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Biological Control of *Fusarium oxysporum* in Tomato Seedling Production with Mexican Strains of *Trichoderma*

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Abstract

The problems and limitations of the control of diseases caused by phytopathogens through the use of fungicides, make the biological control present as an alternative method in the production of tomato plants in greenhouse, which is limited by the incidence of *Fusarium oxysporum* Schlechtend.:Fr., being the most worldwide destructive disease. The objective of the present investigation was to evaluate the effect of three Mexican strains of the genus *Trichoderma* against *F. oxysporum* on the production of tomato seedlings under greenhouse conditions, as well as to determine the antagonistic effect of the strains used. The *Trichoderma harzianum* strain had the highest antagonistic activity (81.50%) and the highest growth rate (1.25 cm/day), proving to be the most aggressive strain to control *F. oxysporum*. In addition the results of the interaction of the dual cultures paired, presented a visible overgrowth zone with hyphae of *Trichoderma* spp. Seeds inoculated with *T. harzianum* showed a survival of 84% and a mortality of 16%, lower than the control group, which present a mortality of 58%; however, the treatment inoculated with *F. oxysporum* had the highest incidence of "disease" with 83%, the lowest survival (17%) and a decrease of the green biomass with respect to the control.

Keywords: antagonistic, greenhouse, phytopathogen, incidence, mortality

1. Introduction

The need to reduce the use of fungicides in phytosanitary control makes it necessary to develop technologies that allow easy, economical and effective ways to obtain products from

endogenous microorganisms with sufficient quality and quantity to their application in the crops areas [1]. In the soil there are microorganisms with antagonistic capacity, the most studied in the world is *Trichoderma* spp [2]; due to its ubiquity, its ability to isolate and present rapid growth on a large number of substrates [3, 4].

In the last 10 years, research work has been carried out, and native species of *Trichoderma* spp. have been isolated, selected and evaluated, with the potential to establish a biological control against different diseases, which have proposed several mechanisms of innovation for the implementation of this fungus with satisfactory results [5–7]. These mechanisms of action may act synergistically on various phytopathogens such as *Septoria triticii* in wheat, *Sclerotinia sclerotiorum* in soybean and lettuce, *Rhizoctonia solani* in soybean, *Sclerotium rolfsii* in cucumber cohombro, *Fusarium oxysporum* in tomato and *Pythium splendens* in beans [8, 9].

The genus *Trichoderma* presents several mechanisms by which they easily move to the phytopathogen, but the most important is based on three types: (a) Direct competition for space or nutrients [10–13], (b) Production of antibiotic metabolites, whether of a volatile or non-volatile nature [14, 15] and (c) Direct parasitism of some species of *Trichoderma* spp [16, 17].

The fungus *F. oxysporum* Schlechtend.: Fr. cause root and neck rot in tomato plants (*Lycopersicon esculentum* M.), causing severe losses that affect the quality and quantity of the production [18]. The most noticeable symptoms produced by using *F. oxysporum* occur in the transplantation of seedlings and at the beginning of flowering [18]. If a transverse section of the stem is made, it is possible to observe a vascular necrosis of brown color, particularly on the smaller lateral roots; which accelerates foliage wilting; after the plant dies and the fungus fructifies on the surface of the stem, under conditions of a humid environment [19]. The vascular wilt of the tomato by *F. oxysporum* was first described by Masse in 1885, on the Isle of Wight and Guernsey, located in the English Channel. In the year 1899, the disease was already in the United States of America, causing severe losses in the areas dedicated to growing tomato in the north of the state of Florida. In 1940 they reported that the disease was disseminated throughout the world and *F. oxysporum* was given greater importance [20].

The tomato (*L. esculentum* M.), is grown in all types of soils for family and commercial use [21]; for the 2016 year, occupied the first place with a total area planted of 4734 million hectares with a production of 163 million tons [22]. To date China is the first producer with 50 million tons, followed by India with 18 million tons, the United States with 12 million tons and Mexico is in the tenth position with 3282 million tons [23]. In Mexico the statistics of the Sistema de Información Agropecuaria [24], reported that in the 2016 year, 52,374 thousand hectares of tomato were planted with a production of 2875.164 tons, with a value of 15,735 million of pesos. While data from the Sistema Producto, they indicated that exports amounted to 20 billion pesos, with the United States and Canada being the main buyers; where the main producers were Sinaloa with 867,832.04 tons, San Luis Potosí with 196,011.25 tons and Michoacán with 169,768.98 tons [25]. Tomato production under greenhouse conditions during 2016 represented 26.2% of the national production, with average yields of 171.82 tons/ha, where Puebla ranked 14th with 75,219.09 tons of tomato [24].

In Mexico, few investigations have been carried out for the biological management of phytopathogens with soil origin through the use of native strains of *Trichoderma* spp [26]. Biocontrol of phytopathogenic fungi and biofertilization using the genus *Trichoderma* is a method used in various crops in different parts of the world; however, the use of commercial strains presents difficulties with their persistence in the soil, due to factors such as the genetics of the isolates, the environmental conditions and others characteristic of phytopathogenic species [27]. For the aftermentioned, the objective of this research was to characterize three native *Trichoderma* strains from the municipality of Tetela de Ocampo, Puebla-Mexico and to evaluate its antagonistic effect on the incidence of root and neck rot in tomatoes caused by *F. oxysporum* in the production of tomato seedlings in greenhouse.

2. Materials and methods

2.1. Strains

Strains native from the state of Puebla-Mexico were used, Th-T4 (3) from *Trichoderma harzianum*, Tav-T7 (2) from *Trichoderma atroviridae*, Tv-T3 (1) from *Trichoderma viridae* and the strain (Fo-A) from *Fusarium oxysporum*, which belong to the Centro de Recursos Genéticos del Centro de Agroecología del Instituto de Ciencias-BUAP and are in culture medium PDA (Potato Dextrose Agar).

2.2. Rate of development and speed of growth

The rate of development and rate growth of Th-T4 (3), Tav-T7 (2), TV-T3 (1) and (Fo-A) strains, was determined in Petri dishes (4.5 cm in diameter) in culture medium (PDA), incubated at room temperature for 7 days; the growth rate was measured every 24 h until the culmination of the total colonization of the strains, the macroscopic morphological characteristics of the colonies were recorded in texture, density, aerial mycelium and color. The rate of development and growth rate were determined using the following formula [28]:

$$TD = VCF - VCI/\text{Number of days} \quad (1)$$

2.3. Antagonistic activity of the strains of *Trichoderma* spp. on *F. oxysporum* in vitro

To evaluate the antagonistic activity of *Trichoderma* spp., Cherif and Benhamou technique was used [29]. For each of the treatments that were performed in Petri dishes with PDA (Agar, Dextrose and Potato) culture medium, was place at one end of the Petri dish a 5 mm diameter agar disc with mycelium of the pathogenic fungus, in this case *F. oxysporum*, due to its slow growth was allowed to develop for 2 days and then another 5 mm disc with mycelium of *Trichoderma* spp. was inoculated at the opposite end, (natives) at a distance of approximately 5 cm between them [30, 31]. The controls consisted of mycelium of the pathogens and

the antagonist, separately cultured in Petri dishes with PDA medium described above. Petri dishes were incubated at a temperature of 26°C. To measure the inhibitory effect of antagonistic fungus to the pathogen, measures colony diameter was recorded every 24 h; until standoff and formation of an inhibition zone between the colonies was formed. Ten repetitions were considered for each comparison, in this case will be three strains of *Trichoderma* spp., a single strain of *F. oxysporum*, evaluating a total of 30 experimental units. The percentage growth inhibition are calculated using the formula given by [32]:

$$\text{PICR} = \frac{[\text{R1} - \text{R2}]}{\text{R1}} \times 100 \quad (2)$$

where:

PICR: Percent inhibition of radial growth.

R1: Diameter of token.

R2: Diameter of organism tested.

Additionally, the strain was compared respect to the antagonistic capacity according to the scale proposed by Bell [33] in 1982 (**Table 1**).

2.4. Greenhouse antagonism tests

Seeds of tomato (*L. esculentum* Mill) var. Ramses were used, these were sterilized in 20% (v/v) sodium hypochlorite solution for 20 min, followed by three 5-minute washes in sterile distilled water. Subsequently the sowing was carried out in disinfected trays of 27 × 17 × 4 cm, containing 60 g of sterilized vermiculite in an autoclave at 121°C for 1 h. In each tray 100 tomato seeds were sown. Three strains of *Trichoderma* spp. were selected in the laboratory for their antagonistic response [Th-T4 (3), Tav-T7 (2), Tv-T3 (1)] plus two commercial strains (Perkins-C21 and Tricovel-25) and a control inoculated with *F. oxysporum* without *Trichoderma* spp. plus an absolute control; for a total of seven treatments, where each tray with 100 tomato seeds represented an experimental unit. The inoculations were carried out at the time of planting the seeds in the trays with 1 mL of a suspension of *F. oxysporum* at a concentration of 5 × 10⁶ conidia mL⁻¹ per well. The trays were installed in a culture chamber at 25 ± 2°C, were irrigated with 80 mL of distilled water per tray every 2 days. After 15 days, 1 mL of the five strains of *Trichoderma* spp. was applied at a total concentration of 83 × 10⁴ conidia with a viability percentage of 96%.

Class	Characteristics
1	Overgrowth of <i>Trichoderma</i> that colonized the entire medium surface and reduced colony pathogen.
2	Overgrowth <i>Trichoderma</i> colonized at least 2/3 of the medium surface.
3	<i>Trichoderma</i> and pathogen colonized medium to medium (more than 1/3 and less than 2/3).
4	Pathogenic fungus colonized at least 2/3 of the medium surface and resist invasion by <i>Trichoderma</i> .
5	Overgrowth of the pathogenic fungus that colonized the entire surface of the medium.

Table 1. Class and characteristics of *Trichoderma* spp. antagonistic capacity.

Scale of severity in root of plants			
Code	Description	Code	Description
a	Circular dry injury.	A	Healthy plant.
b	Light necrosis at the base of the root, lesion greater than 0.5 cm.	B	Plant with some leaves with loss of turgor.
c	Dry necrosis approx 1.5 cm, suberized, adventitious roots.	C	Plants with all leaves with loss of turgor.
d	Root presenting lesions with necrosis of 2 cm.	D	Plants with withered leaves. Planta sana.
e	Lesions with necrosis of 2 cm, roots begin to detach.		
f	Lesions with necrosis of 2 cm, large amount of roots are released.		
g	It gives off epidermis leaving vascular tissue, loss of 50% of roots.		
h	Detachment 80% roots expose tissue loss of epidermis, necrotic roots.		

Table 2. Severity scale of *F. oxysporum* attack on tomato culture.

The variables evaluated were incidence and severity of disease at 30 days after sowing, both for the radical part and for the aerial part (**Table 2**) using the scale proposed by Amaro-Leal [34]. The percentage of mortality and survival of tomato seedlings at 45 days, as well as height, stem thickness and total dry biomass were evaluated.

The results obtained were subjected to an analysis of variance (ANOVA) and test of separation of means by Tukey ($p < 0.05$), using the SPSS version 17 (Statistical Package for Social Sciences) to determine differences between treatments.

3. Results and discussion

3.1. Growth rate and rate of development

The Tv-T3 (1) and Th-T4 (3) strains of *Trichoderma* spp. presented a cottony texture with abundant density and abundant mycelium, a dark green and white coloration, whereas the Tav-T7 (2) strain presented a regular density, regular mycelium and a green/yellow coloration. For the *F. oxysporum* strain in this case (Fo-A) presented a velvety texture with a regular density and mycelium of pink white color, as described by Guzman [35], in addition its mycelium is formed by septate hyphae and the conidiophores present clusters of macroconidia where chlamydo spores are observed.

T. harzianum presented the highest growth rate with a mean of 1.25 cm/day, followed by *T. viridae* with 0.75 cm/day, with the *T. atroviridae* strain being the lowest growth rate with 0.64 cm/day. For *F. oxysporum* the growth was 0.83 mm/day, results similar to those found by Amaro-Leal [36], with a speed between 70 and 73 mm/day in PDA, results similar to those of the present investigation.

Code	Texture	Density	Aerial mycelium	Color	Form	Growth rate (cm/day) *	Development rate(mm/day) *
Tav-T7 <i>T. atroviridae</i>	Cottony	Regular	Regular	Green/yellow	Radial	0.75 b	4.85c
Th-T4 <i>T. harzianum</i>	Cottony	Abundant	Abundant	Green/white	Radial	1.25 a	5.69a
Tv-T3 <i>T. viridae</i>	Cottony	Abundant	Abundant	Green/white	Radial	0.64 c	5.04b
(Fo-A) <i>F. oxysporum</i>	Velvety	Low	Regular	White/pink	Radial	0.83 b	3.80d

*Different lowercase letters indicate significant differences with the Tukey test (P = 0.05)

Table 3. Macroscopic characterization of the colonies of Mexican strains *Trichoderma* spp. and *F. oxysporum* in culture PDA.

The highest development rate was 5.69 mm/day for *T. harzianum*, followed by *T. viridae* at 5.04 mm/day and *T. atroviridae* at 4.85 mm/day; with respect to strain of *F. oxysporum* Fo-A had a rate of 3.80 mm/day, where a significant difference ($p = 0.023$) occurs among strains of *Trichoderma* spp. (Table 3).

3.2. Confrontation of *Trichoderma* spp. on *F. oxysporum* in vitro

The results of the percentage of inhibition of *Trichoderma* spp. strains on *F. oxysporum* by the dual culture method are shown in Figure 1, the Mexican strains of *Trichoderma* spp. inhibited the growth of the pathogenic fungus, where they presented a percentage of inhibition of radial significant growth (PIRG) [$p = 0.056$] at the Fo-A strain of *F. oxysporum*, with *T. harzianum* which showed higher antagonistic activity, with an average value of 81.50% (PIRG), followed

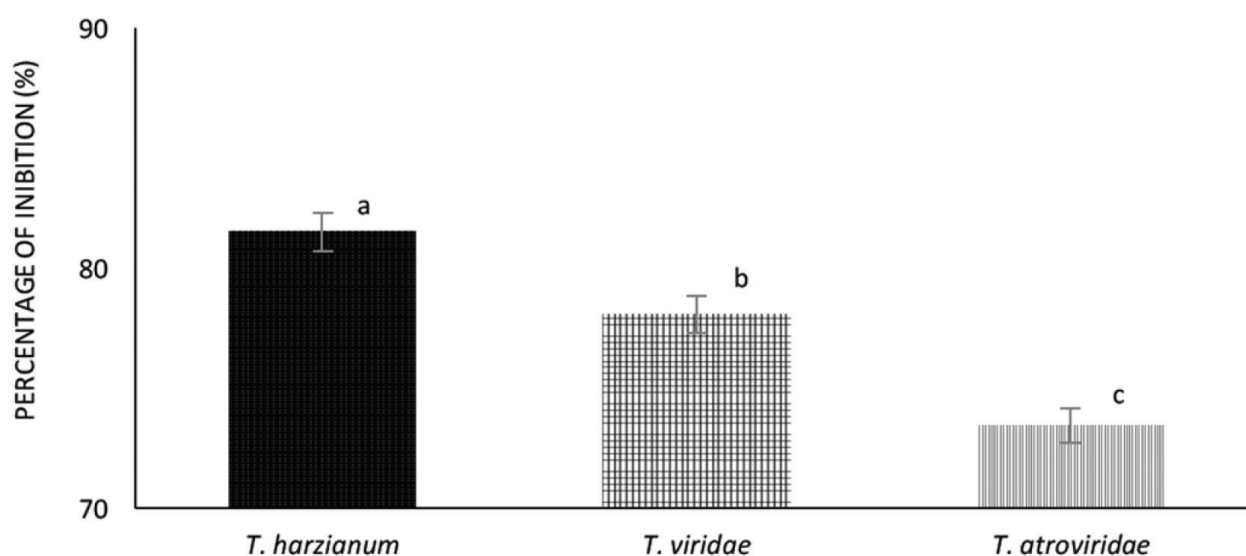


Figure 1. Percentage of radial growth inhibition (PIRG) in replicates of *Trichoderma* spp., on *F. oxysporum* in dual culture. *Different letters in the columns mean statistical differences in percent inhibition with Tukey's test ($p < 0.05$).

by *T. viridae* with (PIRG) 79.61% and finally *T. atroviridae* with (PIRG) 73.41%. Reports of the percentage inhibition of *Fusarium* by *Trichoderma* show values from 22.5 to 86.44% [37]. The values obtained from inhibition are higher than those obtained by Michel [38], who at evaluating the antagonistic effect of native *Trichoderma* spp., on mycelial growth and reproductive potential of *F. oxysporum* and *Fusarium subglutinans*, presented inhibition of 47.6 and 73.0%, respectively. Snyder and Hansen [39], reported a percentage inhibition of 77.8% for *F. oxysporum*, when compared with *Trichoderma viridae* isolates, results lower than those reported in the present investigation.

The results of the interaction of the most representative paired cultures are presented in **Figure 2**. The *F. oxysporum* Fo-A strain was given 2 days advantage because of its slow growth compared to *Trichoderma*; the days after the first contact between hyphae, the behavior was determined, which was very heterogeneous and highly significant ($P = 0.0001$). Most of the *Trichoderma* isolates showed a visible overgrowth zone with the hyphae of *F. oxysporum*; the greater the area of overgrowth, the greater the aggressiveness of the antagonistic fungus [29].

In this sense, Michel [36], reported antagonism 1, 2 and 3 of *F. subglutinans* and *F. oxysporum*, similar results in the present investigation.

3.3. Greenhouse antagonism tests

The treatment inoculated with *F. oxysporum* showed symptoms of the disease in the root and aerial part (**Table 4**), presenting the highest values in incidence and severity, this in comparison with the other treatments evaluated in this study. These results coincide with that observed by Kim [40], who point out the damage caused by *Phytophthora* sp., at the root and crown of the stem of chile plants under greenhouse conditions, similar results in this research.

In the present investigation, the lowest incidence and severity was obtained in the treatment based on *T. harzianum* with 6%, presenting slight dry circular lesions in the root and without symptomatology in the aerial part. *T. harzianum* has the ability to produce enzymes such as cellulases, β -1,3-glucanase and chitinases, which degrade the cell wall of phytopathogens [41].

Treatments based on *T. harzianum*, *T. atroviridae* and *T. viridae*. used in this research work, present antagonistic efficacy against *F. oxysporum* with a survival ranging from 62.7 to 76.4% in comparison to the control treatment (**Figure 3**), which had a survival rate of 46%; while

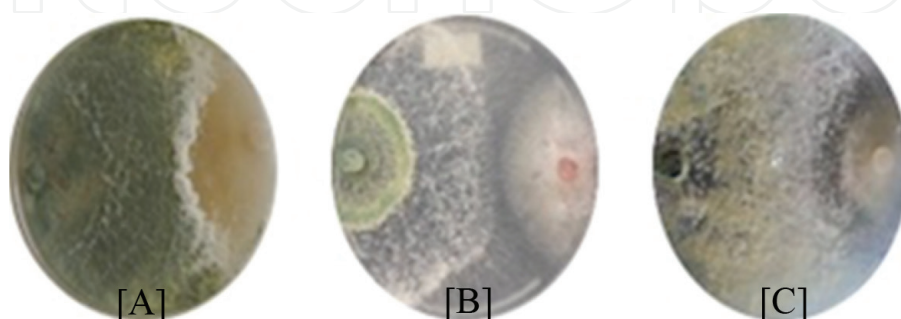


Figure 2. Antagonistic capacity, according to the scale of Bell for *Trichoderma* spp. on *F. oxysporum*. (A and B = *T. harzianum* and *T. atroviridae* overlapping *F. oxysporum*, has a type II interaction, covers 2/3 of the surface of the medium, stops its growth and can overgrow it, and C = *T. viridae* presents an interaction of type I, where it covers the entire surface of the medium and stops its growth).

Treatment	Root damage		Damage in aerial part
	% incidence	*Severity scale	% incidence
Witness	28	e	11
<i>F. oxysporum</i>	70	h	58
<i>T. viridae</i>	13	b	10
<i>T. atroviridae</i>	15	b	13
<i>T. harzianum</i>	6	a	4
Perkins-C21	24	e	14
Tricovel-25	26	e	14

Table 4. Percentage of incidence and severity caused by *F. oxysporum* at 30 days after sowing in tomato plants (*L. esculentum* Mill).

tomato plants inoculated with only *F. oxysporum* achieved the lowest survival with 26.3%. Michel [38] performed antagonistic studies with native isolates obtained from tomato crops planted in Tlayacapan, Morelos-Mexico, with which it confronted *Trichoderma* spp. against *Alternaria solani* and *Phytophthora infestans* achieving a range of inhibition percentage from 16.3 to 85.5%, results that are similar to those obtained in this study.

For the variables height, stem thickness and dry biomass of each treatment, showed significant differences ($p = 0.043$) among the strains of *Trichoderma* spp.; being the treatments based on Mexican strains *Trichoderma* spp. those that presented better results in the evaluated

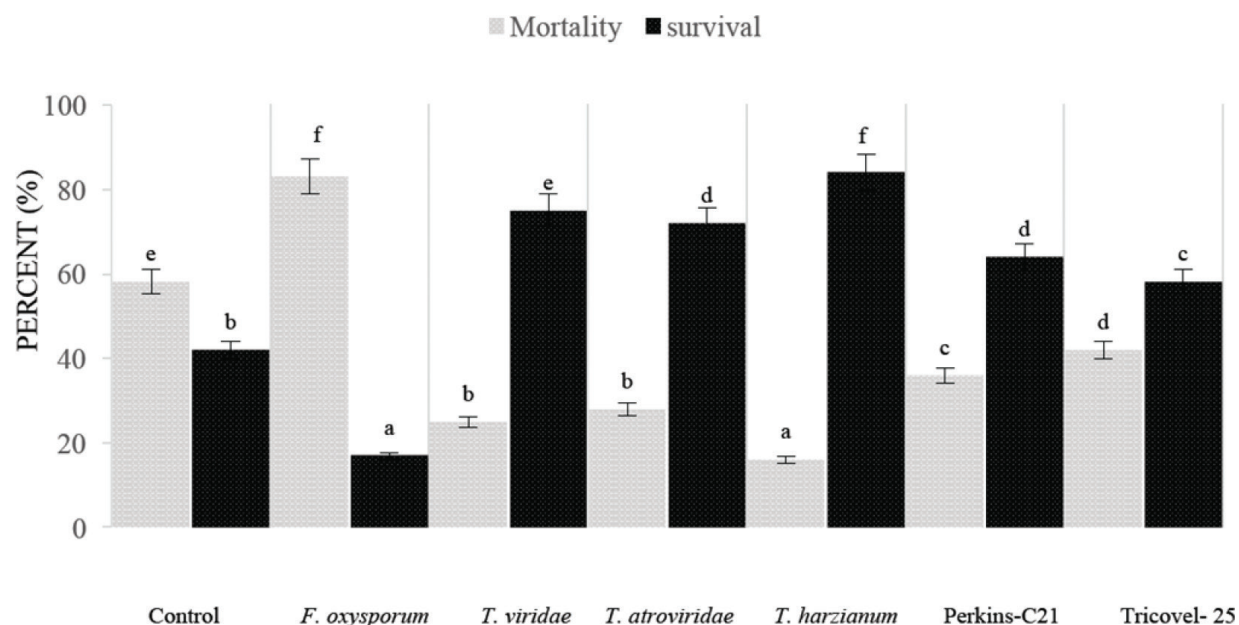


Figure 3. Percentage of mortality and survival caused by *F. oxysporum*, evaluated 30 days after tomato planting (*L. esculentum* Mill). *Different lowercase letters indicate significant differences for % of mortality and survival with Tukey test ($p < 0.05$).

Identification key	Root (cm)	* P. aerial (cm)	* Total height (cm)	* Stem diameter (mm)	* Weight (green)	* Weight (dry)						
Witness	4.53	c	9.04	d	13.57	b	0.20	d	0.19	b	0.02	b
<i>F. oxysporum</i>	3.70	c	8.96	d	12.66	b	0.10	c	0.18	b	0.02	b
<i>T. viridae</i>	9.25	ab	12.18	abc	21.43	a	0.80	a	0.62	b	0.05	b
<i>T. atroviridae</i>	9.04	ab	12.38	abc	21.42	a	0.60	b	0.48	b	0.04	b
<i>T. harzianum</i>	10.58	a	11.05	c	22.64	a	1.00	b	0.97	b	0.18	a
Perkins-C21	8.30	b	13.81	a	20.12	a	0.30	a	0.83	b	0.07	b
Tricovel- 25	7.81	b	12.73	b	20.54	a	0.30	d	0.66	a	0.06	b

*Different lowercase letters indicate significant statistic differences with the Tukey test (P = 0.05)

Table 5. Mean values of height, stem diameter and dry biomass of tomato plants (*L. esculentum* Mill) under greenhouse conditions.

variables, highlighting the treatment based on *T. harzianum*, which showed an average height of 22.64 cm/plant, an average stem thickness of 1.00 mm and an average dry biomass of 0.18 g; denoting a significant increase in comparison with the control, which showed an average height of 13.57 cm/plant, an average stem thickness of 0.20 cm and an average dry biomass of 0.02 g; while the treatment inoculated with the *F. oxysporum* strain Fo-A presented the lowest averages of height (12.66 cm/plant), 0.10 mm of stem thickness and 0.02 g in dry biomass (Table 5). Romo [42], mention that *Trichoderma* spp. has antifungal properties, thanks to the production of substances such as: trichodermine, dermadina, sequisterpeno, suzukacillina, alamethicina, trichotoxina, acetaldehyde, as well as extracellular enzymes such as β -1,3 glucanase, chitinase and cellulase that degrade The host cell walls and allow the penetration of the antagonist's hyphae, reducing its propagation in the root.



Figure 4. Significant statistic differences of each of the treatments in green weight. (a) *T. viridae*, (b) *T. harzianum*, (c) *T. atroviridae*, (d) Perkins-C21, (e) Tricovel-25, (f) control and (g) *F. Oxysporum*.

Harman [43], argues that *T. harzianum* stimulates the growth of plants by producing metabolites that promote developmental processes, which allow greater root development and absorbent hairs, which favors the mobilization of nutrients in the soil, thus improving nutrition and water absorption; also accelerates the decomposition of organic matter and minerals [44].

The native strains of *Trichoderma* spp. presented higher biomass (green), emphasizing the treatment based on *T. harzianum*, which showed a root height of 10.58 cm and a green biomass of 0.97 g; denoting a significant increase in comparison with the control "Figure 4", which showed an average root height of 4.53 cm, and a green biomass of 0.19 g, while the treatment inoculated with the *F. oxysporum* strain Fo-A, presented the lowest root height averages, with 3.70 cm and 0.18 g in green biomass.

4. Conclusions

Plants affected by *F. oxysporum* reduce their growth due to the pathogen's ability to colonize roots, which prevents proper nutrition of the seedling and leads to death, causing losses in the producer in the first stage of tomato crop.

The evaluation of Mexican strains of *Trichoderma* spp. and its antagonistic effect on *F. oxysporum* on tomato seedlings (*L. esculentum* Mill) was determinant to verify their potential for biological control in a crop of great importance in the economy and the country's food.

The *T. harzianum* strain presented the highest growth rate with a mean of 1.25 cm/day, proving to be the most aggressive strain to control *F. oxysporum* with a development rate of 3.80 mm/day.

The three native strains of *Trichoderma* spp. present inhibition of radial growth on the Fo-A (*F. oxysporum*) strain, with *T. harzianum* being the most antagonistic (81.50%), in addition, the results of the interaction of the paired dual cultures to *F. oxysporum*, was very heterogeneous and highly significant statistic differences, where the *Trichoderma* isolates showed a zone of visible overgrowth with the *F. oxysporum* hyphae, which shows more aggressiveness on the part of the antagonistic fungus.

The efficacy shown by the native strains of *Trichoderma* spp. evaluated in this study against *F. oxysporum*, applied to tomato seedlings (*Lycopersicon esculentum* Mill), showed that *T. harzianum* obtained higher height, greater stem thickness and greater production of dry biomass, likewise, the treatment inoculated with *F. oxysporum* obtained the highest incidence (83%) and the lowest survival (17%) of germination in greenhouse conditions.

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