# *IN VITRO* AND *IN VIVO* INHIBITORY EFFECT OF SOLID AND LIQUID *Trichoderma harzianum* FORMULATIONS ON BIOCONTROL OF *Pyrenochaeta lycopersici*

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*SUMMARY*

*The effectiveness of alginate solid formulations of Trichoderma harzianum containing either Th12.A.10.1 or ThF2-1 for the biocontrol of* Pyrenochaeta lycopersici *was compared to that of methyl bromide (60g·m$^{-2}$, five days of soil exposure) and to that of the commercial biofungicide, Trichonativa®, in a commercial tomato crop grown under greenhouse conditions. A single application of any of the solid formulations at 1.7g·l$^{-1}$ soil (8.5-11.9×10$^{5}$ CFU/plant) or of 5ml·l$^{-1}$ soil (5×10$^{9}$ CFU/plant) of Trichonativa immediately before tomato transplantation did not prevent tomato root damage. The formulation containing ThF2-1 was the only that allowed the reduction of the root damage level caused by* P. lycopersici *from 1.6 to 0.2 (Campbell and Shiskoff scale), when applied immediately before transplantation, 15 days after transplanting and one week before sprouting. This effect was coincident with a higher persistence ($t_{0.5}$= 4.7 months) than that of Th12A.10.1 (2.7 months) or to that of the liquid commercial product (0.7 month), suggesting that solid formulations maintain integrity of* Trichoderma *strains in the soil, which appears to be an important factor to prevent corky root of tomato. In addition, persistence of ThF2-1 in the soil after three applications of the solid formulation supports its use for the control of* P. lycopersici*, being an adequate alternative strategy to the application of methyl bromide in commercial tomato crops.*

## Introduction

Tomato corky root, caused by the phytopathogenic fungus *Pyrenochaeta lycopersici* Schneider & Gerlach, is widely present in tomato cultivated under greenhouse and produces important economical loses (Minuto *et al*., 2006). Methyl bromide fumigations are used in Chile for the control of soil phytopathogens in tomato cultures (Apablaza, 2000). However, and according to the established rules in the Montreal Protocol, the use of this agrochemical will be forbidden in Chile in 2015 (Ristaino and Thomas, 1997; Gullino *et al*., 2003). Potential alternatives to the use of methyl bromide, such as the use of ozone (Cicccarese *et al*, 2007), metham sodium and bacterial biocontrol agents based on *Streptomyces gris-*

**KEYWORDS / Biocontrol / Corky Root / Methyl Bromide / *Trichoderma* / Trichonativa® /**
Received: 04/10/2012. Modified: 06/06/2013. Accepted: 06/11/2013.

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## EFECTO INHIBIDOR *IN VITRO* E *IN VIVO* DE FORMULACIONES SÓLIDA Y LÍQUIDA DE *Trichoderma harzianum* EN EL BIOCONTROL DE *Pyrenochaeta lycopersici*

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*RESUMEN*

*La efectividad de dos cepas mejoradas de* Trichoderma harzianum *(Th12A.10.1 y ThF2-1) formuladas como pellets de alginato de sodio (1,7g·l$^{-1}$ suelo u 8,5-11,9×10$^{5}$ UFC/planta) se comparó con bromuro de metilo (60g·m$^{-2}$, 5 días de exposición) y con el biofungicida comercial líquido Trichonativa® (5ml·l$^{-1}$ o 5×10$^{9}$ UFC/planta en pre-transplante y 1l·ha$^{-1}$ post-trasplante), para el control de* Pyrenochaeta lycopersici. *El ensayo se realizó en un cultivo comercial de tomates bajo invernadero frío. Un único tratamiento con cualquiera de las formulaciones no evitó el daño en raíces. Solamente el uso de tres aplicaciones de ThF2-1 disminuyó el nivel de daño de raíces de 1,6 a 0,2 (escala Campbell y Shiskoff); lo que coincidió con la más alta persistencia media ($t_{0.5}$= 4,7 meses) de esta cepa en el suelo en comparación con 2,7 meses para Th12.A.10.1. y con 0,7 meses para la formulación líquida comercial, lo que sugiere que la formulación sólida mantiene la integridad de las cepas de* Trichoderma *en el suelo y parece ser un factor importante para evitar la raíz corchosa del tomate. Además, la persistencia de la cepa biocontroladora ThF2-1 en el suelo tras tres aplicaciones de formulación sólida apoya su utilización para el control de* P. lycopersici*, como una estrategia alternativa al uso de bromuro de metilo en el cultivo comercial del tomate.*

## EFEITO INIBIDOR *IN VITRO* E *IN VIVO* DE FORMULAÇÕES SÓLIDA E LÍQUIDA DE *Trichoderma harzianum* NO BIOCONTROLE DE *Pyrenochaeta lycopersici*

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*RESUMO*

*A efetividade de duas cepas melhoradas de* Trichoderma harzianum *(Th12A.10.1 e ThF2-1) formuladas como pellets de alginato de sódio (1,7g·l$^{-1}$ solo u 8,5-11,9×10$^{5}$ UFC/planta) foram comparadas com metil brometo (60g·m$^{-2}$, 5 dias de exposição) e com o biofungicida comercial líquido Trichonativa® (5ml·l$^{-1}$ o 5×10$^{9}$ UFC/planta e pré-transplante e 1l·ha$^{-1}$ pós-transplante), para o controle de* Pyrenochaeta lycopersici. *O ensaio foi realizado em um cultivo comercial de tomates em estufa fria. Um único tratamento com qualquer das formulações não evitou o dano em raízes. Somente o uso de três aplicações de ThF2-1 diminuiu o nível de dano de raízes de 1,6 a 0,2 (escala Campbell e Shiskoff); o que coincidiu com a mais alta persistência média ($t_{0.5}$= 4,7 meses) de esta cepa no solo em comparação com 2,7 meses para Th12.A.10.1. e com 0,7 meses para a formulação líquida comercial, o que sugere que a formulação sólida mantêm a integridade das cepas com* Trichoderma *no solo e parece ser um fator importante para evitar a podridão corticosa da raiz do tomate. Além disso, a persistência da cepa biocontroladora ThF2-1 no solo depois de três aplicações de formulação sólida apoia sua utilização para o controle de* P. lycopersici*, como uma estratégia alternativa ao uso de metil brometo no cultivo comercial do tomate.*

*eovirides* (Minuto *et al*., 2006), *Streptomyces* spp. and *Bacillus subtilis* (Fiume and Fiume, 2008), have been recently tested for the control of *P. lycopersici.*

One environmentally acceptable alternative to the use of methyl bromide, in addition to the use of solarization (Minuto *et al*., 2006) is the biocontrol based in the use of fungal antagonists belonging to the *Trichoderma* genus (Papavizas *et al*., 1982, Papavizas, 1985; Rey *et al*., 2000; Hasna *et al*., 2009). The improvement of wild *Trichoderma* strains has been mandatory for obtaining more efficient strains. The use of UV radiation and of protoplast fusion have resulted in mutant *Trichoderma* strains, with higher enzyme levels production than parental strains, including chitinases, glucanases and proteases. These enzymes are involved in the biocontrol of fungal pathogens (Rey *et al*., 2000; Pérez *et al*., 2002; Besoain *et al*., 2007; Montealegre *et al*., 2010a).

The present work uses two improved *T. harzianum* strains (Th12.A.10.1 and ThF2-1) included in solid formulations, with the characteristics already mentioned, to test if the improvement in their biocontrol mechanisms (Besoain *et al*., 2007) correlates with the effectiveness in the control of *P. lycopersici* in commercial tomato cv. Fortaleza grown under greenhouse conditions. The work also compares the effectiveness of solid formulations with a commercial liquid formulation (Trichonativa®) containing several *Trichoderma* strains (*T. harzianum, T. virens* and *T. parceramosum*).

### Materials and Methods

*Fungal strains*

Mutant *T. harzianum* strains, Th12A.10.1 (UV irradiation of Th12) and ThF2-1 (protoplast fusion, ThV×Th291) (Besoain *et al*., 2007) and the corresponding parental strains were used. They were activated on potato dextrose agar (PDA) from frozen stored strains. The mutant biocontrol agents were formulated separately in alginate pellets (Montealegre and Larenas, 1995). These contained 5×10$^{4}$ CFU/g of Th12A.10.1 or 7×10$^{4}$ CFU/g of ThF2-1. A commercial liquid formulation of *Trichoderma* spp., Trichonativa® (*T. harzianum, T. virens* and *T. parceramosum*) containing 1×10$^{9}$ CFU/ml, was purchased in the local market.

*Biocontrol activity of the* Trichoderma *strains against* P. lycopersici *in vitro*

Th12A.10.1 (parental strain: Th12), ThF2-1 (parental strains: ThV×Th291) and the corresponding parental strains were compared for direct antagonism against *P. lycopersici* as described by Besoain *et al*. (2007) in Petri dishes containing acidified PDA plates (1ml of 1M lactic acid per liter of PDA medium). For each *Trichodema* strain, an agar disk (0.5cm diameter) with young mycelia of the strains was placed 3cm from the disk with mycelia of *P.*

*lycopersici* (Tewari and Bhanu, 2004). For controls 0.5cm agar disks were similarly paired with the *Trichoderma* strains or *P. lycopersici*. The Petri dishes were incubated for 7 days at 23ºC. The area of the pathogen colony was measured with a milimetric rule and compared to the control. Each experiment was run in triplicates considering four replicates per Petri dish. Growth inhibition of the pathogen (PGI) was calculated as

PGI (%)= 100 - %Control, and

Control (%)= ACP/ACC × 100

where ACP and ACC: areas of colonies of the pathogen in the presence of the biocontrol agent and of the pathogen.

Inhibition of radial growth (IRG) was estimated once control dishes containing the phytopathogen were completely covered by the fungal mycelia, using the formula developed by Dennis and Webster (1971). The experimental unit was one Petri dish. Results, obtained as percent inhibition, were modified through the Bliss angular transformation, and a variance analysis was performed. When significant differences were detected, the Tukey's test was used (at $p \leq 0.05$).

*Biocontrol activity of* Trichoderma *against* P. lycopersici *in a greenhouse*

Eight trials were conducted in a commercial tomato (cv. Fortaleza) monoculture with high levels of *P. lycopersici* inocula, in Olmué, V Region, Chile. The soil was treated either with the *Trichoderma* strain Th12.A.10.1 ($0.85 \times 10^6$ CFU/plant), or ThF2-1 ($1.1 \times 10^6$ CFU/plant) or with Trichonativa ($5 \times 10^9$ CFU/plant). Controls were methyl bromide and sodium alginate pellets without addition of any *Trichoderma* strain, as a positive control, were included. Further details of the treatments are given in Table I.

TABLE I
SOIL TREATMENTS

| Treatment | Applications | Dose |
|---|---|---|
| Th 12A 10.1 | One: Pre-transplantation | 1.7 g/plant ($8.5 \times 10^5$ CFU/plant) |
| Th F2-1 | One: Pre-transplantation | 1.7 g/plant ($11.9 \times 10^5$ CFU/plant) |
| Trichonativa® | One: Pre-transplantation | $5ml \cdot l^{-1}$ ($5 \times 10^9$ CFU/plant) |
| Th 12A 10.1 | Three: Pre-transplantation, 15 days after transplanting and one week before sprouting | 1.7 g/plant each time ($8.5 \times 10^5$ CFU/plant) |
| Th F2-1 | Three: Pre- transplantation, 15 days after transplanting and one week before sprouting | 1.7 g/plant each time ($11.9 \times 10^5$ CFU/plant) |
| Trichonativa® | Three: Pre-transplantation, 15 days after transplanting and one week before sprouting | $5ml \cdot l^{-1}$ pre-transplantation ($5 \times 10^9$ CFU/plant), $1.0l \cdot ha^{-1}$ after transplanting |
| Methyl bromide | Pre-transplantation, five days exposure | $60g/m^{-2}$ |
| Positive control | One: at pre-transplantation with alginate pellets without addition of any *Trichoderma* strain. | 1.0 g/plant |

The experimental design considered a complete randomized block of ten tomato plants in a row, with four replicates for each treatment. Results include tomato root damage and persistence of *Trichoderma* strains in the soil. The experimental unit was ten tomato plants in a row.

*Evaluation and analysis of* P. lycopersici *damage*

The percentage of root damage (% area with corky root symptoms) caused by *P. lycopersici* was established using the Campbell and Shiskoff scale (1990) at the pull out of plants (end of the crop) as 0: no damage, 1: <10% damage, 2: 10-25% damage, 3: 25-75% damage, and 4: >75% damage.

Results were analyzed through the non parametric Friedman's test, and differences between treatments were established using the corresponding tabulated critical value.

*Persistence of* Trichoderma *strains in the soil*

*Trichoderma* content was analyzed in soils before assays. For treatments, samples close to plant crowns were taken at the following times after transplanting: one month, three months (fruit set up to the second fruit bunch), five months (third fruit bunch); and seven months (pull out of plants). The soil samples were assayed in the laboratory for the presence of *Trichoderma* strains using Petri plates containing the corky root agar medium of Grove and Campbell (1987). Soil samples (1.0g) were serially diluted in sterilized distilled water, 0.1ml portions were spread on the agar medium and the Petri dishes were incubated for 72h at 22°C. The colonies of *Trichoderma* were counted and the counts were used to estimate the number of colony forming units (CFU)/g of soil. These values were used to estimate the half survival period of the *Trichoderma* isolates considering the recovered CFUs/g soil and the total CFUs initially inoculated per plant, without considering the initial *Trichoderma* population of $2.5 \times 10^3$ CFU/g soil, which was not significant in relation to the CFU of each *Trichoderma* strain tested.

Soil samples taken from the surroundings of each of the ten tomato plant roots were mixed. The analysis considered five replicates per sample, per replicate of plant treatment and per each time period of sampling. The experimental unit was one Petri dish. Results are presented as mean of the five repeats +SD of each sample.

The half persistence period ($t_{0.5}$) was determined from plots considering these data, where 100% persistence was considered as the initial CFUs inoculated to the soil.

## Results and Discussion

*Biocontrol activity of* Trichoderma *against* P. lycopersici *in vitro*

Th12A.10.1 showed 70.2% inhibition of *P. lycopersici in vitro*, which was not significantly different from the 80.3% inhibition caused by its parental strain Th12. On the other hand, ThF2-1 inhibited in 43% the phytopathogen growth as compared to the 52.3% and 84.8% inhibition of its parental strains ThV and Th291, respectively. Values for growth inhibition of *P. lycopersici* by the parental strains of Th12A.10.1 (Th12) and of ThF2-1 (ThV and Th291) were each similar to those obtained previously (Besoain *et al*., 2007), which suggests that the storage conditions used (frozen storage) did not affect their ability to inhibit pathogen growth. The

data obtained are generally consistent with findings in earlier studies where wild strains of *Trichoderma* spp. inhibited growth of *P. lycopersici* by 20-100% in dual culture assays (Vanachter *et al*., 2008).

*Biocontrol activity of* Trichoderma *against* P. lycopersici *in a greenhouse*

The damage level caused by *P. lycopersici,* (Table II) in tomato roots after a single application of the alginate formulation of Th12A.10.1, of ThF2-1 or of the liquid product (Trichonativa®) did not differ significantly ($p<0.05$) from the positive control in which no *Trichoderma* was employed. These findings indicate that one application of these formulations does not reduce root damage by *P. lycopersici* at the inoculum density employed and with the method used. The high root damage found in tomato roots in soils treated with methyl bromide (Table II) suggests that an inadequate application of the chemical could explain the results obtained for this treatment, because assays previously run in the same conditions showed a 0.63 damage level in tomato roots (Besoain *et al*., 2003). Also, it may be hypothesized that the continuous treatment of the soil used for the trials with methyl bromide could have resulted in the development of more resistant strains of the pathogen (Fiume and Fiume, 2008) or due to the presence of different types of *P. lycopersici* (Bayraktar and Oksal, 2011).

The level of damage caused by *P. lycopersici* was significantly lower in tomato plants treated with three applications of ThF2-1 (i.e. immediately before transplanting, 15 days after transplanting and one week before sprouting) than in plants treated at the same times with Th12A.10 or Trichonativa, and compared to plants of the positive control (Table II). Also, the level of damage observed after three treatments with ThF2-1 was significantly lower than obtained previously with the methyl bromide treatment of the soil (Besoain *et al*., 2003).

Taken together, the data indicates that the strain ThF2-1 of *T. harzianum* derived by protoplast fusion (THV×Th291) is also able to inhibit *in vitro* colony growth of *P. lycopersici* and to effectively reduce root damage by the pathogen in tomatoes grown under commercial greenhouse conditions. The same strain also controlled *Rhizoctonia solani* both under greenhouse and field level conditions (Montealegre *et al*., 2010a).

The biocontrol of *P. lycopersici* under greenhouse conditions has been tested by Varela *et al* (2009), using commercial products based on *Trichoderma* and other biocontrol agents.

TABLE II
DAMAGE LEVEL PRODUCED BY *Pyrenochaeta lycopersici* IN TOMATO CV. FORTALEZA PLANT ROOTS AFTER DIFFERENT TREATMENTS

| Treatment | Damage level[2,3] |
|---|---|
| Trichonativa® (one, at pre-transplantation) | 1.8 a |
| Control (no treatment) | 1.6 a |
| Th F2-1 (one, at pre- transplantation ) | 1.4 a |
| Trichonativa® (three treatments[1]) | 1.4 a |
| Th 12A 10.1 (one, at pre- transplantation ) | 1.2 a |
| Th 12A 10.1 (three treatments[1]) | 1.0 a |
| Methyl bromide | 0.8 a |
| Th F2-1 (three treatments[1]) | 0.2 b |

[1] Soil treatment at pre-transplantation, 15 days after transplanting and one week before sprouting.
[2] Damage level measured as 0: no damage, 1: <10% damage, 2: 10-25% damage, 3: 25-75% damage, and 4: >75% damage.
[3] Identical letters in the column mean no significant differences between treatments, after variance analysis of results and Friedman's test at $p\leq0.05$.

*Persistence of* Trichoderma *strains in the soil*

Estimated density (CFU/g soil) of the tested strains of *Trichoderma* each decreased following a single application before transplanting (Figure 1). Density of Trichonativa® declined to zero in less than one month while Th12A.10.1 persisted for at least one month, and ThF2-1 for at least three months. Rapid loss of CFU of the Th12A-10.1 and ThF2-1 was found also when the agent was applied three times (Figure 2). When Th12A10.1 and ThF2-1 were applied three times CFU densities in the soil increased and subsequently

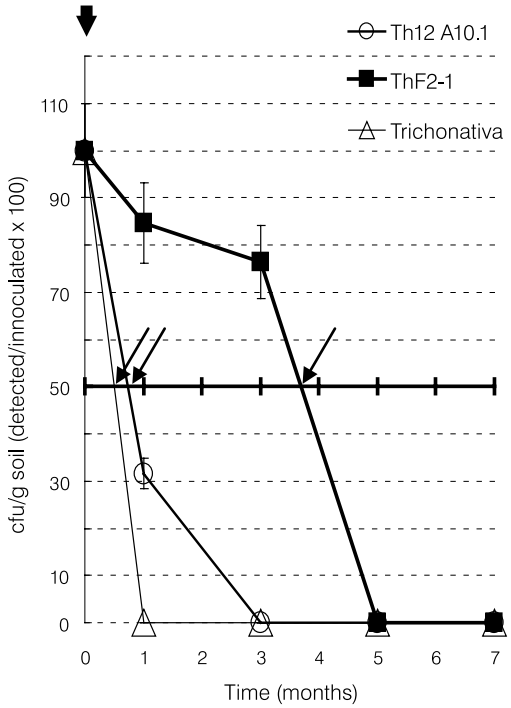


Figure 1. Density of colony forming units of *Trichoderma* strains Th12A.10.1, ThF2-1 and Trichonativa® in field soil as a function of time after one application immediately before tomato plants were transplanted (top arrow). Small arrows in 50% CFU/g soil show the half persistence period. Values are means of five replicates ±SD.

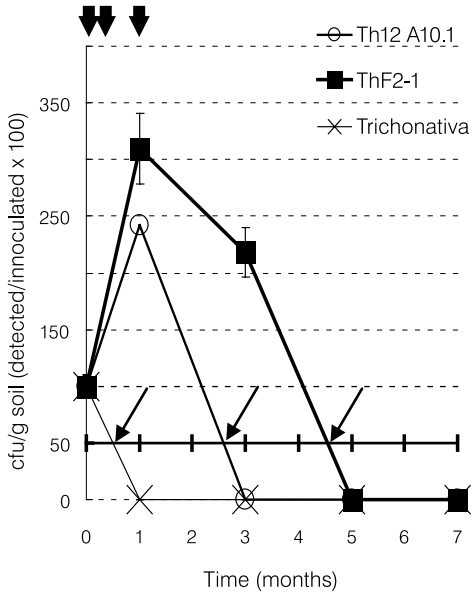


Figure 2. Density of colony forming units of *Trichoderma* strains Th12A.10.1, ThF2-1 and Trichonativa® in field soil as a function of time after three applications: immediately before transplanting, 15 days after transplanting and one week before sprouting (top arrows). Small arrows in 50% CFU/g soil show the half persistence period. Values are means of five replicates ±SD.

decreased, ultimately to zero. The markedly longer persistence of Th12A.10.1 and ThF2-1 in the soil compared to Trichonativa was probably related, in large part, to protection of the spores by the solid formulation used against adverse soil conditions, including soil organisms. The same formulation of Th12A.10.1 tested *in vitro* for storage with different soils and temperatures was persistent against *R. solani* at least for three months (Montealegre *et al*., 2009). Thus, the solid formulation appears to favor stability and persistence of *Trichoderma* in the soil, reflected in the half-persistence period (50% of the CFU applied to the soil (Figures 1 and 2, small arrows). However, this half persistent period could be less than the calculated one if zero density occurred at an earlier time than the last chosen for sampling, a fact that also applies to Trichonative.

Results obtained by Lewis and Papavisas (1985) using two different *T. harzianum* strains, *T. hamatum* and *Gliocladium virens*, to control *R. solani*, showed that conidial suspensions were less effective than dried mycelia plus base nutrients. On the other hand, differences between the two solid formulations that differ in the included *Trichoderma* strains (Th12A10.1 or ThF2-1) might also be attributed to specific strain characteristics, accounting for a higher persistence of ThF2-1 in the soil. Thus, the poor persistence in soil of the *Trichoderma* strains contained with Trichonativa could be attributed to the type of formulation rather than to the ability of the fungal strains to persist in the soil (Montealegre *et al*., 2010b).

The three soil applications with ThF2-1 that resulted in a significant increase of its half-persistence periods correlates with the decrease in disease symptoms caused by *P. lycopersici* (Table II, Figure 2), while the three applications with Th12A10.1, which also increased its half-persistant period (Figure 2), was ineffective to control root damage of tomato plants (Table I). The persistence of a high density of specific *Trichoderma* strains in the soil appears to be very important to prevent diseases that take long time periods for symptoms to be noticeable, as is the case of corky root caused by *P. lycopersici*. In fact, it has been described that corky root symptoms appears once half of the culture time has elapsed (Crüger *et al*., 2002).

Therefore, it may be concluded that the strategy of three applications of a solid formulation containing a ThF2-1 is more adequate to maintain a high concentration of the microorganism in the soil for the control of *P. lycopersici*, being an adequate alternative strategy to the use of methyl bromide in commercial tomato crops.


## ACKNOWLEDGEMENTS

This study was funded through PROYECTO FONDECYT: 1040531-04.


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