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# Bio-controller Effect of Four Native Strains of *Trichoderma* spp., on *Phytophthora capsici* in Manzano Chili (*Capsicum pubescens*) in Puebla-Mexico

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## Abstract

**Diversity of the different types of chilies in Mexico has been scarcely studied, and a large variety have been found to be, such as Manzano chili. Root rot caused by oomycete *Phytophthora capsici* is a severe disease that affects Manzano chili production in Mexico, detracted from its production and quality. The use of biological control agents such as *Trichoderma* native's species, represents an efficient alternative to reduce losses and control the disease. For this reason, the objective of the present investigation was to evaluate the antagonistic effect *In vitro* and *In vivo* of four native strains of *Trichoderma* spp., on *Phytophthora capsici* in seedlings of Manzano chili from Puebla-Mexico was evaluated. Dual culture technique was used to determine the percentage of inhibition of radial growth (PICR) of the PC-A strain of *P. capsici*. Analysis of the percentage of germination was also carried out, as well as the incidence of root rot at 20 days after inoculation with the pathogen (dai) in the nursery. *T. harzianum* strain presented the highest PICR (42.86%) of antagonistic level *in vitro* and class I in the Bell scale, in addition, it obtained 88% germination in the nursery and 10% mortality at 20 dai, higher than the other native strains of *Trichoderma*. The bio-controlling effect of strains of *Trichoderma* spp., offers an effective alternative for root necrosis caused by *P. capsici* in the cultivation of Manzano chili in Puebla-Mexico.**

**Keywords:** Antagonism, incidence of rot, PICR, nursery, mortality

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## INTRODUCTION

Chili is a fundamental and representative ingredient of Mexican diet, and which is considered the first domesticated crop in the American continent. The Manzano chili (*Capsicum pubescens*) is native to upper parts of Peru and Bolivia, recently introduced in Mexico, favored by its ability to adapt to cold places, where no other type of chili thrives[1].

The Manzano chili is distributed in Sierra Norte of state of Puebla, specifically in the municipalities of Tlatlauquitepec, Zacapoaxtla and Teziutlan; as well too in Tacambaro and Zitacuaro in Michoacan; Huatusco, Coscomatepec and Zongolica in Veracruz; Coatepec, Villa Guerrero and San Miguel Tlaixpan in the Edo. Mexico; Finally, San Cristobal and Motozintla in the State of Chiapas[2], in addition the Manzano chili has great gastronomic, economic and social importance as it is a basic ingredient of traditional food[3].

Currently the production of the Manzano chili in the region of Tlatlauquitepec is compromised by presence to fungal diseases. The diseases with the highest incidence in seedling stage of genus *Capsicum* are caused by pathogens such as *Rhizoctonia* sp., *Fusarium* spp. and *Phytophthora* sp., which cause the loss of more than a third of the crop production, where the main symptom is premature plant death, damage to reproductive structures and irregular root-rot[4,5]. However, basal root production caused by oomycete *P. capsici*, Leonian is the most lethal[6]. The disease occurs in roots, stems, leaves and fruits[7], being one of the most destructive diseases worldwide for the cultivation of chili[8].

Chemical synthesis products have been used for decades to control these diseases[9]. However, its use is related to generation of resistance fungal, damage to environment and human health[10]. Under this premise, biological control is considered an efficient and friendly with the environment practice, for the development of sustainable agriculture[11].

The use of filamentous fungi as biocontrol agents represents an effective alternative for agricultural production systems because it reduces numerous applications of various types of fungicides. For more than eight decades, research has been conducted on the use of different species of *Trichoderma*[12,13]. Multiple mechanisms of action have been described, but the most relevant are (a) the production of secondary metabolites that strengthen the plant's immune system[14,15,] (b) the action of natural mycoparasites and antagonistic agents and (c) the promotion of seed germination and plantgrowth, as well as an increased root and foliage biomass and mineral assimilation; thus, different indigenous species of *Trichoderma* play an important role in agroforestry and natural ecosystems; they are considered biological control agents[16] because they act against pathogens of agroecology importance.

Consequently, the objectives of this research work were: A) Determine antagonistic capacity and percentage of inhibition of radial growth *in vitro* of four native strains of *Trichoderma* spp., upon PC-A strain of *P. capsici*; B) Analyze percentage of germination and root rot of Manzano chili seedlings at 20 days after pathogen inoculation in nursery.

## MATERIALS AND METHODS

### Antagonism of native strains of *Trichoderma* spp., on *P. capsici* in dual cultures

Evaluation of antagonism was carried out with the native strains of *T. harzianum*, *T. viridae*, *T. atroviridae* and *T. hamatum*, likewise the PC-A strain of *P. capsici* was used. All biological material is protected in laboratory 204 of Eco-Campus Valsequillo of Instituto de Ciencias, Benemérita Universidad Autonoma de Puebla (BUAP).

For evaluation of mycelial development, fragments of 5 mm diameter native strains of *Trichoderma* spp., as well as the PC-A strain of *P. capsici* (5 mm diameter), were inoculated in Petri dishes with potato and dextrose agar (PDA) incubated under darkness at 28 °C for 10 days. Mycelial diameter was measured every 12 h to estimate the growth speed (cm), which was calculated with the linear growth function y = mx + b (where 'y' is the distance, 'x' is the time and 'b' the constant factor) and was expressed in centimeters per day (cm $d^{-1}$)[17]. Diameter was determined with a digital Vernier (CD-6 Mitutoyo) always in the same direction in triplicate, which was established at random for each repetition.

Dual culture technique was used according to Andrade-Hoyos et al.[10] in triplicate to determine percentage of inhibition of radial growth with formula PICR = [(R1-R2/R1) x 100]

for each assay evaluated for a period of 10 days. To complement the evidence of antagonism, each trial was compared and classified in one of the 5 levels of grade scale (Table 1) established by Bell[18]. And thus, obtain qualitative evidence of the antagonistic effect.

**Nursery tests to analyze the percentage of germination and incidence of root rot**

Manzano chili 3,600 seeds were used provided by producers from Huaxtla community belonging to the municipality of Tlatlauquitepec, Puebla-Mexico. The trial was carried out under controlled conditions in nursery of Eco-Campus Valsequillo, ICUAP-BUAP. Prior to seed germination; these were disinfected with 0.3% sodium hypochlorite (v/v) for 10 min, rinsed three times with sterile water and dried with sterile paper[19].Was infested strain PC-A of *P. capsici* in 600 seeds per palate for each treatment under study; was prepared in a sterile saline suspension at a concentration of $1\times10^8$ zoospores $mL^{-1}$ by means of a Neubauer chamber (PAUL MARIENFELD)[20]. Once pathogen was soaked and the seeds were dry, of native strains of *Trichoderma* spp., were inoculated. This was carried out with a suspension of teliospores at a concentration of $1x10^8$ conidia $mL^{-1}$for all separate treatments, each one with 600 seeds.

Once the treatments were inoculated, seeds were placed in germinating trays with Peatmoss and Agrolita (1:1 v/v), previously sterilized at 121 ºC and 15 lb pressure[21]. Seeds were sown through a standardized mechanical procedure; the wet substrate was deposited at three quarters of total cavity capacity, one seed per cavity was placed at a depth of 1 cm[22]. A control group was used; which consisted of seeds disinfected in a sterile solution of distilled water and 3% sucrose. Finally, the trays were covered with black plastic for 20 days at 27 °C for germination[22]. Seed germination percentages were calculated according to following formula: Seed germination (%) = (number of germinated seeds / total number of seeds) × 100[23]. The data were taken when the control group presented ≥ 90% germination.

The degree of incidence of root rot was quantified by the damage that the seedlings presented throughout 20 days after inoculation (dai) for this a scale (Table 2) of general aspects modified from the one used by Sobrino[24]is formulated,where each treatment consists a germinating tray of 200 cavities in triplicate (n = 600).

**Statistical analysis**

The analysis of variance (two-way ANOVA) of response variables and comparison tests of means by LSD Tukey-Fisher (α=0.05) was carried out, using statistical package IBM SPSS Statistics version 25.

## RESULTS AND DISCUSSION

There were zones of interaction between native strains of *Trichoderma* spp., against *P. capsici*, where parasitism was observed at 142 h. Development rate and growth speed showed significant differences ($p \geq 0.05$), where *T. harzianum* obtained highest value (Table 3) with 1.27 ± 0.08 mm/h and 1.52 ± 0.02 cm $\delta^{-1}$, respectively. Results similar to those reported by Morales et al.[25] where it reports a growth speed for *T. harzianum* (TH-4) of 1.86 ± 0.22 cm $d^{-1}$ and 1.67 ± 0.01 mm/h. The pathogen *P. capsici* obtained lowest growth speed (0.67 ± 0.06 cm $d^{-1}$), higher results than those reported by Andrade-Hoyos et al.[10] where they show that growth rate is different in species of pathogens, including *P. capsici*.

Percentage of inhibition radial growth (PICR) shows significant differences ($p$<0.014), however, antagonistic strains show between 23 and 42% inhibition (Table 3). It was observed that *T. harzianum* and *T. hamatum* were more efficient than *T. viridae* and *T. atroviridae*, for which they obtained values of 33.33 and 23.63% respectively; however, *T. harzianum* presented a class I classification and *T. hamatum* presented a class II classification according to the scale established by Bell[18].

The present investigation coincides with a study carried out by Ezziyyani et al.[26] where it inhibited 50% of mycelial growth of *P. capsici* in pepper plants, proving the antagonistic effect *in vitro* of *T. harzianum*. Similarly, Zegeye et al.[27] found that *T. viride* showed a complete inhibition in radial growth of *P. infestansin vitro* tests. In addition, they mentioned that foliar application of *T. viride* has good potential to control *P. infestans* under greenhouse conditions. Stefanova et al.[28] reported presence of non-volatile metabolites with antifungal activity in four isolates of *Trichoderma*

and concluded that they reduce the mycelial growth of *P. nicotianae*. This indicates that probably the species of the genus *Trichoderma* may have a higher nutrient incorporation rate and metabolism than *P. capsici*, as well as different secretion mechanisms of hydrolytic enzymes; among them cellulases, chitinases, glucanases, xylases and proteases, which may be involved in biocontrol mechanisms, allowing it to take better advantage of the nutrients in the environment and deprive the pathogen of using resources[29].

Mycoparasitism is another of action mechanisms of the genus *Trichoderma*, in this research, it was possible to observe granulation of the PC-A strain of *P. capsici* and mycoparasitism between hyphae (strangulation) induced by *T. harzianum* (Fig. 1), this reaffirms the high mycoparasitic capacity of TH-4 strain. *T. harzianum*, which is capable of inhibiting the growth of phytopathogens because it produces numerous antibiotics such as trichodermine, suzukacillin, alameticin, dermadin, penicillin, trichothecenes, trichorzianines, among others[16]. In other strains of *Trichoderma* spp., it was not possible to observe any interaction mechanism.

The results corroborated what was reported by González et al.[30] and Hyder et al.[31] on enzymatic degradation of cell wall of phytopathogenic fungi during the mycoparasitic action; which causes lysis and with-it disorganization of cytoplasmic content and lysis of the cells.

**Table 1.** Bell1[17] antagonism scale.

| Class | Features |
|---|---|
| I | Growth of *Trichoderma* spp., which covered entire surface of medium and reduced pathogen colony. |
| II | Growth of *Trichoderma* spp., which covered at least 2/3 parts of medium. |
| III | *Trichoderma* spp., and pathogen they grew 1/2 and 1/2 surface of medium, one did not overlap other. |
| IV | Pathogenic fungus grew at least 2/3 parts of medium and resisted invasion of *Trichoderma* spp. |
| V | Growth of pathogen that covered the entire surface of medium. |

**Table 2.** Incidence scale of root rot in Manzano chili seedlings caused by *P. capsici*.

| Value | Percentage(%) | Hurt |
|---|---|---|
| 0 | 0 | Healthy plant |
| 1 | 10 | Mild symptoms |
| 2 | 50 | Severe symptoms |
| 3 | 75 | Very severe symptoms |
| 4 | 100 | Dead plant |

*Evaluation carried out on a scale graduated from 0 (no symptoms in 600 plants) to 4 (600 dead plants).

**Table 3.** Development rate, growth speed, percentage of radial growth inhibition and antagonism classification on Bell Scale[18]

| Name | Development rate (mm/hour)* | Growth rate (cm $d^{-1}$)* | PICR * | Class Bell |
|---|---|---|---|---|
| *T. harzianum* | 1.27±0.08$^{a}$ | 1.52±0.02$^{a}$ | | |
| *T. viridae* | 0.78±0.12$^{b}$ | 1.23±0.06$^{c}$ | | |
| *T. atroviridae* | 0.83±0.08$^{c}$ | 1.19±0.06$^{d}$ | | |
| *T. hamatum* | 0.61±0.12$^{d}$ | 1.46±0.02$^{b}$ | | |
| *P. capsici* | 0.59±0.14$^{e}$ | 0.67±0.06$^{e}$ | | |
| *P. capsici vs T. harzianum* | | | 42.86±0.27$^{a}$ | I |
| *P. capsici vs T. viridae* | | | 33.33±0.012$^{c}$ | II |
| *P. capsici vs T. atroviridae* | | | 23.63±0.042$^{d}$ | III |
| *P. capsici vs T. hamatum* | | | 37.62±0.40$^{b}$ | II |

*Different letters mean significant difference between treatments according to Tukey-Kramer ($p \leq 0.05$).

Antagonism tests reflect the ability and genetic variability of antagonist and phytopathogen to resist antagonism, this allows preliminary selection to be evaluated under field conditions, as well as to complement and determine their bio-controlling capacity[25].

Germination of seeds of Manzano chili in control group was presented at 14 days with 93%, where significant differences ($p$=0.023) were presented with respect to the other treatments (Fig. 2), where the effect of inoculation was observed of *P. capsici*, which affected germination percentage (23.45%). Huallanca et al.[32] mention that *P. capsici* pseudo-fungus inoculated in chili seedlings produced secondary symptoms of wilting, yellowing and ascending death; as well as primary symptoms of neck rot and root rot. Madhavi et al.[33] reported a germination percentage of 90.3% in chili (*Capsicum annuum*) seedlings inoculated with *T. harzianum* in greenhouse, results similar to those found in the present investigation.

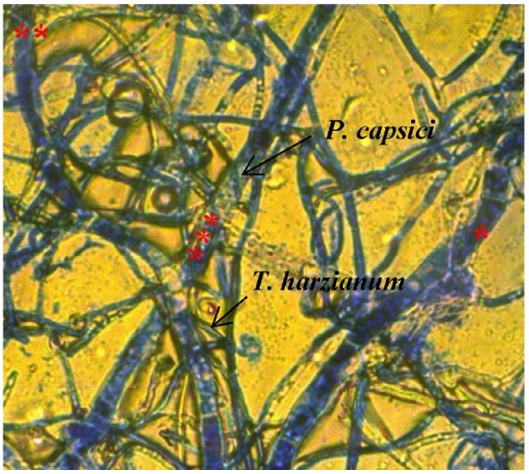


**Fig. 1.** Mycoparasitism of *T. harzianum* on the hyphae of *P. capsici*, *hypha swelling, **hypha curl, ***granulations within hypha

Microbial antagonists significantly reduced seed infection in Manzano chili caused by *P. capsici*, likewise a significant increase in seed germination was recorded in treatments of *T. harzianum* and *T. hamatum*, while treatment with strains of *T. viridae* and *T. atroviridae*, reduced seed mortality by 70% compared to the pathogen (PC-A strain).

The results showed treatments presented bio-controller activity were effective in reducing incidence of wilt and root rot disease in Manzano chili plants under induced infection (Figure 3). Treatments with native strains of *Trichoderma* reduced the disease 90% in relation to the pathogen, which reached very severe symptoms, in which incidence obtained was 1.6, 1.4, 1.3, 1.2 and 3.8 respectively.

In untreated seedlings (Control) they presented a natural incidence of the pathogen, which reached an average of 1.6 on scale used,

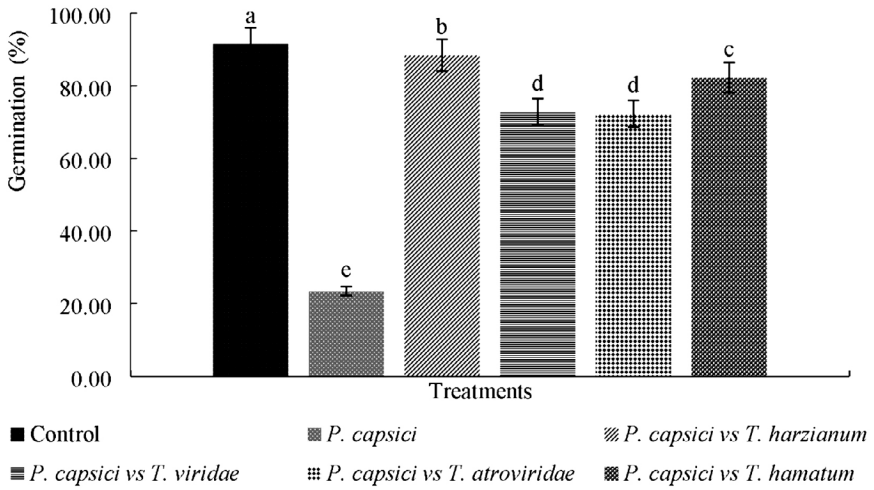


**Fig. 2.** Comparison of Germination Percentage of Manzano chili seeds inoculated with native strains of *Trichoderma* spp., and *P. capsici* in nursery.*Different letters mean significant difference between treatments according to Tukey-Kramer ($p \leq 0.05$)

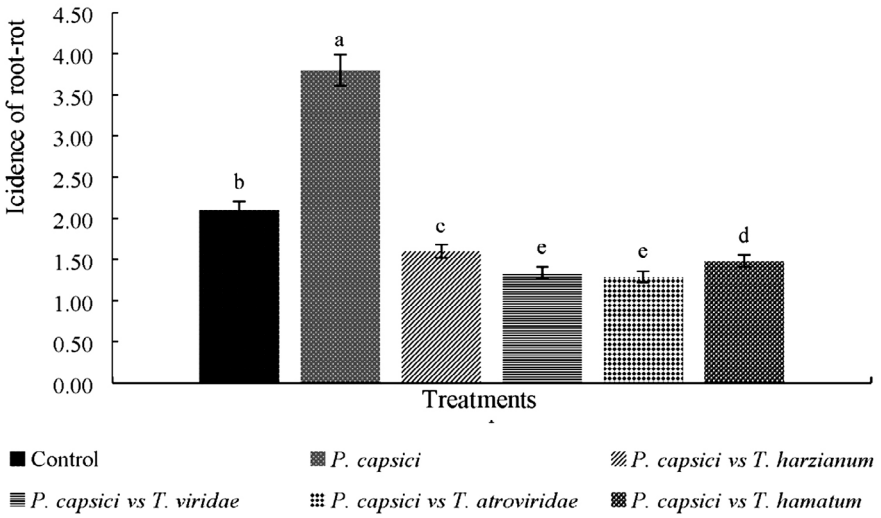


**Fig. 3.** Comparison of incidence of root rot in Manzano chili(*Capsicum pubescens*) seedlings germinated from seeds treated with native strains of *Trichoderma* spp. and *P. capsici* in nursery 20 dai.*Different letters mean significant difference between treatments according to Tukey-Kramer ($p \leq 0.05$)

which tells us that plants used by the producers present some natural contamination and are mostly affected with symptoms ranging from mild to severe, that is why, they the producers of the study region are looking for an efficient control method.

The Manzano chili seedlings that were inoculated with *P. capsici* showed root rot at 20 dai, this coincided with that described by Agrios[34] when discovering rot caused by *F. solani*for the cultivation of chili. Ezziyyani et al.[26] applied *T. harzianum* on *P. capsici*, reducing wilting between 22 and 56%. Results inferior to those reported in present investigation.

Andrade-Hoyos et al.[16], where they attribute effect on growth not only to protection against pathogens, but also to presence of a growth regulating factor in plants[30].*T. harzianum* possesses the ability to solubilize various plant nutrients from their insoluble or poorly soluble mineral phases through chelating and reducing mechanisms; due to these mechanisms it is that phosphates (mainly calcium), $Fe_2O_3$, $MnO_2$, Cu and Zn, tend to be more assimilated by plants[30].

The genus *Trichoderma* can parasitize pathogenic fungi and produce antibiotics, in addition, it has positive effects on plant growth, yield, nutrient absorption, efficiency in use of fertilizers and systemic resistance to plant diseases[16,30].

## CONCLUSIONS

Native strains of *T. harzianum* and *T.hamatum* showed greater antagonism on *P. capsici* in the Bell's scale, however, *T. harzianum* showed higher PCIR *in vitro* conditions.

Under nursery conditions, all antagonistic strains showed a better response in germination of Manzano chili plants. However, *T. harzianum* presented the highest germination percentage (88%) followed by *T. hamatum* (82%).

PC-A strain of *P. capsici* caused up to 90% root rot to the 20 days after inoculation with the pathogen in nursery.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

## ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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