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Effect of *Trichoderma* on Growth and Sporangia Production of *Phytophthora capsici*

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Abstract

Phytophthora capsici is the main agent of chili pepper root rot, causing significant yield losses in commercial fields worldwide. Management of this plant pathogen has been difficult due to its ability to develop fungicide resistance, therefore biocontrol of *P. capsici* appears to be a promising alternative. In this study we evaluated the effects of three species of *Trichoderma*: *T. harzianum* (Th-7), *T. koningiopsis* (Tk NRRL50190) and *T. asperellum* (Ta NRRL50191) on two mexican isolates of *P. capsici* (RDP-1 and RDP-2) obtained from commercial pepper fields in Aguascalientes, Mexico. The ability to inhibit mycelia growth of *P. capsici* was tested with *in vitro* assays, followed by the quantification of sporangia after dual confrontations. All *Trichoderma* isolates inhibited the mycelia growth of the phytopathogen, but only Ta and Tk showed mycoparasitism. Sporangia production of *P. capsici* RDP-1 and RDP-2 was significantly reduced by *Trichoderma* isolates, compared to the non-confronted control, except for the Th-RDP-2 confrontation. Sporangia of RDP-1 significantly decreased by 95.6%, 81.3% and 78% after confrontations with Tk, Ta and Th, respectively; and by 76% and 85.4% in RDP-2 by Tk and Ta, respectively. The results suggest that Ta, Tk and Th could be used as potential biocontrol agents of *Phytopthora root rot*, however, further studies are necessary to test the *in vivo* protection of Ta, Tk and Th and their synergistic effects against *Phytophthora root* rot of chile peppers and other *P. capsici* susceptible solanaceous and cucurbitaceous crops such as tomatoes, squash, melons, and cucumbers.

Keywords: biocontrol, *Phytophthora capsici*, sporangia, *Trichoderma*

1. Introduction

The oomycete *Phytophthora capsici* is a highly destructive pathogen affecting mainly solanaceous and cucurbitaceous crops. In Mexico, *P. capsici* is the most devastating of the soil-borne pathogens that affect chili peppers (Robles-Yerena et al., 2010). It has the capability of infecting subterranean and aerial plant tissues, causing root, stem and fruit rot, and also leaf blight (Quesada-Ocampo & Hausbeck, 2010). When favorable environmental conditions occur under rainfall or irrigation, *P. capsici* produces thousands of sporangia in free water, releasing 20 to 40 zoospores each. All zoospores have the potential to start a new infection (Granke et al., 2012).

If the conditions are favorable for disease development, fungicide application may not be sufficient to control this pathogen (Marín et al., 2014), because *P. capsici* is resistant to a variety of fungicides such as mefenoxam (Silva-Rojas et al., 2009; Foster & Hausbeck, 2010), copper sulphate (Fernández-Pavía et al., 2004) azoxystrobin, metalaxyl and propamocarb (Pérez-Moreno et al., 2003). Therefore, new and more promising and sustainable alternatives are required such as genetic resistance and biological control. The biological control using antagonistic organisms is a valuable and more sustainable non-chemical tool, which has stimulated efforts to detect microorganisms with potential as biocontrol agents. Among the vast variety of microorganisms that have shown to suppress the growth of *P. capsici*, the most explored have been rizhobacteria (Jong-Hiu & Sang-Dal,

2010) and *Trichoderma* (Segarra et al., 2013; Jiang et al., 2016), both individually and combined for synergistic effects (Chemeltorit et al., 2016).

The beneficial effects of *Trichoderma* have been attributed to its capacity to antagonize pathogens like *Phytophthora*, due to a combination of antibiosis, mycoparasitism and competition for substrate and space (Bae et al., 2016). Antibiosis entails the production of compounds such as gliotoxin and viridina which have an antibiotic activity (Osorio-Hernández et al., 2011), and inhibit the development of other microorganisms. However, these metabolites inhibit the germination and root growth of mustard and other dicotiyledons *in vitro*, while in monocots their effect is minor (Bailey & Lumsden, 1998). Other metabolites produced by *Trichoderma* are viridiol, which has an herbicide activity, and the variant 9-epi-viridiol wich has a cytotoxic effect (Phuwapraisisan et al., 2006).

Several species of microorganisms are commercially available as biocontrol agents against soil-borne pathogens, nevertheless, it has been reported that some of these products are not effective against *P. capsici* in tomato (Fernández-Herrera et al., 2007). Also, plants treated with *Trichoderma* spp. may not be protected against *Phytophthora* attacks because no mechanisms to inhibit zoospores is yet known (Harman, 2000).

Most investigations have been focused in demonstrating the effectiveness of various *Trichoderma* strains, especially under laboratory conditions in order tp elucidate the antagonistic mechanisms, and in most cases, the effects and responses of *P. capsici* during this interaction. The aim of this work was to evaluate the antagonistic effects of three species of *Trichoderma* (*T. asperellum*, *T. koningiopsis* and *T. harzianum*), on the in vitro mycelia growth and sporangia production of two *P. capsici* isolates from chile pepper.

2. Materials and Methods

2.1 Strains

The two *P. capsici* strains were isolated from infected roots of commercial chili pepper in Central Mexico, and maintained on V8 juice agar (V8-A) media. Morphological identification of *P. capsici* was based on Erwin and Ribeiro (1996). *T. koningiopsis* (Tk NRRL50190), and *T. asperellum* (Ta NRRL50191) were provided by the Experimental Biotechnology Lab, Centro de Biotecnologia Genomica, Instituto Politecnico Nacional (CBG-IPN) and *T. harzianum* (Th-7) was provided by the Microbiology Lab, Universidad Autonoma de Aguascalientes (UAA), Mexico. The *Trichoderma* and *P. capsici* strains were maintained on PDA and V8-A media, respectively.

2.2 DNA Isolation and Molecular Identification of P. capsici

Mycelia of the *P. capsici* isolates were grown in 50 ml of potato dextrose broth for 72 h in a shaker at 200 rpm under 25 °C, followed by centrifugation at 3500 rpm for 10 min. The supernatant was discarded and the mycelia biomass was frozen at -20 °C until the analysis. Genomic DNA was extracted using a chloroform-octanol (24:1) procedure adapted from the protocol of Reader and Broda (1985). DNA was resuspended in nuclease-free water.

PCR reactions were conducted on a thermocycler (GeneAmp® PCR System 9600 PE Appled Biosystem); the amplifications were performed using three specific primers (PHYTO-primers): one Forward (5'-CTTTCCACGTGAACCGTWTC-3'), and two Reverse primers (5'-CAAAATGGATCGACCCCTCG-3' and 5'-CCAAATGGATCGACCCTCG -3'). A volume of 25 µl was prepared by mixing 1µl of the DNA with 2.4 µl of Buffer 10X (final concentration 1X), 0.75 µl of magnesium chloride of 50 mM, 1 µl of each primer (5 µM), 0.2 µl of dNTPs 10 mM, 0.2 µl of Taq polymerase 5U/µl, and adjusted to a final volume of 50 µl with Milli-Q sterile water. The PCR conditions were as follows: denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 59 °C, and 60 s at 72 °C, with a final extension time of 5 min at 72 °C. Electrophoresis analysis was applied to PCR products in a 1% agarose gel at 85 V during 1h, with Syber Gold and Orange as stainers. The amplified products were visualized in a Bio-Rad Molecular Imager® Gel Doc™ XR. The sequencing was executed by automated DNA sequencing (ABI Prism ®) using Big Dye ® technology. Sequence alignments and corrections were done using the CLC Sequence Viewer program version 7. Product sequences were compared with similar sequences in the GenBank using BLASTn.

2.3 In vitro Assays

Antagonistic activity of *Trichoderma* species against *P. capsici* strains were determined by dual culture in V8-A. A 5 mm diameter disc of active mycelia from each antagonistic species (Tk, Ta or Th) was placed opposite to each isolate of *P. capsici* mycelia disc on a Petri plate. All dual culture plates were incubated in darkness at 30 °C. Individual plates containing the specific antagonistic and pathogen strains were used as controls. All combined confrontations and control strains had four replications. The linear growth of mycelia was registered during 60 h for both, antagonistic and pathogen every 12 h in dual and individual culture plates. Registration of mycelia growth stopped when mycelia in dual cultures reached contact.

Scanning Electron Microscopy (SEM) was used to analyze and verify the antagonistic and pathogen interaction. Disc samples of 5 mm diameter were obtained from the mycelia contact zone of selected dual petri plates with Tk and *P. capsici*. Selected areas of contact were fixed in FAA (formaldehyde-acetic acid-alcohol), dehydrated with ethanol, dried with CO to a critical point, coated in gold and observed. Images of selected areas were obtained for visual analysis of antagonistic and pathogen interaction.

2.4 Sporulation Tests

Sporangia formation was induced for each dual confrontation using standard procedures for *P. capsici.* Four discs of 5 mm diameter each were obtained from the mycelia contact zone of each dual confrontation. The discs with mycelia were placed on a Petri plate, with 10 ml of distilled water and incubated in a chamber for 48 h at 30 °C. A millimetric transparent grid sheet was placed on top of the discs and a 2 × 2 mm area was selected and examined under the light microscope to register the number of full or empty sporangia. The same procedure was applied to induce and quantify sporangia formation in the *P. capsici* controls.

3. Results

3.1 Phytophthora Identification

The two pathogen isolates were identified as *Phytophthora* sp. based on morphological characteristics of asexual structures, and designated with the codes RDP-1 and RDP-2. Additionally to the taxonomic identification, molecular analyses were performed to confirm the species identification. The PCR products resulted in a band of 595 bp, and the sequence alignment with the NCBI database confirmed close homology (96%) to *P capsici*.

3.2 Antagonism of Trichoderma Against P. capsici

Dual culture assays showed that mycelia reached contact after 60 h. Both *P. capsici* isolates stopped growing in presence of the three *Trichoderma* isolates, however, only Ta and Tk overgrew the pathogen (Figure 1). The contact zone of *P. capsici* (RDP-1 and RDP-2) and Th (Figure 2) only exhibited thickening of mycelia.

Mycelia of the two *P. capsici* strains grew significantly less at 60 h in presence of Th ($p < 0.01$) compared to the control (Table 1). Also, *P. capsici* RDP-1 grew significantly less at 60 h in presence of Tk ($p < 0.05$) compared to the control.

The interaction between mycelia of the antagonistic and pathogen was observed using SEM, which revealed mycoparasitism of Tk on *P. capsici*. Mycoparasitism appears in two different ways: coiling and penetration of *P. capsici* hyphae by Tk (Figure 3).

Table 1. *P. capsici* lineal mycelia growth (mm) in confrontation with *Trichoderma* isolates every 12 hours

Trichoderma strain	*P. capsici* strain	12 h	24 h	36 h	48 h	60 h
Tk-NRRL50190		4.15*	9.85**	14.00*	19.63*	21.35*
Ta-NRRL50191	RDP1	4.23*	8.95	13.58	19.23	23.10
Th-7		3.63	9.53*	13.70	18.90	20.95**
None (Control)		3.33	8.65	13.25	18.55	23.05
Tk-NRRL50190		3.58	8.65	13.25	19.00	22.75
Ta-NRRL50191	RDP2	3.63	9.13	13.33	19.50*	22.65
Th-7		3.45	8.43	13.00	18.18	20.55**
None (Control)		3.20	9.20	13.53	18.35	23.85

Note. * Indicates a significant ($p < 0.05$) difference from the control mean within same column.

** Indicates a highly significant ($p < 0.01$) difference from the control mean within same column.

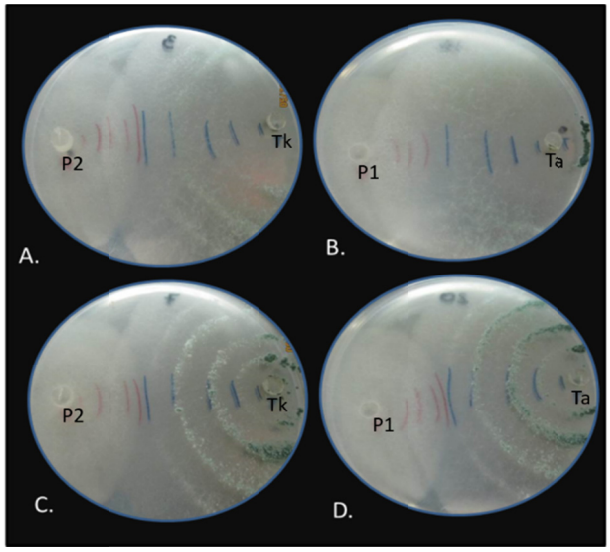

Figure 1. Overgrowth and growth inhibition of *P. capsici*-RDP1 (P1) and RDP2 (P2) by *T. koningiopsis*-NRRL50190 (Tk) and *T. asperellum*-NRRL50191 (Ta)

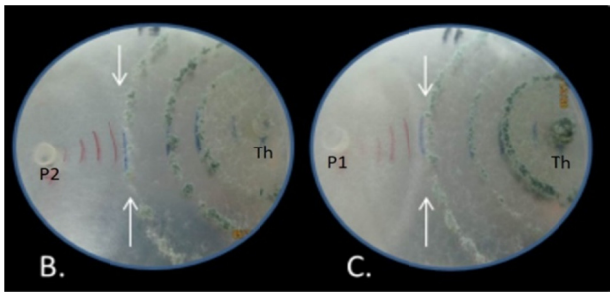

Figure 2. Dual growth of *T. harzianum*- Th7 and *P. capsici* strains RDP1 (P1) and RDP2 (P2). The arrows indicate the thickening of the mycelia at the contact zone

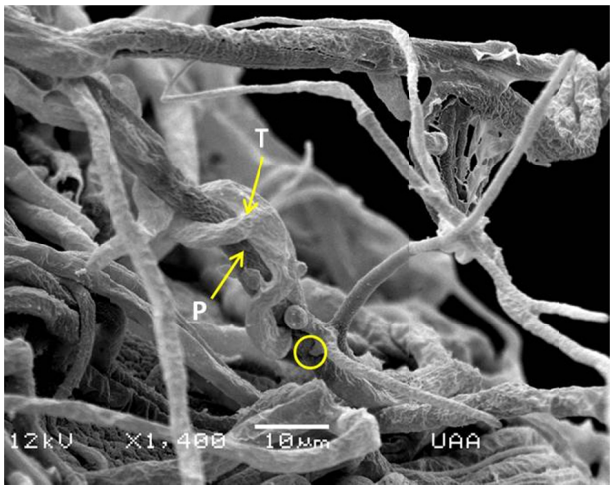

Figure 3. Mycoparasitims of *T. koningiopsis* (Tk NRRL50190) on *P. capsici* captured with a Scanning Electron Microscope. Hyphae of Tk (T) is tightly coiled around the hypha of *P. capsici* (P). Also, the hypha of *P. capsici* appears penetrated by Tk (encircled)

3.3 Effect of Trichoderma on P. capsici Sporangia Production

Confrontations with Tk, Ta and Th strongly reduced sporangia production of RDP1 by 95.6, 81.3 and 78% of the control ($p < 0.01$; Table 2). Similar levels of sporangia reduction were observed with strain RDP-2 of *P. capsici*, except for Th, which reduced sporangia by only 28.2% with no significant difference from the control ($p > 0.05$). These results showed that the three strains and species of *Trichoderma* inhibit sporangia production of *P. capsici* under lab controlled conditions.

Microscopic observations displayed the apparent invasion of sporangia by hyphae of Tk and Ta (Figure 4). Also it is worth mention the mean difference of 60.8 sporangia per unit area (68%) between the two strains of *P. capsici* in the absence of *Trichoderma* (Figure 5).

Table 2. Inhibition effect of *Trichoderma* strains on *P. capsici* sporangia production

Trichoderma strain	*P. capsici* strain	No. sporangia	Sporangia inhibition
Tk-NRRL50190	RDP1	5.6 **	95.6%
Ta-NRRL50191		23.6 **	81.3%
Th-7		27.6 **	78.0%
Control		125.8	0%
Tk-NRRL50190	RDP2	15.6 **	76.0%
Ta-NRRL50191		9.5 **	85.4%
Th-7		46.7 ns	28.2%
Control		65.0	0%

Note. The values in the table are the average of four replicates.

* Significant ($P \leq 0.05$), and ** highly significant ($P \leq 0.01$) difference from the control.

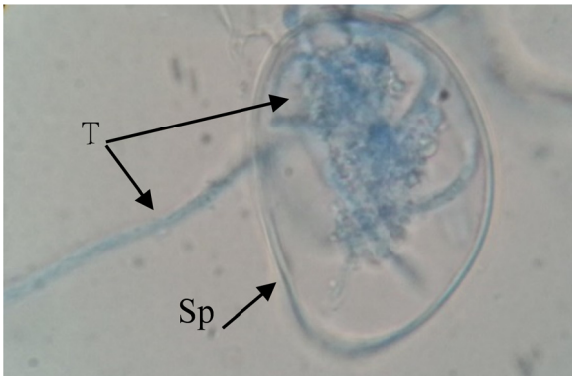

Figure 4. Hyphae of Tk NRRL50190 (T) invading a *P. capsici* sporangium (Sp)

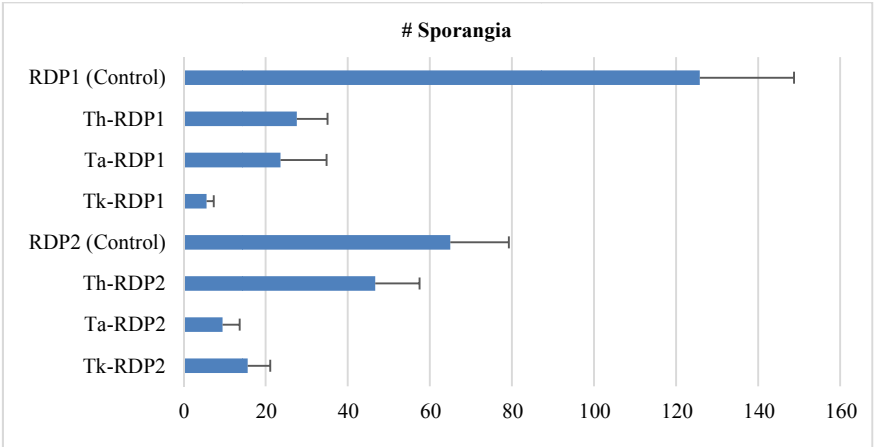

Figure 5. Mean sporangia countings (in 2 mm^2) of *P. capsici* strains (RDP1 and RDP2) in presence of three species of *Trichoderma* (Tk, Ta and Th). Bars denote standard errors of the mean

4. Discussion

When using a *Trichoderma* isolate as a biological control agent it is important to consider its ability to inhibit the growth and development of the target pathogen. According to the results obtained in this study, different species of *Trichoderma* are capable to halt the mycelial growth of *P. capsici*. *T. harzianum* (Th-7), showed certain ability that may be associated to fungistatic effects, while *T. koningiopsis* (Tk NRRL50190) and *T. asperellum* (Ta NRRL50191) showed some fungicidal effects that were captured when hyphae and sporangia of the pathogen were parasitized by Tk and Ta hyphae. Similar results have been reported for *T. koningiopsis* against *Macrophomina phaseolina* (Hernández-Mendoza et al., 2015). Osorio-Hernández et al. (2011) also reported 31 Mexican *Trichoderma* isolates with the ability to produce volatile compounds with inhibitory properties against *P. capsici*. Therefore biocontrol effects of *Trichoderma* might be due to direct interactions with the pathogen, such as in mycoparasitism, which involves the synthesis of certain compounds acting as antibiotics (Benítez et al., 2004). However, in this work we did not attempt to evaluate the microbial production of specific compounds.

To the authors' knowledge, the effect of *Trichoderma* sp. on sporangia production of *P. capsici* has not been documented. Some have focused on the possible inhibition of zoospore production (Ayobi et al., 2010), and germination of *P. capsici* (Segarra et al., 2013) by *Trichoderma*. Because different forms of inoculum (mycelia, sporangia, zoospores, oospores) may contribute to the spread of *P. capsici*, accurate detection and quantification of these propagules is essential for disease prevention and management (Larkin et al., 1995). Considering our results, the three antagonistic species and isolates: Tk NRRL50190, Ta NRRL50191, and Th-7 are able to inhibit mycelia growth and sporangia production of *P. capsici*, therefore the three species have the potential to directly or indirectly reduce different sources of primary inoculum - such as mycelia, sporangia and zoospores, and thus the probability of infection (Granke & Hausbeck, 2010). Zoosporogenesis depends on mature sporangia (Maltese et al., 1995), which live longer than the short lived water motile zoospores (Segarra et al., 2013).

5. Conclusion

Molecular studies of DNA confirmed and complemented the morphological identification of two strains of *P. capsici* that were isolated in 2015 from commercial cultivated pepper roots in Aguascalientes, Mexico. The three *Trichoderma* isolates showed promising applications for biological control of *P. capsici*. *T. koningiopsis* (Tk NRRL50190) and *T. asperellum* (Ta NRRL50191) showed fungicidal activity, while *T. harzianum* (Th-7) only showed fungistatic activity. Given the different antagonistic effects on *P. capsici* by *Trichoiderma* species, further studies should be conducted to examine, for example, possible synergistic effects among *Trichoderma* species.

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