Article

Trichoderma harzianum antagonism against chickpea fusariosis and its biofertilizing effect

Talina Olivia Martínez-Martínez[1]
Brenda Zulema Guerrero-Aguilar[1]
Víctor Pecina-Quintero[1]
Patricia Rivas-Valencia[2]
Enrique González-Pérez[1]
Juan Gabriel Angeles-Núñez[1§]

[1]Bajío Experimental Field-INIFAP. San Miguel de Allende-Celaya highway km 6.5, Celaya, Guanajuato, Mexico. CP. 38110. Tel. 5538718700, ext. 85242. (martinez.talina@inifap.gob.mx; guerrero.brenda@inifap.gob.mx; pecina.victor@inifap.gob.mx; gonzalez.enrique@inifap.gob.mx. [2]Experimental Field Valle de México-INIFAP. Los Reyes-Texcoco highway km 13.5, Coatlinchán, Texcoco, Mexico. CP. 56250. Tel. 5538718700, ext. 85214. (rivas.patricia@inifap.gob.mx).

[§]Corresponding author: angeles.gabriel@inifap.gob.mx.

Abstract

Chickpea is a legume, which is grown in two regions of Mexico mainly, Northwest (Sonora, Sinaloa and Baja California) and El Bajío region (Guanajuato, Michoacán and Jalisco); however, each year the production of the culture is compromised with vascular fusariosis, one of the main diseases that affect the culture and that is associated with the fungal complex *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Sclerotium rolfsii*. An alternative of biological control is the application of *Trichoderma*, which also has an indirect effect on the nutrition of the plant. The objective of this study was to determine the *in vitro* antagonism of two strains of *Trichoderma harzianum* (T1 and T2) and its effect as a biofertilizer. *In vitro* confrontations were carried out against strains of the *Fusarium oxysporum* f. sp. *ciceris* (Foc 0, 1B/C, 5 and 6), *Fusarium solani*, *Macrophomina phaseolina* (M-Sonora and M-GTO) and *Sclerotium rolfsii*. The effect of T2 as a biofertilizer (TB) was evaluated by measuring the variables: number of flowers, pods, plant height, stem diameter, root length and grain yield. The two strains of *T. harzianum* showed antagonism on different scales against pathogens. Additionally, with the treatment where *T. harzianum* (TB) was applied, there were increases in the number of flowers (30%), pods (24%), height (3%), plant diameter (3.5%), as well as root length (13%) and grain yield (23%).

Keywords: crop growth, grain yield, mycoparasitism.

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Introduction

The diseases represent one of the causes in the decrease in yields in the chickpea crop, among the most important in the world, are those caused by soil fungi, such as: *Fusarium oxysporum* Schltdl., *Fusarium solani* (Mart.) Sacc., *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi) Goid and *Sclerotium rolfsii* Sacc.; however, due to the damage they cause and the frequency with which they occur, *Fusarium* spp. It is of greater importance, especially the special form *ciceris*.

This disease called fusariosis, wilt or rabies of chickpea appears, mainly, in clay soils or with drainage problems, a condition that favors the development of these pathologies (Jiménez-Gasco *et al*., 2004). The yield losses quantified with the weight of 100 seeds can be 82% and up to 100% in susceptible varieties (Navas-Cortés *et al*., 2000). *Fusarium oxysporum* f. sp. *ciceris* (FOC) produces chlamydospores that can survive in the soil in the absence of the host for more than six years (Haware *et al*., 1996).

This pathogen spreads rapidly, attacks the root of the plant and causes water stress and nutrient deficiency due to occlusion of the xylem-conducting vessels, in addition to chlorosis, necrosis, and leaf abscission (Jiménez-Gasco *et al*., 2004). Eight races of have been described FOC: 0, 1A, 1B/C, 2, 3, 4, 5 and 6 (Haware and Nene, 1982; Jiménez-Diaz *et al*., 1993) and two pathogenicity biotypes: yellowing and wilt.

In the first, foliar yellowing and vascular coloration appear, the plant dies 40 days after inoculation of the pathogen. In wilt, severe chlorosis, flaccidity and vascular coloration are observed, the plant dies 20 days after inoculation (Jiménez-Gasco *et al*., 2004). Races 0 and 1B/C correspond to yellowing pathotypes, while races 1A, 2, 3, 4, 5 and 6 to wilt pathotypes (Arvayo-Ortiz *et al*., 2011).

Meanwhile, *Macrophomina phaseolina* is commonly present when there are high temperatures and low soil moisture, due to its ability to form sclerotia, allowing the fungus to survive adverse environmental conditions, it can cause losses due to the decrease in root length (44 -49%), sprouts length (5-16%) and fresh weight (55-63%) (Khan *et al*., 2017). *Sclerotium rolfsii* can cause a mortality of 55-95% of chickpea seedlings, high rainfall and temperatures above 25 °C are optimal conditions to cause disease, it survives as a mycelium in plant remains and as sclerotic structures in soil (Sharma and Ghosh, 2017).

Due to the above, the identification of biocontrol agents is required to counteract the effects of these pathogens, alternative to conventional control with chemical products, which represents a severe risk to human health and contributes to increased contamination of the environment. (Abdel-Monaim *et al*., 2011), in addition to the fact that they have given rise to highly resistant microorganisms that lead to fungal diseases with a higher incidence (Hoyos-Carvajal *et al*., 2019).

An alternative is the use of fungi of the *Trichoderma* genus, which is recognized as a biocontrol agent due to its capacity for antibiosis, mycoparasitism, competition for space and nutrients, as well as the production of secondary metabolites (Hernández-Melchor *et al*., 2019). Most

Trichoderma species have accelerated growth and development, can tolerate extreme environmental conditions, and are capable of parasitizing, controlling, and destroying fungi, nematodes, and other plant pathogens (Ruiz Cisneros *et al*., 2018), in addition to tolerating the presence of agrochemicals.

Chickpea *Trichoderma* has been noted for its antagonistic capacity (Rajput *et al*., 2010) and the control of pathogens such as *Rhizoctonia solani*, *Fusarium solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum* f. sp. *ciceri* (Abdel-Monaim *et al*., 2011; Khan *et al*., 2014). Different investigations show that the use of biological control is a viable alternative for the control of fusariosis in chickpea, in addition to improving the microbiological characteristics of the soil and therefore its physicochemical characteristics, at the same time promoting growth through the production of growth hormones, mineral solubilization, and induction of systemic resistance (Hernández-Melchor *et al*., 2019).

Given the information that has been developed by various investigations, the possibility of using *Trichoderma* strains for the control of fusariosis and its possible effect on the development of chickpea cultivation has been considered. In this context, the following objectives were set: 1) To determine the antagonistic effect *in vitro* of two strains of *T. harzianum* (T1 and T2) for the biological control of phytopathogenic fungi that cause chickpea fusariosis; 2) Evaluate the *in vivo* effect of the application of T2 on the development of the chickpea crop.

Materials and methods

Biological material

Four isolates of the *Fusarium oxysporum* f. sp. *ciceris* (Foc) breed were used. The Foc 5 breed from the Northwest zone and the Foc. 0, Foc. 1 B/C and Foc 6 breeds of El Bajio Guanajuatense area, the strains were previously identified by means of molecular markers type SCARs and RAPDs described by Jiménez-Gasco *et al*. (2004). One strain of *F. solani* and *Sclerotium rolfsii*, and two strains of *Macrophomina phaseolina* isolated from Sonora (M-Sonora) and Guanajuato (M-GTO).

The *Trichoderma harzianum* strains (T1 and T2) were donated by the Bajio Experimental Field (CEBAJ) of the National Institute for Forest, Agricultural and Livestock Research (INIFAP), isolated from agricultural soils in Valle de Santiago, Guanajuato. White chickpea seed var. Blanoro (spring autumn 2013 harvest) provided by the CEBAJ germplasm bank, whose protection was at -4 °C with 80% RH.

In vitro antagonism tests (dual cultures)

The antagonistic mechanisms of action of T1 and T2 against the eight phytopathogens involved in chickpea fusariosis (Foc 250. Foc. 71, Foc 27, Foc 12, *Macrophomina phaseolina* and *Sclerotium rolfsii*) were evaluated using the dual culture technique (Dennis and Webster, 1971). The evaluation was performed by observing the mechanisms of competition, mycoparasitism and antibiosis according to the scale described by Bell *et al*. (1982) (Table 1).

Table 1. *Trichoderma* antagonistic capacity according to the Bell *et al*. (1982).

Class	Antagonistic capacity
1	*Trichoderma* completely colonizes the plant pathogen and completely covers the surface of the medium.
2	*Trichoderma* colonizes two thirds of the culture medium, limiting the growth of the plant pathogen.
3	*Trichoderma* and the pathogen each colonize half of the surface, the growth is similar.
4	The plant pathogen colonizes two thirds of the surface of the culture medium and limits the growth of *Trichoderma*.
5	The plant pathogen completely colonizes the culture medium and grows on *Trichoderma*.

The discs were placed at the equidistant ends of the box and incubated at room temperature (22 °C). The evaluation of the antagonistic capacity was calculated estimating the percentage of radial growth inhibition (PRGI). This was obtained from the growth of each pathogen in dual culture, with respect to the controls, using the formula used by Suárez *et al*. (2008).

The tests were performed with five replications (plates), in a two-factor factorial design (Trichoderma strain and phytopathogens), an analysis of variance and comparison of Tukey means ($p \leq 0.05$) M was performed. The level of mycoparasitism was estimated considering the criteria established by Widyastuti (2006), for the microscopic observations the imprinting technique with diurex was used, a Carl Zeiss Axiostar plus compound microscope with a 40X field of view was used.

Effect of *Trichoderma harzianum* on chickpea cultivation

A chickpea plot of the Blanoro variety was established with a randomized block design with three experimental treatments corresponding to TC: control without fertilization, TQ: application of 60 kg of nitrogen ha^{-1} and 40 kg of phosphorus ha^{-1}, using urea (50-00-00) and triple superphosphate (50-00-00) and TB: application of the T2 strain ($1x10^8$ CFU g^{-1}).

For the effect of *Trichoderma* application in the field, the T2 strain was used, since the time for obtaining conidia was faster. Each treatment consisted of three repetitions, for a total of 9 experimental units, each with 10 plants distributed in 39 rows of 100 m and 0.8 m wide. At 45 days after sowing (DDS) the 'in drench' applications of the TQ and TB treatments were performed.

Two days after the application, weekly measurements were started up to the 108 DDS of the plant height, stem diameter, number of flowers and pod and at the end of the cycle the length of the root, plant weight and yield were measured. The height of the plant was measured from the neck to the apex using a flexometer; the diameter was considered from the middle part of the plant and was evaluated with a digital vernier.

After harvest, the length of the root was determined with a flexometer; the grain yield was estimated by obtaining the weight of the grains of the plants harvested per experimental unit and transformed into yield per hectare at a humidity of 12%. The data obtained was analyzed with SAS v. 9, using an analysis of variance and comparison of Tukey means ($p \leq 0.05$).

Results and discussion

In vitro confrontations with *Trichoderma harzianum* (dual cultures)

According to the scale described by Bell *et al*. (1982), the level of T1 antagonism against *Fusarium* strains was class 1 since the T1 strain invaded the box surface. While in the confrontations with *S. rolfsii*, *M. phaseolina* (M-Sonora) and (M-GTO) the level of T1 antagonism was found in class 2, because it only covered two thirds of the growth area of the pathogens (Figure 1).

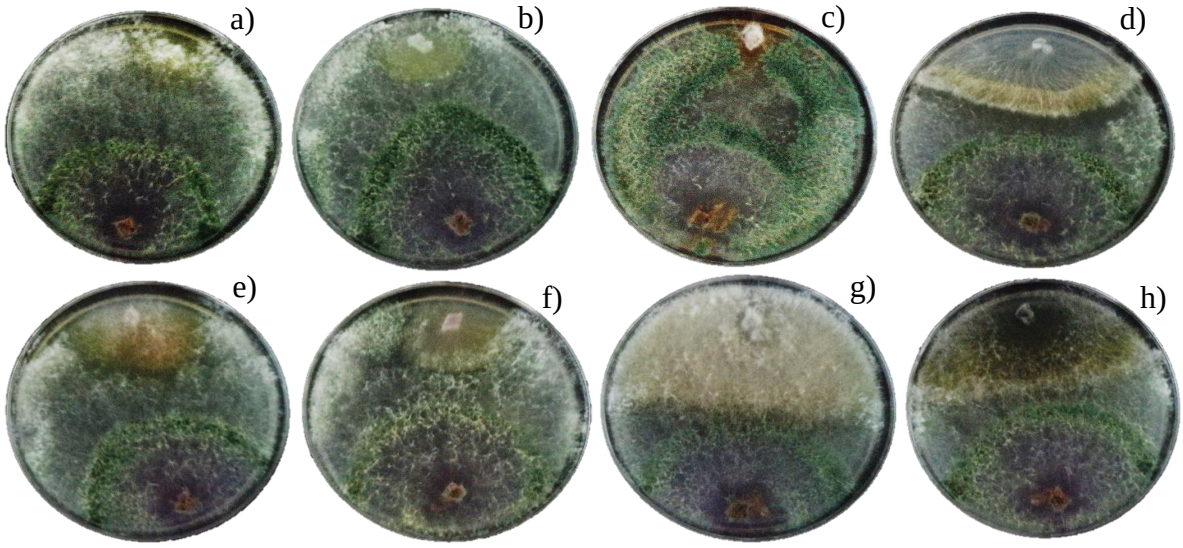

Figure 1. *In vitro* antagonistic evaluation of T1 strain (dual cultures). Evaluation of the interaction 7 days of growth a) Foc 250, mycoparasitism and competition for nutrients; b) Foc 71, mycoparasitism and competition for nutrients; c) *F. solani*, mycoparasitism and possible antibiosis; d) *S. rolfsii*, mycoparasitism and competition for nutrients; e) Foc 27, mycoparasitism and presence of antifungal enzymes; f) Foc 12, mycoparasitism and competition for nutrients; g) M-Sonora, mycoparasitisms and competition for nutrients and h) M-GTO, mycoparasitisms and competition for nutrients.

According to the criteria of Widyastuti (2006), the T1 strain exhibited greater growth with respect to the growth of pathogens associated with vascular fusariosis of chickpea. Compared to the Foc 250, Foc 71, Foc 12 strains, there was mild mycoparasitism and competition for nutrients; with *F. solani* possible antibiosis, while with Foc 27 there was production of antifungal enzymes due to the change of pale pink coloration at the contact site.

As for *S. rolfsii*, it presented moderate mycoparasitism and competition for nutrients, the two strains of *M. phaseolina* (M-Sonora) and (M-GTO) maintained moderate mycoparasitism and competition for nutrients. Regarding the T2 strain, a similar behavior of T1 colonization was obtained, the antagonism against the Foc 250, Foc 27, Foc 71 and Foc 12, *F. solani*, *S. rolfsii* and *M. phaseolina* (M-GTO) strains, was found in class 1, while colonization of half the surface of the medium was observed with the M-Sonora strain, which corresponded to a level of class 2 antagonism (Figure 2).

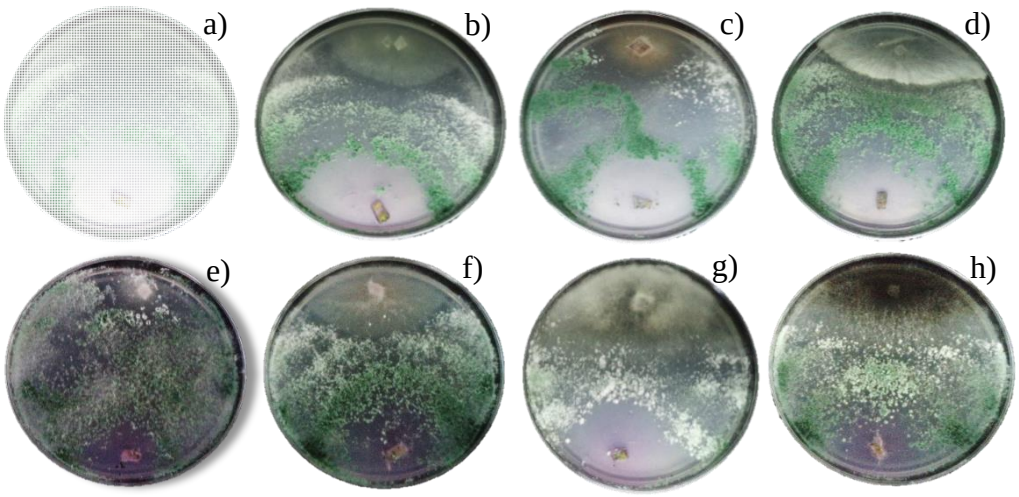

Figure 2. *In vitro* antagonistic evaluation of the T2 strain (dual cultures). Evaluation of the interaction 7 days of growth a) Foc 250, mycoparasitism and competition for nutrients; b) Foc 71, mycoparasitism and competition for nutrients; c) *F. solani*, mycoparasitism and competition for nutrients; d) *S. rolfsii*, micoparasitism and competition for nutrients; e) Foc 27, mycoparasitism; f) Foc 12, mycoparasitism and competition for nutrients; g) M-Sonora, micoparasitism and competition for nutrients; and h) M-GTO, mycoparasitism and competition for nutrients.

Regarding the antagonism mechanism, seven of the strains of the pathogen presented mycoparasitism and competition for nutrients, except for the Foc 27 strain that exhibited mycoparasitism and rapid growth (Widyastuti, 2006). Other studies have agreed that *Trichoderma* species maintain mycoparasitism values against plant pathogens on a scale of 1 (complete invasion); 2 (invasion two thirds); and 3 (*Trichoderma* and the plant pathogen each colonize half of the surface) (Michel-Aceves *et al*., 2009).

According to Guedez *et al*. (2012) a main characteristic in the choice of *Trichoderma* strains to be used in biological control is the aggressiveness for mycoparasitization and the growth rate, which must exceed that of the pathogen to be controlled. Invasion of the mycelium exerted *Trichoderma* confirmed its hyperparasitic effect act on reducing mycelial growth of pathogens, to respect, have been indicated different mechanisms antagonism ranging from competition for nutrients and space, production of antifungal metabolites and hydrolytic enzymes.

The antagonistic properties of this fungus are based on the activation of multiple mechanisms that also promote the production of specific compounds and metabolites that function as plant growth factors and improve their systemic resistance against diseases (Hernández-Melchor *et*

al., 2019). Microscopically, mycoparasitism mechanisms T1 and T2 strains on the eight strains phytopathogenic generally observed through the winding, penetration and vacuolization (Figure 3).

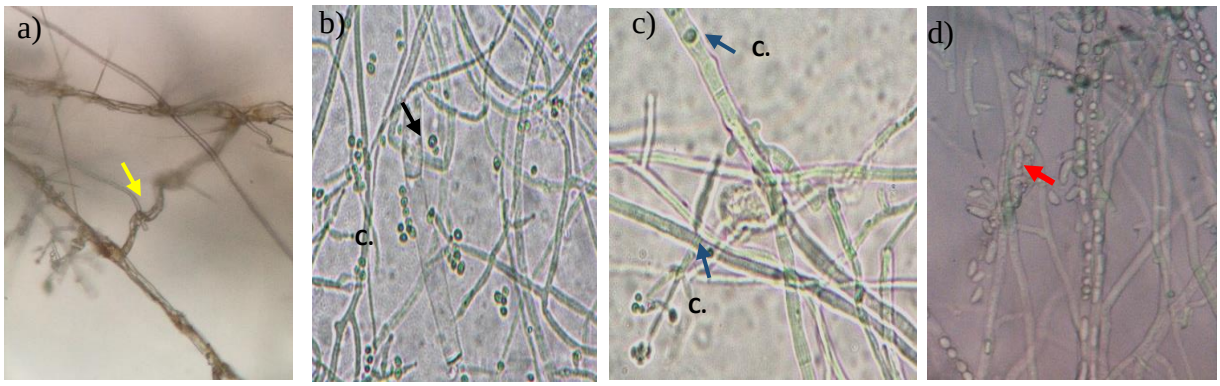

Figure 3. Mechanisms T2 micoparasitism against a Foc 250. a)= curl (yellow arrow); b) penetration (black arrow); c) penetration arrow blue; and d) vacuolization (red arrow).

Different studies indicate that the process of mycoparasitism is best antagonistic mechanism shown by *Trichoderma*, it starts when recognizes the host and joins hyphae by appressoria then degrades the cell wall by secreting enzymes, particularly chitinase and β-1, 3-glucanases, cellulases, proteases and phosphatases (Qualhato *et al*., 2013).

The values of the percentage of radial growth inhibition (PRGI) in the eight strains evaluated were found in a range of 49 to 85% (Table 2). According to the analysis of variance ($p \leq 0.05$) a significant difference was observed in PRGI regarding T1 and T2 and its effect on the eight pathogenic (Table 2). T1 strain showed greater inhibition against *F. solani*, Foc 250, Foc Foc 71 and 12.

Table 2. Percentage of radial growth inhibition (PRGI) of the pathogens evaluated against the *Trichoderma harzianum* T1 and T2 strains.

Pathogen	PRGI (%)	
	T1	T2
Foc 250 (race 5)	78.5 b	78.3 a
Foc 71(race 0)	72.6 bc	70.9 c
Fusarium solani	85.8 a	80.8 a
Sclerotium rolfsii	63.1 de	74.3 ba
Foc 27 (race 1B/C)	67.5 d	80 a
Foc 12 (race 6)	72.9 bc	76.6 ba
M-Sonora	49 f	75.3 ba
M-GTO	60 de	65.3 d

Means with the same literal between columns, are statistically equal, Tukey ($p \leq 0.05$).

A similar effect was observed with T2 against *F. solani*, Foc 250 and Foc 71; however, the inhibition increased to 27 Foc, *S. rolfsii*, M-GTO and M-Sonora, ultimately it was the considerable PRGI the observed an increase of 53% compared to T1. These results coincide with those documented by authors like Michel-Aceves *et al*. (2009) who indicated PRGI in a range of 16.4 to 77.8% when evaluated *Trichoderma* against *F. oxysporum* f. sp. *lycopersici* and 13.1 to 94.4% for *S. rolfsii*.

In another study by Rudresh *et al*. (2005) using *T. harzianum*, obtained inhibition values in a range from 12.2 to 59.9% for *S. rolfsii* and from 65.2 to 77% for *F. oxysporum* f. sp. *ciceri*. Meanwhile, for *M. phaseolina* Salazar *et al*. (2012) determined PRGI intervals of 67 to 91%; while Cubilla-Rios *et al*. (2019) obtained inhibition between 55.6 to 52.8%.

Effect of *T. harzianum* in the chickpea

Plants inoculated with TB (application of the *Trichoderma* T2 strain) presented height increases of 2.2, 3 and 2.3% on days 66, 73 and 81, respectively, compared to the control (Figure 4a); while with TQ (application of chemical fertilizer) the values were 1.3% lower. Regarding the stem diameter, the main differences were observed from day 94, during this period, the plants with the application of *Trichoderma* and chemical fertilization presented increases of 3.3 and 3.5%, respectively (Figure 4b).

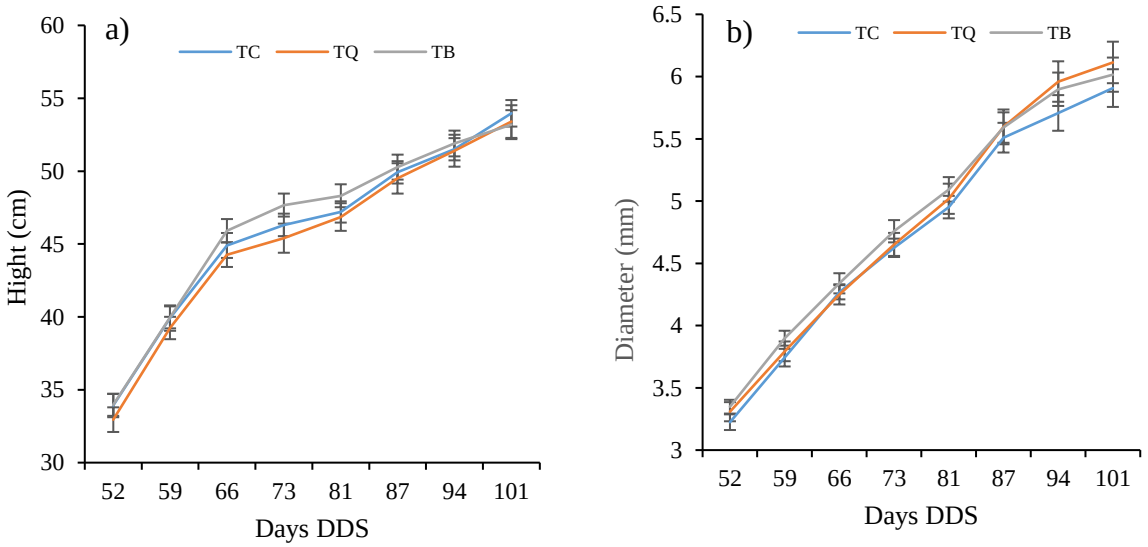

Figure 4. Evaluation of height (a) and stem diameter (b) in chickpea plants. n= 30.

The effect on the height and diameter of the plants found in this study were less compared to other studies, such as the one documented by Boureghda and Bouznad (2009), who applied *T. harzianum* to chickpea plants, their results indicated increases in average of 8% in the height of the plants. Meanwhile Oliva-Ortiz *et al.* (2017) to evaluate the effect of the *Trichoderma* strains obtained increases of 26% over the control additionally, they observed an increase in the diameter of the stem of 8% over the control.

Regarding the variable number of flowers and pods, it was observed that TB stimulated bloom from day 56, thus, for the day 66 increased 30% over estimated to control, whereas treatment TQ 10% less flowers were determined in relation to the control. In general, no significant differences were observed ($p< 0.05$). After day 81, both treatments showed similar behavior (Figure 5a).

As pod formation TB there was an increase of these from day 73 compared to control, whereas TQ this showed a slight increase from the day 87, the average increase was 24 and 11%, with TB and TQ, respectively (Figure 5b). Authors like Mukherjee *et al*. (2019) found advancement of flowering (7-10 days) in chickpea and lentil when they applied *T. virens*, in addition to observing a 26% increase in production.

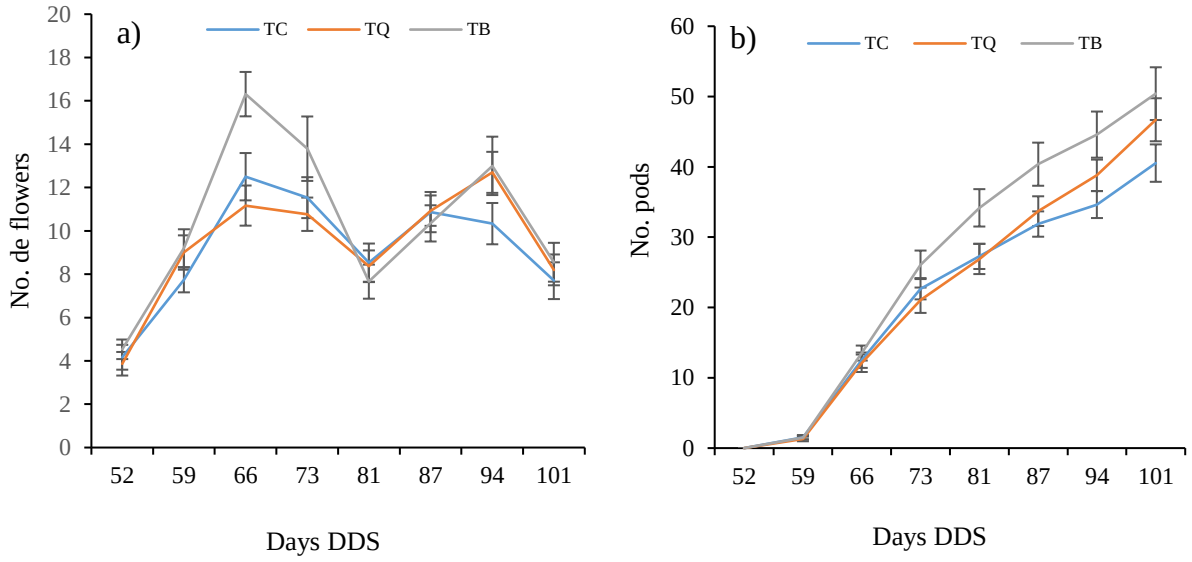

Figure 5. Evaluation of the number of flowers (a) and pods (b) in chickpea plants. n= 30.

In this regard Mishra and Nautiyal (2018) obtained in 20% increments production chickpea pods when applied *T. viride*. In another study conducted by Ávila-Miramontes *et al*. (2015) where they evaluated different treatments based on *T. harzianum*, *B. subtilis*, *Mesorhizobium ciceri* and nitrogen fertilization, they were able to observe that with the *T. harzianum* + fertilization combination there was higher earliness and constant flower emission, as well as a long period of fruiting bodies.

For the variable root length, a significant difference ($p\leq 0.05$) was determined between TB and TQ treatments, causing an increase of 40 and 13%, respectively, with reference to the control (Table 3). This behavior has been presented in various studies in which *Trichoderma* is applied and which register increases in root length, due to the stimulation of the production of hormones, mainly auxins, which are synthesized from a source of tryptophan that is naturally secrete in exudates from plant roots (Hoyos-Carvajal *et al*., 2009).

Table 3. Agronomic variables determined after harvest.

Treatments	Root length (cm)	Yield (t ha^{-1})
Control (TC)	10.3 b	2.1 a
Chemical (TQ)	11.7 b	2.4 a
Biological (TB)	14.4 a	2.6 a
DMS	2.7	1.7

Means with the same literal between columns, are statistically equal, Tukey ($p \leq 0.05$).

It is then that the increase of the root allows the plants to cover large volumes of soil and increase the absorption of available nutrients and those that have been solubilized by the same fungus (Jyotsna *et al.* 2008; Hoyos-Carvajal *et al*., 2009). In this regard, Kumar *et al*. (2014) obtained similar results when using 5% of *T. harzianum* in relation to one kg of chickpea seed, additionally, it reduced the incidence of wilt associated with the *F. oxysporum* f. sp. *ciceris*, *M. phaseolina* and *S. rolfsii*.

Similarly, Jyotsna *et al*. (2008) detected increases in the size of the root between 25 to 27% and the decrease of diseases in 40 to 60%, the authors indicated that the stimulation of the production of hormones in early stages of growth can help the growth of the plant and root development. Hoyos-Carvajal *et al*. (2009) recommends that growth promoting strains of *Trichoderma* can be used to formulate new products that are beneficial for agriculture.

Regarding performance, it was determined that with TQ there was an increase of 13% and with TB of 23%, both data with respect to TC. These results coincide with different authors who point out increases in crop yield (Oliva-Ortiz *et al*., 2017; Ávila-Miramontes *et al*., 2015; Ruiz-Cisneros *et al*., 2018).

In chickpea, most of the studies carried out with *Trichoderma* are focused on the control of pathogens, mainly, *F. oxysporum* f. sp. *ciceris* and secondarily estimate the effects on the growth and production of plants, in such a way that their results indicate a decrease in the severity of the disease, as well as increases in yield, such is the case of Oliva-Ortiz *et al*. (2017) who found an increase of more than 70% when applying the HRG-060 strain.

As well as the decrease in incidence and root wilt, the authors obtained different results between strains, noting that not all maintain the same effects with respect to the variables studied. Meanwhile, Khan *et al*. (2014) showed that when applying *Trichoderma* in soil, they obtained a reduction in the severity of the disease from 25% to 67%, while obtaining increases in yields in an interval of 8 to 24%. Also, Kumar *et al*. (2014) showed that the application of *T. harzianum* increased the yield by 68% and at the same time the incidence decreased by 91%.

The increases in production can be attributed, on the one hand, to the antagonistic protection of *Trichoderma* sp. Against causal agents of plant diseases and, on the other hand, to the synthesis of compounds that regulate growth or that intervene in the assimilation of nutrients for the plant (Hoyos-Carbajal *et al*., 2009; Hernández-Melchor *et al*., 2019).

Likewise, in some crops such as beans it is recognized that *Trichoderma* sp. It favors the germination of the seed through the production of lytic enzymes that degrade the episperm, which is a seminal covering that constitutes the testa; additionally, the fungus is capable of promoting the development of primary meristematic tissues, which are responsible for plant growth in relation to height, weight, and root development (Zuñiga-Silgado and Velez-Vargas, 2016).

According to the results obtained with T1 and T2, both strains had the potential to be used to control pathogens, as well as to increase the agronomic characteristics that were evaluated; however, considering the experience of the producers, it is necessary to carry out evaluations to determine the optimal dose of *Trichoderma* application (CFU g^{-1}) considering, in the conditions of each production unit, mainly the initial level of inoculation of phytopathogens, the availability of nutrients and the climatic and edaphological conditions of the area.

Conclusions

According to the results of this research, both strains of *T. harzianum* (T1 and T2) had the potential to be used to control vascular fusariosis in chickpea cultivation. The antagonism mechanisms exhibited by both strains were competition for nutrients, mycoparasitism, production of antifungal enzymes and possible antibiosis. The T1 strain showed greater inhibition against the FOC 250, FOC 71 and *Fusarium solani* strains, while T2 for *Sclerotium rolfsii*, FOC 27, FOC 12 and for the *Macrophomina phaseolina* isolates. Additionally, the application of *Trichoderma* allowed increases in pod production (24%), root length (40%) and yield (23%).

Acknowledgments

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