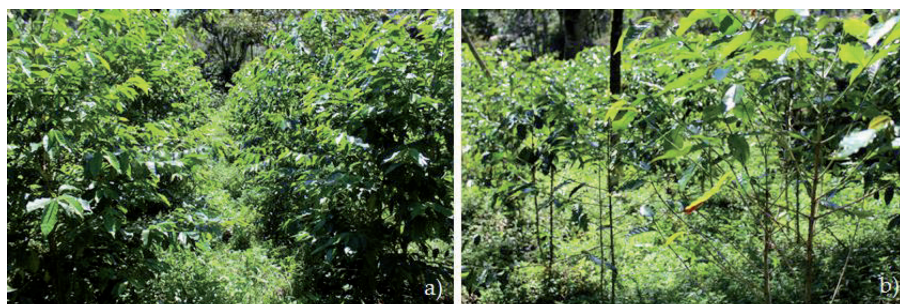

Figure 13.
Expression of incidence of coffee rust. (a) Plants with treatment based on bio formulate based on Bacillus *spp., and (b) plants without treatment, where leaf defoliation is clearly expressed.*

as transformed percentages ($p \leq 0.05$). It can be seen that *Trichoderma* spp.-based treatments alone or in combination have lower severity values.

The effects on yield as the weight and size of the fruit showed by the use of microbial agents applied alone or in combination as shown in **Table 8**. When *Trichoderma* is used, the yield increased; for example, its increase in the production was 62% when used alone and up to 76% when used as a mixture in comparison with the control.

This behavior of positive effect has already evidenced with the use of different *Trichoderma* species on habanero pepper plants (*Capsicum chinense*) [46], lettuce (*Lactuca sativa*), and radish (*Raphanus sativus*) [47]. In the same context, Cubillos-Hinojosa et al. [48] tested *T. harzianum* in the passion fruit crops (*Passiflora edulis*) where they were able to determine an antagonist to *F. oxysporum* and *F. solani*, in addition to stimulating germination, increased biomass, and root length.

In other field tests with *Bacillus* spp. bioformulate prototypes, a reduction in incidence and severity of coffee rust (*Hemileia vastatrix*) was observed. It was observed that the control presented 38% of incidence; nevertheless, it showed defoliation compared with the prototype treatments, which present an incidence between 5 and 15%, while with the chemist, the incidence was 9% (**Figure 13**).

The positive interaction between *Trichoderma* spp. and the host plant is attributed to a complex chemical activity of volatile and diffusible secondary metabolites and release of phytohormones and antibiotics in the rhizosphere, which promote root development and increased nutrient absorption, which helps control phytopathogens and increase yield [49]; this effect explains the results produced in this research. Microbial extracts as biofertilizers can generate hormones that stimulate development and increase yield, which are verified with the application of exudates from consortium *Trichoderma* spp. and *Bacillus* spp., which showed an effect on disease control and crop development in the same or better percentage than when using microorganisms.

8. Resistance induction by *Trichoderma* spp. and *Bacillus* spp.

In addition to the above aspects, plants can develop an increase in resistance to pathogen infection by treatment with a wide variety of biotic and abiotic inducers. Among the biotic inducers, we have the same phytopathogens, the growth-promoting rhizobacteria, and the microbial agents of the species of the genera *Bacillus* spp., *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* and nonpathogenic microorganisms such as *Trichoderma* species (antibiotics or siderophores that lead to induction of resistance).

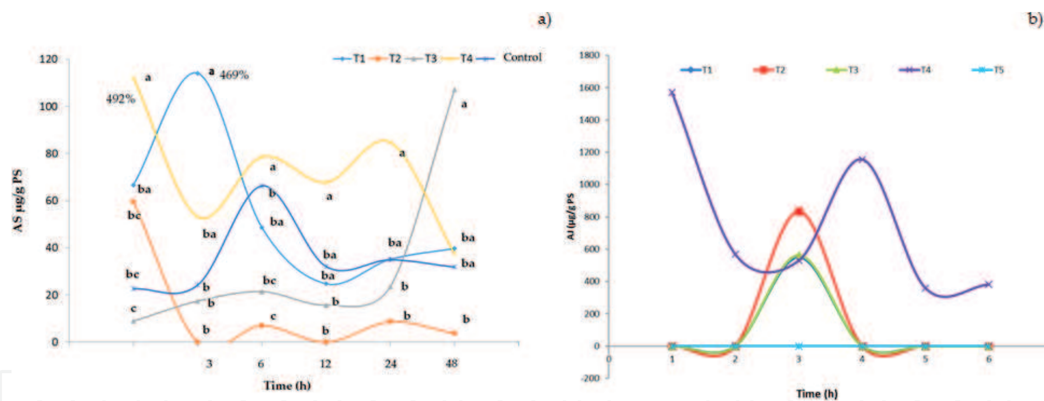

Figure 14.
(a) Salicylic acid production on potato leaves in a different time. T1 = Bacillus *spp. and* Pseudomonas fluorescens*, T2 = jasmonic ac. 1500 ppm, T3 = mezcla T1 0.5% + T2 0.1%, T4 = Milor®, and T5 = control (agua). Different letters indicate significant difference. (b) Jasmonic acid production on potato leaves in different time. T1 =* Bacillus *spp. and* Pseudomonas fluorescens*, T2 = jasmonic ac. 1500 ppm, T3 = mezcla T1 0.5% + T2 0.1%, T4 = Milor®, and T5 = control.*

Among the abiotic inducers are salicylic acid (SA), jasmonic acid (JAS), β-aminobutyric acid, ethylene, chitosan, potassium, sodium or magnesium phosphate, acibenzolar-S-methyl (ASM), menadione, sodium bisulfite, and phosphites. The application of these inducers causes specific biochemical changes that occur after their application such as expression of genes that code for PR proteins; the increase of certain defense-related enzymes such as polyphenol oxidase, lipoxygenase, peroxidase, superoxide dismutase, and phenylalanine ammonia-lyase (PAL); the accumulation of phytoalexins and phenolic compounds; and the reinforcement of the cell wall with lignin deposition.

In this regard, we have observed changes in the endogenous levels of salicylic acid and jasmonic acid in potato plants in response to foliar application of microbial consortiums based on *Bacillus* spp. and *Pseudomonas fluorescent*. The microbial consortium of *Bacillus* spp. significantly increased the production of SA 3 h after spraying raising to 114.02 µg/g DW. This is 496% more than the control (**Figure 14a**). Jasmonic acid is not detected in control plants but detected in plants treated with the microbial consortium. The level of jasmonic acid, 6 h later, reached a level of 550 µg/g DW (**Figure 14b**).

The resistance induction is associated with some defense gene expression as encoding pathogenicity-related proteins (PR), for example, phenylalanine ammonia-lyase, which is crucial in the synthesis of phytoalexins, because these constitute highly toxic compounds to the pathogen. On the other hand, PAL is part of the synthesis of salicylic acid and phenolic compounds that reduce the incidence of diseases in plants. It has also shown that *B. amylolicheniformis*, *B. subtilis*, *B. pumilus*, and *B. cereus* are capable of eliciting and activating the induced systemic resistance by increasing the levels of biochemical compounds related to resistance induction. Besides, it reported that some *Pseudomonas* species could induce systemic resistance in plants.

9. Conclusions

The results shown in this chapter allow to demonstrate the efficacy of *Bacillus* and *Trichoderma,* as agents of biological control of fungi and stramenopiles that are causatives of plant diseases; these beneficial microorganisms can be used under a sustainable agriculture program or under integrate management pest program in a conventional agriculture. The microbial agents also express other advantages due

to their beneficial effects on the increase of the yields, growth, and development of plants, as well as the induction of systemic resistance in plants to phytopathogens. Currently our workgroup has any projects on the development of prototypes based on these microbial agents, alone or in consortium, as well as micro- and nanoencapsulated formulations.

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Conflict of interest

The authors declare no conflict of interest.

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