



Agronomía Costarricense

ISSN: 0377-9424

Universidad de Costa Rica. Colegio de Ingenieros y Agrónomos. Ministerio de Agricultura y Ganadería

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Agronomía Costarricense, vol. 43, no. 1, 2019, January-June, pp. 85-100

Universidad de Costa Rica. Colegio de Ingenieros y Agrónomos. Ministerio de Agricultura y Ganadería

DOI: <https://doi.org/10.15517/rac.v43i1.35671>

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USE OF DIFFERENT *Trichoderma* SPECIES IN CHERRY TYPE TOMATOES (*Solanum lycopersicum* L.) AGAINST *Fusarium oxysporum* WILT IN TROPICAL GREENHOUSES

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Palabras clave: Biocontrol; hongos; control biológico; antagonismo; micoparasitismo.

Keywords: Biocontrol; fungi; biological control; antagonism; mycoparasitism.

Recibido: 12/04/18

Aceptado: 29/06/18

RESUMEN

Utilización de diferentes especies de *Trichoderma* en tomates cherry (*Solanum lycopersicum* L.) contra la marchitez del *Fusarium oxysporum* en invernaderos tropicales. El uso *Trichoderma* spp. se ha incrementado en la actualidad para el control de hongos patógenos de plantas. En Venezuela los productos comerciales a base de *Trichoderma* son formulados casi exclusivamente a partir de esporas de *T. Harzia-num*, pero muchos experimentos han reportado en la literatura un mejor desempeño de otras especies nativas no comerciales en comparación con los productos formulados en el país. En este trabajo, 4 especies nativas de *Trichoderma* Persoon fueron evaluadas en condiciones de laboratorio y en invernaderos comerciales para el control de la marchitez del *Fusarium* en una variedad de tomate tipo “cherry” de crecimiento indeterminado. Diferentes experimentos fueron realizados para evaluar el antagonismo, mico-parasitismo, endofitismo, efecto de metabolitos secundarios producidos por *Trichoderma* y el efecto agronómico de esas especies en los invernaderos comerciales. Las especies evaluadas fueron:

ABSTRACT

The use of *Trichoderma* species is increasing worldwide over the years to control different plant pathogenic fungi. Four indigenous *Trichoderma* Persoon were tested in laboratory and in a commercial greenhouse conditions to control *Fusarium* wilt in an undetermined variety of cherry tomatoes. In Venezuela available commercial products are formulated almost exclusively with *T. harzianum* but experimental trials indicate failure to control *Fusarium* compared to noncommercial strains. Different experiments were set to assess antagonism, mycoparasitism, edophytism, effect of secondary metabolites produced by *Trichoderma*, and the agronomic effect of those species in a commercial farm greenhouse. Species tested were *T. koningiopsis* Samuels, Suárez & Evans, *T. virens* Mill., Giddens & Foster, *T. spirale* Bissett and *T. harzianum* Rifai and among them, *T. spirale* showed the best performance in all experiments. *Trichoderma spirale* was able to inhibit the growth of *Fusarium* up to 79% in PDA plates; its secondary metabolites reduced the pathogen growth in 64%. On the other

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T. koningiopsis Samuels, Suárez & Evans, *T. virens* Mill., Giddens & Foster, *T. spirale* Bissett y *T. harzianum* Rifai. De todas estas, *T. spirale* mostró los mejores resultados en todos los experimentos. *Trichoderma spirale* fue capaz de inhibir el crecimiento de *Fusarium* hasta el 79% en placas de PDA; mientras que sus metabolitos secundarios redujeron el crecimiento del patógeno en un 64%. Por otro lado, mostró un efecto protector contra *Fusarium* en condiciones in planta en pruebas de laboratorio, reduciendo la enfermedad en 70%. También demostró un efecto positivo en el crecimiento de la planta, posiblemente la acción edofítica de *T. spirale*. En condiciones de invernadero *T. spirale* produjo los mejores resultados luego de 90 días post-transplante con el aumento del tamaño de las plantas, la producción de tomates por racimo junto con la protección de las plantas contra la marchitez del *Fusarium*. Los resultados obtenidos con la cepa nativa de *T. spirale* usada en este trabajo demostraron que este organismo posee un gran potencial como agente de control biológico.

INTRODUCTION

Fusarium fungi are cosmopolitan plant pathogens which lead to important yield and economical loss in many crops. Among this genus, many species have saprophytic life cycles and do not represent any risk, but some are plant pathogens and even can act as human and animal opportunistic pathogens (Harmann *et al.* 2004). *Fusarium* wilt on tomatoes (*Solanum lycopersicum* L.) is caused by *Fusarium oxysporum* f.s. *lycopersici* Schltdl. which leads to a severe wilt of the vascular system and a general plant chlorosis, necrosis, premature leaf drop, etc., as consequences. Exudates from plant roots trigger germination of the fungal resting structures which are in the soil, penetrating the host plant's root tips, root wounds, or lateral roots. The fungi then colonize cortical cells and migrate intercellularly

hand, the given species showed a protective effect against *Fusarium* in planta and field situations reducing the incidence of the disease in 70% and also showed positive effects in plant growth possibly because of improved water and nutrient intake by the plants or endophytism. In greenhouse experiment, *T. spirale* produced the best results after 90 days post-transplanting both by production and by protecting plants against *Fusarium* wilt. Plants inoculated with *T. spirale* were significantly higher, produced more fruits and reduced the incidence of *Fusarium* wilt in a commercial greenhouse conditions. The indigenous strain of *T. spirale* used in this work has a good potential to become a regular biocontrol agent in the near future.

toward the vascular parenchyma and invade the xylem vessels (Yadeta & Thomma 2013).

In the last decades, concerns about the overuse of chemical pesticides has increased and countless attempts to incorporate biological control agents in the fields have been done. *Trichoderma* spp. Persoon are well known biological control agents to plant pathogenic fungi (Harman 2006), specially soil-borne diseases. The beneficial effects of *Trichoderma* have been attributed to many factors such as antagonism, mycoparasitism (Kubicek *et al.* 2001), production of secondary metabolites (Reino *et al.* 2008), promotion of plant growth by improvement on water and nutrient uptake (Chet 2001, Harman 2006, Li *et al.* 2018), promotion of plant resistance (Jogaiah *et al.* 2018) and effects mediated by environmental factors (Zhang *et al.* 1998),

Trichoderma species and the host plant genotypes (Tucci *et al.* 2011). These mechanisms have been favoured by the ability of *Trichoderma* for colonizing plant roots (Howell 2006) which can even reduce foliar diseases (Harman *et al.* 2004). Some *Trichoderma* species are capable to improve soil conditions for root development, reducing plant stress (Shores *et al.* 2005) and by activation of physiological and biochemical mechanisms to induce resistance to pathogens by plants (Hanson & Howell 2004, Harman *et al.* 2004, Hoitink *et al.* 2006).

In Venezuela, more than 11.900 ha are dedicated to tomato production and up to date, nearly 1000 ha of greenhouses have been installed to grow tomato, sweet pepper, lettuces and flowers mainly. As many places in the world, Venezuelan crops are highly affected by *Fusarium* species, but in many cases the management of the disease is inefficient and losses are important. Some authors have calculated the severity of *F. oxysporum* in Venezuelan tomato plantations and have determined more than 60% of yield losses (Anzola & Román 1982) in many places across the country. Currently the vast majority of control measures worldwide to tackle *Fusarium* are related to plant resistant varieties and chemical fungicides applications; however, the pathogen remains an enormous problem. The excessive and indiscriminate use of chemical fungicides also have increased detrimental effects on the environment, water sources, food, and people (especially farm workers, and final consumers).

The use of *Trichoderma* strains in Venezuelan fields has increased since 1990 but many aspects regarding their mode of action, plant protection effect or yield increase remain unknown (Perdomo *et al.* 2007, Guédez *et al.* 2009, López *et al.* 2010). At the beginning, some alien species were introduced to the country but with less than good results (probably, due to ecological differences between the place of origin of the strain and the tropical environment of Venezuela). Isolation and production of native species has increased during the years and now around 20 commercial *Trichoderma*-based products are

being sold in the country while research goes on. The objectives of this work were i) to test the effect of 4 *Trichoderma* species isolated in the country on *F. oxysporum* in in vitro conditions ii) to evaluate the potential of these *Trichoderma* spp. as biological control agents in controlled conditions and iii) to evaluate their performance in commercial greenhouses for the production of tomatoes.

MATERIALS AND METHODS

Biological material

F. oxysporum was isolated directly from tomato diseased plants found in different horticultural farms at El Jarrillo, Estado Miranda, Venezuela. Pieces of stem of the plants were surface sterilized using sodium hypochlorite at 3% for 10 minutes. After sterilization, the stem pieces were cut longitudinally and damaged tissues were separated and plated in Potato Dextrose Agar (PDA). Plates were incubated for 5 days at 28°C and growing mycelia were re-plated and purified following the same protocols. Identification was done by both light microscopy observation of conidia and molecular tools (Rep *et al.* 2004) (done in the Molecular Phytopathology laboratory at Instituto de Estudios Avanzados).

Trichoderma spp. were obtained from the collection of the Molecular Phytopathology laboratory that houses isolates from different agricultural soils across the country. Four species were used in the experiments (*T. koningiopsis* Samuels, Suárez & Evans (VT16), *T. virens* Mill., Giddens & Foster (VT43), *T. spirale* Bissett (VT56) and *T. harzianum* Rifai (CT6)).

Antagonism tests

Dual culture assay

Twenty five PDA plates were prepared and inoculated with *F. oxysporum* or individually with each *Trichoderma* species and incubated as explained earlier. After 5 days the plates were used as inoculum for antagonism experiments.

A plug of growing mycelium (5 mm) of *F. oxysporum* was placed in a side of a PDA plate and incubated for 48 h at 28°C. Then a plug of a single *Trichoderma* species was placed in the opposite side of the petri dish and returned to the incubator (control plates were inoculated only with *F. oxysporum*). The daily growing rate of *F. oxysporum* was measured every 24 h, measuring the diameter of the fungus in 2 perpendicular directions and the experiment was finished when the control PDA plate (only *F. oxysporum*) was totally covered by the fungus.

The perpendicular measurements of the *F. oxysporum* mycelium were averaged. The antifungal index was determined in comparison with the control plates, and calculated by the Wang *et al.* (2009) equation seen below. Where Da is the diameter of the growing mycelium of the *F. oxysporum* in the antagonism plates, and Db is the diameter of the growing mycelium of the *F. oxysporum* in the control plate (Wang *et al.* 2009).

Antifungal index (%) = $[1 - (Da - 0.5) / (Db - 0.5)] \times 100$
The experiment had 5 replicas and was repeated three times.

Micro-culture of fungi for microscopic observations

The microscopic effects of the 4 *Trichoderma* species on *F. oxysporum* mycelium, and conidia were evaluated using the protocol developed by Riddell (1950) with few modifications. A PDA square (10 mm diameter x 20 mm height) was placed upon a sterile glass slide. Using a microbiological loop, tiny pieces of *F. oxysporum* mycelium were placed in the opposite lateral sides of the agar block (i.e. north and south sides) and placed in a moist chamber for 48 h at 28°C in an incubator. The chamber consisted on a Petri dish half filled with 10 ml of 10% glycerol solution and some glass rods used as support to avoid the slide glass to sink. After *F. oxysporum* inoculation, tiny pieces of *Trichoderma* were attached to the other free sides of the agar block

(i.e. east and west sides). A sterile cover glass was then set on upon the top of the block and the slide was returned to the moist chamber and incubated for 96 h at 28°C. After incubation, the glycerol solution in the Petri dish was substituted by a solution of 10% formaldehyde for 2 h to fix fungal structures. The cover glass was withdrawn out from the PDA block and set in a new microscope glass slide with a drop of cotton blue/lactophenol. The new slides were sealed and evaluated using a Leica® DM2500 compound microscope fitted with a differential interference contrast system. The experiment was replicated 5 times and repeated 3 times.

***Trichoderma* antibiosis**

To study the effect of secondary metabolites, experiments were done following Vinale *et al.* (2009). Twenty five 50 ml Erlenmeyer flasks were filled with Potato Dextrose Broth (PDB) and sterilized by autoclaving. Five plugs of 5 mm of growing mycelium of every *Trichoderma* species were transferred aseptically into the flasks (1 plug per flask) and 5 flasks remained as control broths. The flasks were placed in a shaker (100 rpm) for 9 days at room temperature (25±2°C). After incubation, the flask content was pre-filtered to separate the mycelium from the broth using filter paper Whatman N°6 (3 µm pore). The resulting broth was vacuum filtered using Millipore® membranes (0.22 µm) to extract only metabolites. An aliquot of the sterilized metabolite suspension was plated in PDA to confirm the fungus absence.

A 5 mm plug of plated *F. oxysporum* was soaked in the *Trichoderma* metabolite suspensions for 15 min (a control plug was soaked in the control broth) and plated in PDA. The mycelium growth was recorded by measuring the diameter of the fungus in 2 perpendicular directions and the experiment was finished when the control PDA plate (only *F. oxysporum*) was totally covered by the fungus. The experiment was repeated 3 times and replicated 5 times. The antifungal index was calculated as before.

Effect of different *Trichoderma* species on tomato plants infected or non-infected with *F. oxysporum* in laboratory conditions

Inoculum preparation

F. oxysporum PDA plates were prepared as before and incubated for 7 days. Sterile water was applied after incubation into the plates and the mycelium was scraped with a sterile glass rod. The suspension of spores was filtered and poured into tubes and the concentration of spores were adjusted using a hemocytometer to $1,0 \times 10^6$ spores.ml⁻¹. *Trichoderma* species inoculum was set in the same way than *F. oxysporum* but 3 different concentrations of spores were prepared ($1,0 \times 10^6$, $1,0 \times 10^7$ and $2,0 \times 10^6$ spores.ml⁻¹).

Tomato seed treatments

Thirty grams of tomato seeds (undetermined hybrid Camelia, Hazera®, resistant to *F. oxysporum* f. sp. lagenariae (Race1)) were surface sterilized by soaking them in a 20% solution of sodium hypochlorite for 10 min. Seeds were washed 3 times with sterilized distilled water for 5 min. Sterile seeds were separated into 12 groups (2 g each) and inoculated accordingly with the 4 *Trichoderma* species using 3 different spore concentrations ($1,0 \times 10^6$, $1,0 \times 10^7$ and $2,0 \times 10^6$ spores.ml⁻¹). Seeds were soaked in the *Trichoderma* spore inoculum (5 ml water and 1 ml of spore suspensions) for 15 min and placed in a filter paper to remove water excess. Two groups of sterile seeds were kept sterile to serve as control (one group as negative control using sterile water as inoculum and the remaining as positive control inoculated with *F. oxysporum* as before).

After 30 days post-emergence, plantlets were transplanted in 1 l pots (10 plants per treatment) containing a sterile mixture of peat moss and sand (3:1 v/v) and placed randomly in a table under controlled temperature ($25 \pm 2^\circ\text{C}$) and 12 h photoperiod. Irrigation was done manually

with sterile water. After 7 days post-transplant, the plantlets were inoculated with *F. oxysporum* by applying the spores ($1,0 \times 10^6$ spores in 5 ml of water) directly on the substrate (except negative controls), the rest of *Trichoderma* inoculated plants were used as positive control (without *F. oxysporum*) and to measure the development of plants inoculated only with *Trichoderma*. After 60 days post emergence, 5 plants per treatment were chosen for further measurements: stem height, stem and roots fresh weight, stem and roots dry weight, severity of *F. oxysporum* infection. The experiment was replicated 3 times.

Greenhouse study

The study was carried in “Finca Frutiagro PTM” at El Jarillo, Estado Miranda, Venezuela (N $10^\circ 20' 42''$; W $67^\circ 9' 35''$) in commercial greenhouses in which cherry tomatoes were grown (undetermined hybrid Camelia, Hazera®) in which a plant death outbreak of 34.7% was attributed to *F. oxysporum* (such plants were the *F. oxysporum* source for this work).

Three weeks before transplanting, seed were set in trays with sterilized peat moss to ensure disease –free plantlets. The greenhouse soil was prepared, beds were rose, and a fungicide based on 77% copper hydroxide was applied, following manufacture instructions by drench (1 kg per ha). Beds then were covered with plastic and drip irrigation was installed. Soil physic chemistry is as follows: (pH = 6.2; electric conductivity = 0.2; %clay = 17; %silt = 27; %sand = 56; %RH inside the greenhouse = 75%; photoperiod = 12:12, mean temperature inside the greenhouse = 33°C).

Free disease tomato plantlets, using sterile substrate and distilled water were produced in a local nursery. Before transplanting, the germination trays with the plantlets were soaked in a suspension containing the given *Trichoderma* species (5 liters of distilled water with 5 ml of a suspension of $5,0 \times 10^8$ spores.ml⁻¹) for 15 min. Control trays were soaked in distilled water. From

the trays, 40 plantlets per *Trichoderma* species and 40 control plantlets were chosen randomly and transplanted in 4 blocks distributed randomly inside the greenhouse in lines of 10 plants (1 line per treatment). The plantlets were transplanted directly in the greenhouse soil (with no treatment) to allow the natural infections of *F. oxysporum*. The *Trichoderma* inoculations were repeated once a week (100 ml of water with 1 ml of the spore suspension of $5,0 \times 10^8$ spores.ml⁻¹) after transplanting for 4 week using a manual spray pump. At day 90 after transplanting, the first harvest of tomatoes was done; randomly 3 plants per line per treatment were chosen and their height, number of fruits of the harvested first cluster and fruit weight were measured. If any plant showed symptoms of *F. oxysporum* wilt, samples of stem tissues were taken to microscopically confirm *F. oxysporum* presence in PDA plates.

Statistical analysis

Data collected were evaluated using Mini-tab[®] statistical program. Prior analysis, normality tests were performed. Experimental differences between treatments when normality was achieved

were determined by ANOVA. If significant differences were found, Tukey family comparisons were done. If collected data were in percentage, the results were angular transformed and presented untransformed (% $\pm$ s.d.).

RESULTS

Antagonism tests

Dual culture assay

The antifungal index of *Trichoderma* spp. to *F. oxysporum* was different according to the species ($p \leq 0.001$; $\alpha = 0.05$) (Table 1). Along the experiment until 168 h, *T. koningiopsis* showed the highest antifungal indexes, but there was a delay in its effect in the last measurement. *Trichoderma spirale* was the most effective inhibitory fungus in plates at the end of the experiment (192 h) by 78.3 $\pm$ 4.9%, followed by *T. koningiopsis* (76.6 $\pm$ 5.5%). *Trichoderma virens* and *T. harzianum* showed less inhibitory capacity than the previous ones (73.3%).

Table 1. Atifungal effect caused by different *Trichoderma* species against *Fusarium* growth at different time in Petri dishes.

Treatment	Percentage of radial growth inhibition (%)							
	24 h	48 h	72 h	96h	120 h	144 h	168 h	192 h
D94	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d
D94+VT56	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	23.2 $\pm$ 3.9 ^c	34.4 $\pm$ 5.2 ^c	51.9 $\pm$ 4.9 ^c	62.1 $\pm$ 4.9 ^c	68.5 $\pm$ 4.9 ^c	78.3 $\pm$ 4.9 ^c
D94+VT43	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	16.9 $\pm$ 3.8 ^b	24.2 $\pm$ 6.2 ^b	42.2 $\pm$ 5.0 ^b	54.4 $\pm$ 5.0 ^b	62.0 $\pm$ 5.0 ^c	73.3 $\pm$ 5.0 ^b
D94+VT16	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	21.1 $\pm$ 3.8 ^a	27.5 $\pm$ 6.4 ^a	48.8 $\pm$ 5.6 ^a	54.6 $\pm$ 5.7 ^a	65.2 $\pm$ 5.7 ^a	76.6 $\pm$ 5.5 ^c
D94+CT6	0 $\pm$ 0 ^d	0 $\pm$ 0 ^d	17.7 $\pm$ 4.3 ^b	24.2 $\pm$ 7.0 ^b	42.4 $\pm$ 5.5 ^b	54.6 $\pm$ 5.5 ^b	62.2 $\pm$ 5.5 ^b	73.3 $\pm$ 5.5 ^b

D94 = *Fusarium oxysporum* (control); VT56 = *Trichoderma spirale*; VT16 = *Trichoderma koningiopsis*; VT43 = *Trichoderma virens* and CT6 = *Trichoderma harzianum*.

Different letters indicate significant differences within the same time of evaluation according to Tukey test ($p < 0.05$).

Micro-culture of fungi for microscopic observations

Interactions between *Trichoderma* and *F. oxysporum* were different according with the studied species. All *Trichoderma* species showed mycoparasitism against *F. oxysporum*. First, the *Trichoderma* hyphae entered in contact with the pathogen hyphae and, after recognition, the antagonist hyphae adhered and penetrated the *F. oxysporum* hyphae destroying them. On the other hand, interactions between spores were observed. *Trichoderma* conidia surrounded the *F. oxysporum* ones causing deformation and posterior lysis.

Trichoderma antibiosis

When secondary metabolites were tested, the antifungal index to *F. oxysporum* also differed according to the *Trichoderma* species ($p \leq 0.001$; $\alpha = 0.05$) (Table 2). Metabolites coming from *T. spirale* were more effective in fungal inhibitory capacity ($64.28 \pm 0.8\%$) followed by *T. virens* ($61.42 \pm 0.6\%$). Metabolites from *T. harzianum* and *T. koningiopsis* were less effective against *F. oxysporum* in in vitro conditions, with percentages of growth inhibition of $60 \pm 1.3\%$ and $57.2 \pm 0.5\%$, respectively.

Table 2. Atifungal effect caused by metabolites produced by different *Trichoderma* species against *Fusarium* growth at different time in Petri dishes.

Treatment	Percentage of radial growth inhibition (%)							
	24 h	48 h	72 h	96h	120 h	144 h	168 h	192 h
D94	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c	0 $\pm$ 0 ^c
D94+VT56	5 $\pm$ 0.7 ^c	16.6 $\pm$ 0.6 ^d	15.5 $\pm$ 1.2 ^d	18.6 $\pm$ 1.0 ^d	33.8 $\pm$ 0.7 ^d	48.4 $\pm$ 0.7 ^d	56.2 $\pm$ 0.7 ^d	64.2 $\pm$ 1.2 ^d
D94+VT43	5 $\pm$ 0.7 ^c	16.6 $\pm$ 0.6 ^d	18 $\pm$ 0.8 ^c	18.6 $\pm$ 1.0 ^d	33.8 $\pm$ 0.7 ^d	44.8 $\pm$ 1.0 ^c	51.1 $\pm$ 1.1 ^c	61.4 $\pm$ 0.3 ^c
D94+VT16	8.8 $\pm$ 0.8 ^c	16.6 $\pm$ 0 ^d	12.5 $\pm$ 1.5 ^b	16.6 $\pm$ 0.6 ^c	32.3 $\pm$ 0.5 ^c	43.2 $\pm$ 1.0 ^b	49.1 $\pm$ 0 ^b	57.2 $\pm$ 0.5 ^b
D94+CT6	0 $\pm$ 0 ^c	13 $\pm$ 1.3 ^c	7 $\pm$ 0.8 ^a	18.6 $\pm$ 1.0 ^d	33.8 $\pm$ 0.7 ^d	44.8 $\pm$ 1.0 ^c	51.3 $\pm$ 1.2 ^c	60 $\pm$ 1.3 ^c

D94 = *Fusarium oxysporum* (control); VT56 = *Trichoderma spirale*; VT16 = *Trichoderma koningiopsis*; VT43 = *Trichoderma virens* and CT6 = *Trichoderma harzianum*.

Different letters indicate significant differences within the same time of evaluation according to Tukey test ($p < 0.05$).

Effect of different *Trichoderma* species on tomato plants infected or non-infected with *F. oxysporum* in laboratory conditions

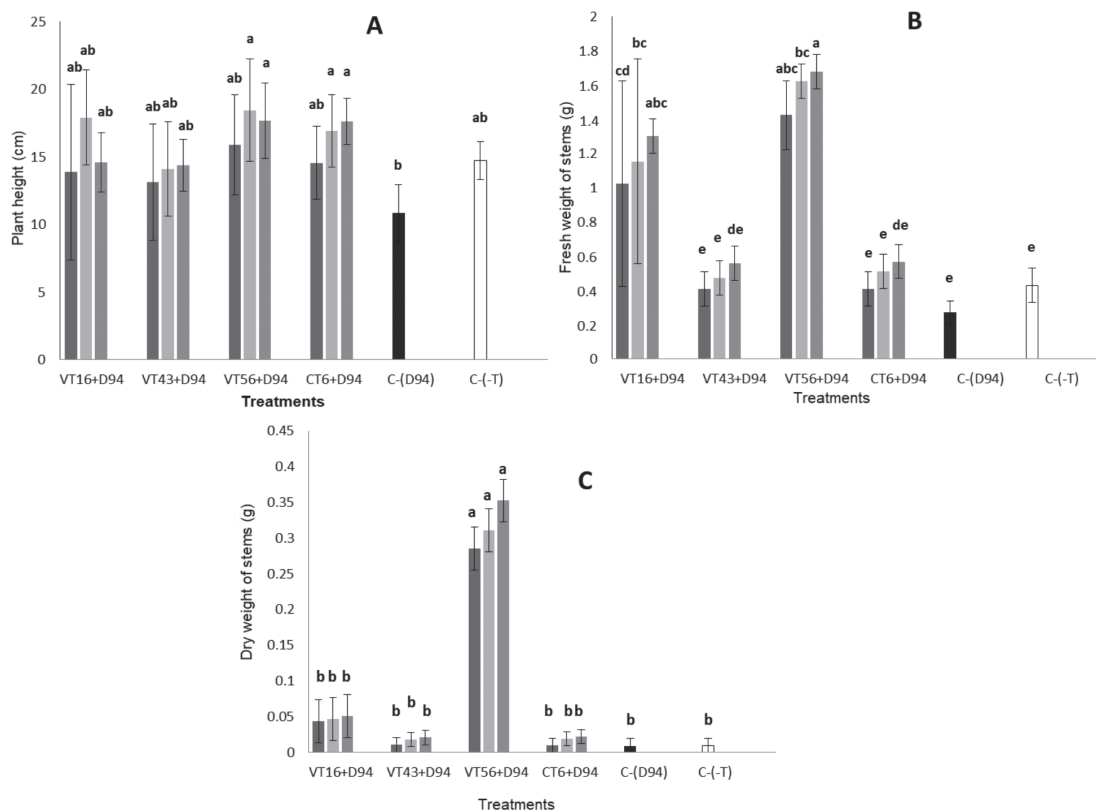
Plants infected with *F. oxysporum*

The disease presented 30% of incidence when tomato plants were treated with 1.0×10^6

spores of *T. koningiopsis* whereas the rest of the *Trichoderma* species treatments showed an incidence of 20%. When the dose was increased to 1.0×10^7 and 2.0×10^6 spores of *Trichoderma* per ml, the incidence lowered to 10% in all treatments. Plants treated only with *Trichoderma* or only with *F. oxysporum* produced 0% and 70% of incidence of the disease, respectively, although symptoms of the disease were not severe.

Measured variables such as plant height were significantly different when compared with positive control (plants infected with *F. oxysporum*) ($p \leq 0.001$; $\alpha = 0.05$). Plants inoculated with *Trichoderma* species and *F. oxysporum* kept the same

height than the control plants, around 14.7 ± 1.4 cm (Figure 1A). However, significant differences were noticed depending on the *Trichoderma* species under test, when fresh and dry weight of the roots and stems of the infected plants were measured.



A) Plant height, B) Fresh weight of stems, C) dry weight of stems. VT16 = *T. koningiopsis*; VT43 = *T. virens*; VT56 = *T. spirale*; CT6 = *T. harzianum*; D94 = *Fusarium oxysporum*; C-(D94) = control with *Trichoderma* but without *Fusarium*; C-(-T) = control without *Trichoderma* or *Fusarium*. Dark gray = 1×10^6 spores per ml; light gray 1×10^7 ; medium gray 2×10^6 .

Different letters indicate significant differences ($p < 0.05$) among treatments. Data is presented in form of means $\pm$ s.d. ($n = 15$).

Fig. 1. Effect of different doses of 4 *Trichoderma* species on tomato plants inoculated with *Fusarium oxysporum* in aerial measurements after 60 days post-emergence.

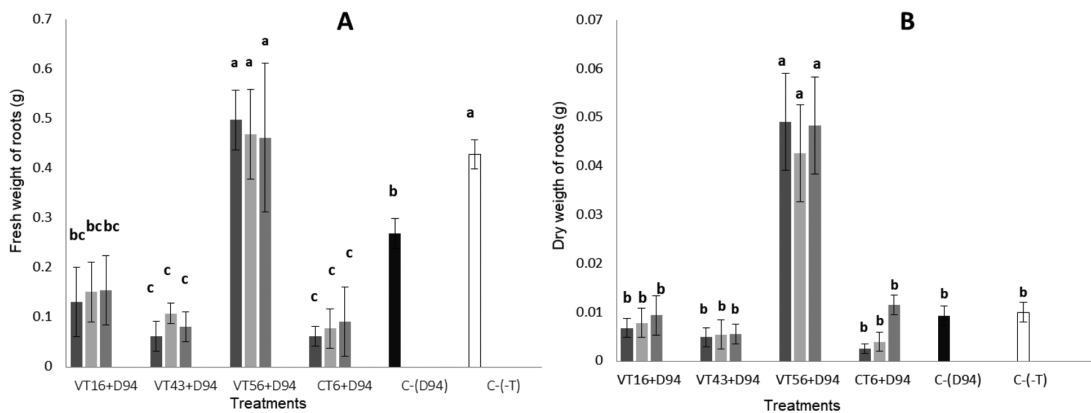
Fresh weight of stems (Figure 1B) revealed that plants inoculated with *T. virens* and *T. koningiopsis* weighed 66% and 57% more than all other treatments, including *T. spirale*, *T. harzianum* and both controls ($p \leq 0.001$; $\alpha = 0.05$). Spore numbers were the same when

fresh weight of the stems was measured. Dry weight of stems also showed significant differences among treatments ($p \leq 0.001$; $\alpha = 0.05$). Dry stems coming from plants treated with *T. spirale* were heavier (0.5 ± 0.03 g) than those produced by all other treatments, including

controls (in which none of them was heavier than 0.05 ± 0.01 g) (Figure 1C).

Fresh weight of plant roots showed significant differences between treatments ($p \leq 0.001$; $\alpha = 0.05$). *Trichoderma spirale*, and control plants (without *F. oxysporum* or *Trichoderma* sp.) were

heavier (0.5 ± 0.06 and 0.43 ± 0.03 respectively) (Figure 2A) compared to all other treatments. When the roots' dry weight was calculated, plants infected with *F. oxysporum* and inoculated with *T. spirale* showed differences comparing with other treatments ($p \leq 0.001$; $\alpha = 0.05$) (Figure 2B).



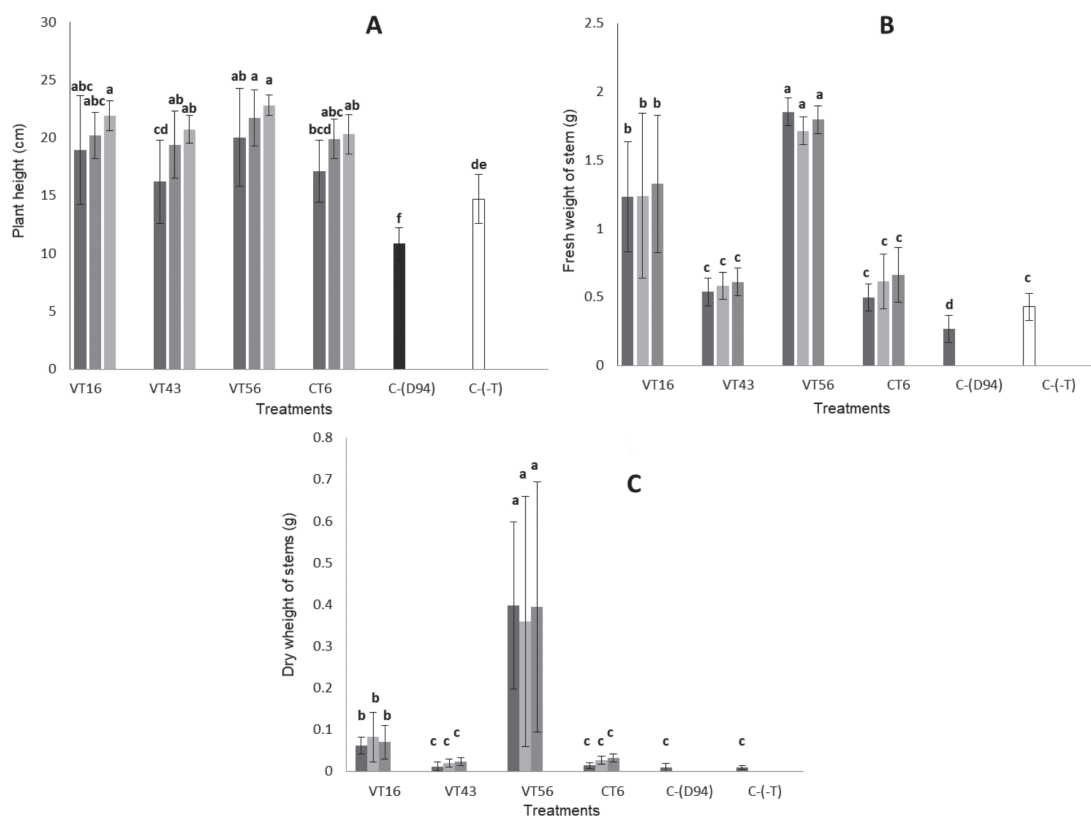
A) Fresh weight of roots, B) dry weight of roots. VT16 = *T. konongiopsis*; VT43 = *T. virens*; VT56 = *T. spirale*; CT6 = *T. harzianum*; D94 = *Fusarium oxysporum*; C-(D94) = control with *Trichoderma* but without *Fusarium*; C-(T) = control without *Trichoderma* or *Fusarium*. Dark gray = 1×10^6 spores per ml; light gray 1×10^7 ; medium gray 2×10^6 . Different letters indicate significant differences ($p < 0.05$) among treatments. Data is presented in form of means $\pm$ s.d. ($n = 15$).

Fig. 2. Effect of different doses of 4 *Trichoderma* species on tomato plants inoculated with *Fusarium oxysporum* in root measurements after 60 days post-emergence.

Effect of *Trichoderma* species on the development of tomato plants

Plants inoculated only with *Trichoderma* spp. showed differences in plant height ($p > 0.001$; $\alpha = 0.05$) (Figure 3A). Plants inoculated with 1.0×10^7 and 2.0×10^6 spores of *T. spirale* and 2.0×10^6 spores of *T. koningiopsis* showed the highest

growing rate among treatments as 22.8 ± 2.4 , 21.7 ± 0.9 and 21.9 ± 2.0 cm, respectively. The shortest height went to control plants infected with *F. oxysporum* (10.83 ± 1.4 cm). Control plants (with neither *F. oxysporum* nor *Trichoderma*) were also reduced when compared to every *Trichoderma* treatment (14.7 ± 2.1 cm).



A) Plant height, B) Fresh weight of stems, C) dry weight of stems. VT16 = *T. koningiopsis*; VT43 = *T. virens*; VT56 = *T. spirale*; CT6 = *T. harzianum*; D94 = *Fusarium oxysporum*; C-(D94) = control with *Trichoderma* but without *Fusarium*; C-(-T) = control without *Trichoderma* or *Fusarium*. Dark gray = 1×10^6 spores per ml; light gray 1×10^7 ; medium gray 2×10^6 .

Different letters indicate significant differences ($p < 0.05$) among treatments. Data is presented in form of means $\pm$ s.d. ($n = 15$).

Fig. 3. Effect of different doses of 4 *Trichoderma* species tomato plants (*Fusarium* free) aerial measurements after 60 days post-emergence.

Fresh stem weight also varied significantly among treatments ($p > 0.001$; $\alpha = 0.05$) (Figure 3B); 4 different groups were clearly identified. The maximum weight was recorded when plants were inoculated with 1.0×10^7 and 2.0×10^6 spores of *T. spirale* (1.85 ± 0.1 g and 1.8 ± 0.1 g respectively). Plants treated with *T. koningiopsis* formed a second group with a maximum fresh weight of 1.32 ± 0.5 g. The third group was formed by *T. harzianum*, *T. virens* and control plants (without *F. oxysporum* infections) where maximum weight were 0.66 ± 0.2 , 0.61 ± 0.1 and

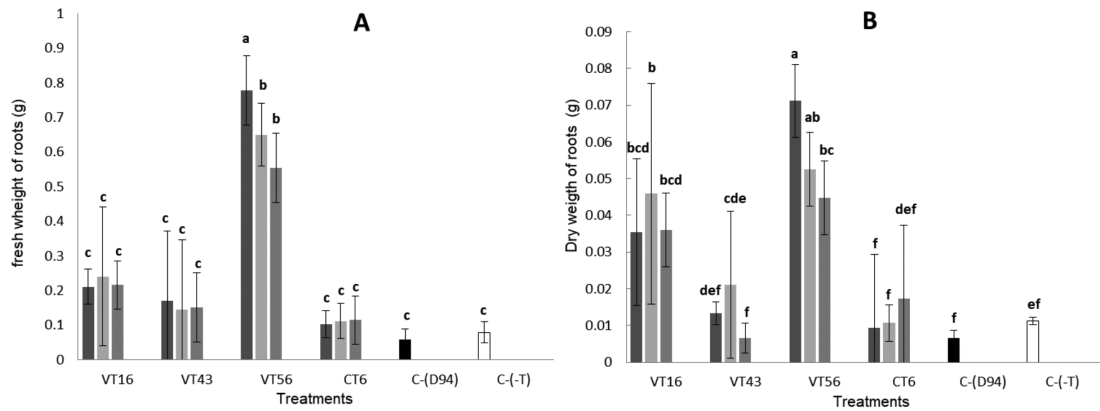
0.43 ± 0.1 g respectively. Control plants inoculated with *F. oxysporum* had lighter fresh stems (0.42 ± 0.1 g).

As before, dry weight of stems were significantly different among treatments ($p < 0.001$; $\alpha = 0.05$) (Figure 3C). However in this case, only plants treated with *T. spirale* and *T. koningiopsis* showed such differences. Plants treated with *T. spirale* registered the heaviest stems with a maximum of 0.40 ± 0.2 g whereas *T. koningiopsis* formed a second group with a maximum weight

of 0.08 ± 0.2 g, all other treatments including both controls weighted equally, forming a third group.

Fresh and dry weight of roots also showed significant differences among treatments ($p > 0.001$; $\alpha = 0.05$ and $p > 0.001$; $\alpha = 0.05$ respectively), plants

treated with *T. spirale* performed the best (Figure 4A and 4B). Patterns were similar compared to the fresh and dry weight of stems, plants treated with *T. spirale* had heavier stems than all other treatments ($p < 0.001$; $\alpha = 0.05$ and $p < 0.001$; $\alpha = 0.05$ respectively).



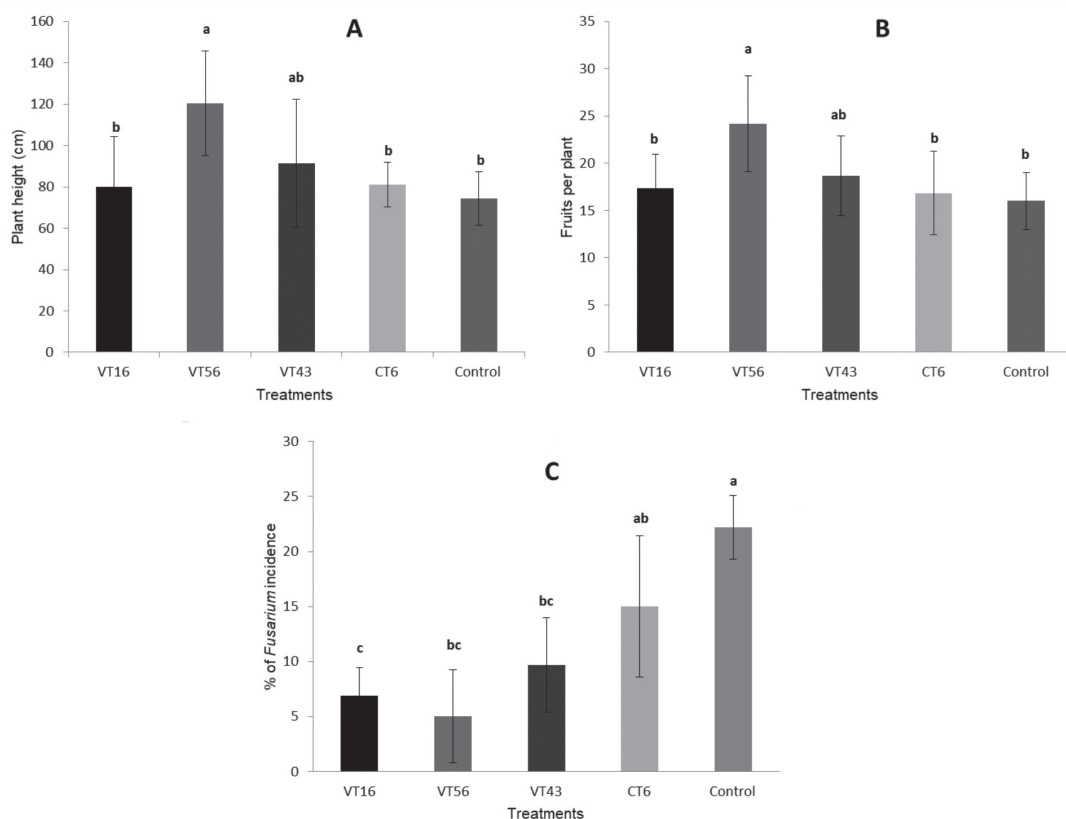
A) Fresh weight of roots, B) dry weight of roots. VT16 = *T. konongiopsis*; VT43 = *T. virens*; VT56 = *T. spirale*; CT6 = *T. harzianum*; D94 = *Fusarium oxysporum*; C-(D94) = control with *Trichoderma* but without *Fusarium*; C-(-T) = control without *Trichoderma* or *Fusarium*. Dark gray = 1×10^6 spores per ml; light gray 1×10^7 ; medium gray 2×10^6 . Different letters indicate significant differences ($p < 0.05$) among treatments. Data is presented in form of means $\pm$ s.d. ($n = 15$).

Fig. 4. Effect of different doses of 4 *Trichoderma* species on tomato plants (*Fusarium* free) in root measurements after 60 days post-emergence.

Field study

Plant height at 90 days varied according to treatment ($p < 0.001$; $\alpha = 0.05$). Plants inoculated with *T. spirale* were significantly

higher (120.4 ± 25.3 cm) followed by plants treated with *T. virens* (91.4 ± 31 cm), all other plants were similar but smaller (74.4 ± 12.82 cm) (Figure 5A).



A) plant height, B) number of fruits harvested per plant, C) percentage of *Fusarium* incidence. VT16 = *T. konongiopsis*; VT43 = *T. virens*; VT56 = *T. spirale*; CT6 = *T. harzianum*. Different letters indicate significant differences ($p < 0.05$) among treatments. Data is presented in form of means $\pm$ s.d. ($n = 12$).

Fig. 5. Effect of 4 *Trichoderma* species on tomato plants in a commercial farm greenhouse after 90 days post-transplant.

The number of harvesting fruits followed the same pattern that the plant height according to the applied treatment (Figure 5B). The number of fruits harvested in the period of the experimental trial (90 days after transplanting) was significantly higher ($p=0.04$; $\alpha=0.05$) on those plants treated with *T. spirale* (24 ± 7 fruits), followed by plants treated with *T. virens* (19 ± 4 cm), remaining treatments produced similar number of fruits as controls (16 ± 3 cm). Individual weight per fruit was not statistically different between treatments

or the control ($p=0.58$; $\alpha=0.05$), mean weight value being 18.8 ± 3.9 g per fruit.

Plants affected by *F. oxysporum* wilt differed according to the *Trichoderma* species ($p>0.001$; $\alpha=0.05$). Control plants and those inoculated with *T. harzianum* were affected by *F. oxysporum* in $22.3 \pm 2.9\%$ and $15 \pm 6.4\%$, respectively. Plants inoculated with *T. virens* and *T. konongiopsis* showed medium rates of infection ($9.7 \pm 4.3\%$ and $6.9 \pm 2.6\%$, respectively), whereas plants treated with *T. spirale* were less affected by the pathogen ($5 \pm 4.22\%$) (Figure 5C).

DISCUSSION

According to the results, in vitro antagonism tests showed an evident effect of *Trichoderma* against *F. oxysporum* fungus. This antagonistic activity has been reported in a number of studies in which authors have proposed several mechanisms to explain the biocontrol effect of *Trichoderma* (Lorito *et al.* 1993, Latunde-Dada 1993; De Boer *et al.* 2003, Howell 2003, Asran-Amal *et al.* 2005, Hoyos-Carvajal *et al.* 2008, Sempere & Santamarina 2008). Kullnig *et al.* (2000) mentioned mycoparasitism, antibiosis and competence for both space and nutrients as the most important mechanisms of *Trichoderma* species for reducing or inhibiting the growth of phytopathogenic fungi. The inhibition percentages in the present work between *Trichoderma* species tested in in vitro experiments and the pathogen indicates biocontrol potential in all *Trichoderma* species, however, *T. spirale* performed better inhibitory capacity against *F. oxysporum*.

All 4 species tested in this work, presented different levels of antagonism against *F. oxysporum*, and mycoparasitism was observed in all species. The mycoparasitic process includes localization through chemotrophic growth, recognition, adhesion to target hyphae, coiling and lysis (Chet & Inbar 1994, Bruce *et al.* 1995). *Trichoderma* species were able to deform and produce septation in *F. oxysporum* conidia, as well as to lysate and destroy conidiophores and spores. These effects may be related to enzyme productions such as chitinase, α -glucanase, cellulase, proteases, among others (Harman 2006). Similar processes were reported against *Pythium oligandrum* Dreschler, *Rhizoctonia solani* Kühn, *F. oxysporum*, *Phytophthora megasperma* Dreschler and *Pythium ultimum* Trow (Benhamou *et al.* 1999).

Antibiosis of *Trichoderma* is augmented because of the competition with other pathogens. The application of culture filtrates containing secondary metabolites produced by the 4 *Trichoderma* species in agar plates, reduced the growth of *Fusarium* mycelia. These results coincide with many other works and depend on the species tested

(Bolar *et al.* 2000, Reino *et al.* 2008, Li *et al.* 2015, Moosa *et al.* 2016).

Competition for nutrients and space, also depend on the *Trichoderma* species and the pathogen (Infante *et al.* 2009). Martínez *et al.* (2008), evaluated 59 *Trichoderma* isolates and reported a strong competition for the substrate, especially competing for nitrogen, carbohydrates and macronutrients. The beneficial effect of *Trichoderma* on the protection of tomato plants in vivo under laboratory conditions, demonstrate that *T. spirale* strain used in the experiments, protected the plants against the pathogen and also improve their agronomic variables, possibly because improving water and nutrient intake by the plants (Santander *et al.* 2003, Patel & Saraf 2017). The increased dry weight tomato plant stems and roots by different *Trichoderma* species has been reported previously (i.e. *T. koningiopsis* (Moreno *et al.* 2009), *T. harzianum* (Li *et al.* 2015) and *T. asperellum* (Li *et al.* 2018)). Altomare *et al.* (1999) established in in vitro conditions that *T. harzianum* was able to solubilize phosphates and micronutrients such as Zn and Mn in liquid cultures. In other experiments, the use of nitrogen fertilizers was reduced to 33% by applying different *Trichoderma* species (Guigón-López & González 2004, Harman 2006).

In field experiments, *T. spirale* produced best results after 90 days post-transplanting both by production and by protecting plants against *Fusarium* wilt (Figure 5). Plant height and number of harvested fruits indicated the benefit of using *T. spirale* over the other 3 used species. Kleifeld & Chet (1992) and González-Cárdenas *et al.* (2005) indicated an increase of production in tomato plants and a good control of *Fusarium* using different isolates of *T. harzianum*; however the results did not show any improvement on the measured variables when *T. harzianum* was used. Bal & Altintas (2008) also demonstrated that application of *T. harzianum* did not increase yield in tomato. On the other hand, integrated management of *Fusarium* wilt in tomatoes using cow manure and a combination of *T. viride* and *T. harzianum* produced the best inhibition and

showed lower levels of disease severity in *in vivo* experiments (Moosa *et al.* 2016). The use of *T. spirale* as biological control agent is uncommon and few reports on its biological characterization are available in the literature. Hakim *et al.* (2015) proved a high antifungal index against *Fusarium*, endophytic behavior and phosphate solubilizing effect of a *T. spirale* strain from Indonesia but in *in vitro* conditions. Conversely to the results, *T. spirale* had low effect controlling *F. oxysporum* in eggplants under fields conditions compared to *T. hamatum* and *T. virens* (Abdel-Monaim *et al.* 2014). As it has been mentioned, the responses varied according to the species/strain of *Trichoderma* and even the beneficial effect of *Trichoderma*, is also modulated by the tomato plant genotype (Tucci *et al.* 2011).

In Venezuela commercial products available are formulated almost exclusively with *T. harzianum* but in experimental trials have failed to control *Fusarium* compared to noncommercial species (Salazar *et al.* 2011). The authors attributed that lack of effectiveness of the commercial products to problems related to shelf life or in store handling, however in many cases, some *Trichoderma* isolates commercialized in the country have been imported from other latitudes and probably their performance is compromised due to different environmental conditions. According to the results, the native *T. virens* strain tested in this work showed a superior performance in both protecting and improving plants production, compared to the other species tested. More research is mandatory in order to evaluate this strain against other pathogens and crops in order to improve development of a formulated product.

ACKNOWLEDGEMENTS

To the staff of “Finca Frutiagro PTM”, to Dr. Daynet Sosa (IDEA, Venezuela) for providing the *Trichoderma* isolates and to Prof. Gioconda San-Blas (IVIC, Venezuela) for her comments and suggestions. This work was financially founded by the regular budget of the Venezuelan Institute of Scientific Research.

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