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RESEARCH PAPER

In vitro and glasshouse biocontrol of *Rhizoctonia solani* with improved strains of *Trichoderma* spp.

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Abstract

J.R. Montealegre, F. Ochoa, X. Besoain, R. Herrera, and L.M. Pérez. 2014. *In vitro* and glasshouse biocontrol of *Rhizoctonia solani* with improved strains of *Trichoderma* spp. Cien. Inv. Agr. 41(2):197-206. The potential of *Trichoderma* spp. fusants for the biocontrol of *Rhizoctonia solani* was compared with the ability of their corresponding parental strains. Their effect was tested *in vitro* using two *R. solani* strains, 509 (AG 2-1) and 618 (AG 4). The highest inhibitions in growth in dual cultures were obtained with the ThF2-1 (89.79%), ThF3-3 (90.55%), ThF4-15 (91.75%) and ThF5-8 (77.67%) fusants on *R. solani* 509; only ThF2-1 was able to inhibit the growth of *R. solani* 618 (60.19%). The inhibitory effect on growth was mainly due to diffusible metabolites. Percent mortality and canker level in tomato plants were evaluated in glasshouse experiments where all of the evaluated fusants suppressed plant mortality, but only ThF2-1 and ThF5-8 significantly decreased the canker level.

Key words: Biological control, methyl bromide, tomato, *Solanum lycopersicum.*

Introduction

Tomato (*Solanum lycopersicum* Mill.) fruit production under greenhouse conditions can be reduced by several pathogens, which thrive due to monoculture conditions that concentrate microorganisms, such as *Rhizoctonia solani*, in the soil (Latorre, 2004). *R. solani* attacks different crops, but only isolates belonging to AG 4 and AG-2-1 anastomosis groups have been found to attack tomatoes in Chile (Montealegre *et al*., 2003). The control of this pathogen still depends on methyl bromide (MeBr).

Research on alternatives to the use of agrochemicals has increased not only because of their beneficial effects for the environment but also due to global regulations and consumer demand. Biocontrol microorganisms (BCM), either wild or improved, have arisen as alternatives for the control of phytopathogens. Protoplast fusion of wild *Trichoderma* strains has allowed for new strains to be obtained (Stasz *et al*., 1988) that show increased antagonistic effects (Ogawa *et*

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al., 2000), faster growth and abundant sporulation (Prabavathy *et al.,* 2006). Specifically, antagonistic activity against *Pyrenochaeta lycopersici* has increased (Besoain *et al*., 2007), and it is important to know whether the improved strains could be used for the biocontrol of other phytopathogens.

Taking into account that *R. solani* is responsible for losses of tomato crops in Chile and considering the availability of new *Trichoderma* strains obtained by protoplast fusion (Besoain *et al*., 2007), it is important to test whether the biocontrol activity of these strains against this phytopathogen is improved compared to their corresponding parental strains. This work describes the biocontrol activity, both *in vitro* and in glasshouse conditions, of several *Trichoderma* fusant strains against the two anastomosis groups of *R. solani* that infect tomato crops in Chile.

Materials and methods

Fungal strains

R. solani strains 509 (AG 2-1) and 618 (AG 4), both of which were isolated from tomato crops in Chile and are sensitive to pencycuron, were obtained from the laboratory collection. Additionally, the following *T. harzianum* fusants obtained by protoplast fusion (Besoain *et al*., 2007) were used for the *in vitro* tests: ThF2 Series (ThVxTh291): ThF2-1,ThF2-10, ThF2-12, ThF2-18; ThF3 Series (ThVxTh11): ThF3-2, ThF3-3, ThF3-4, ThF3-6, ThF3-7; ThF4 Series (ThVxTh12): ThF4-13, ThF4-15; ThF5 Series (Th11xTh12): ThF5-8; ThF6 Series (Th12xTh291): ThF6-2. The following *T. harzianum* fusants were used for *in vivo* (glasshouse) tests: ThF2 Series: (ThVxTh291): ThF2-1, ThF2-18; ThF3 Series (ThVxTh11): ThF3-3; ThF4 Series (ThVxTh12): ThF4-15; ThF5 Series: ThF5-8 along with the parental *T. harzianum* strains, ThV, Th291, Th11 and Th12 that were used as controls.

Tomato plants

Tomato plant cultivars (cvs.) 92.95 and Gondola, which are susceptible to *R. solani*, were used for the glasshouse assays.

In vitro assays for R. solani development inhibition

Dual cultures. One disk (5 mm) containing four days culture of *R. solani* 509 or *R. solani* 618 was placed at one side of a Petri dish containing Potato Dextrose Agar (PDA - Difco), and one disk (5 mm) with four days culture of one of the fusant *Trichoderma* strains was placed at the other side of the plate and in front of the phytopathogen (Dennis and Webster, 1971a). Plates were prepared under the optimal pH conditions and then incubated at the optimal temperatures for *R. solani* 509 (pH 7.0, 20 °C) or *R. solani* 618 (pH 6.5, 22 °C). Controls were run on PDA plates inoculated with either *R. solani* 509 or *R. solani* 618 and mock-inoculated with a 5 mm disk of PDA. The growth diameter of the pathogen in the controls was measured with a ruler and compared to its corresponding growth in the presence of the fusant *Trichoderma* strain over the same time period as the controls. Percent inhibition of radial growth (IRG) was established using the Dennis and Webster formula (1971a): $IRG\ (\%) = (R_1 - R_2)\ 100/\ R_1$, where R_1 is the farthest distance covered by the pathogen in the control plates, and R_2 is the distance covered by the fusant towards the antagonist. Each experiment was run in five replicates and repeated twice. The results (as percentages) are presented as the mean for each treatment and were first transformed using the Bliss Angular Transformation before testing with an ANOVA. The Tukey's test was used if significant differences were obtained ($P \leq 0.05$).

Effect of volatile metabolites

A 5 mm disk of pure culture of *R. solani* 509 or *R. solani* 618 or one of the *T. harzianum* fusants

was placed in the middle of a Petri dish containing PDA. One half plate containing one of the T. *harzianum* fusant strains and another containing one of the *R. solani* strains were placed face to face to avoid any physical contact between the fungi (Dennis and Webster, 1971b). The half plates were sealed to isolate the atmosphere inside and prevent the loss of the volatiles formed. Plates were incubated at the same temperature and pH conditions as described for the dual cultures above. Controls were run with one half plate containing one of the pathogen strains while the other was mock-inoculated with a 5-mm disk of PDA. The growth diameter of the pathogen in the control treatment was measured and compared to those in the presence of the corresponding fusant *Trichoderma* strain. Percent IRG was established as IRG (%) = (1 – R2/ R1) 100, where R1 is the distance covered by the pathogen in the controls, and R2 is the distance covered by the pathogen in its half plate in the presence of the antagonist in the other half plate. Each experiment was run in five replicates and was repeated twice. The results (as percentages) are the mean of each treatment and were first transformed using the Bliss Angular Transformation before testing with an ANOVA. The Tukey's test was used if significant differences were obtained ($P \leq 0.05$).

Effect of diffusible metabolites

A 5 mm disk of each of the antagonistic *Trichoderma* strains was inoculated in the middle of Petri dishes containing PDA covered with a sterile dialysis membrane (3.0 in. Sigma-Aldrich). After incubation for 72 h at 28 °C, the membrane with the *Trichoderma* was removed, and the plate was inoculated in the middle with a 5-mm disk of pure *R. solani* 509 or *R. solani* 618 culture. Plates were further incubated at the same temperatures and pH conditions as for the dual cultures. Controls were run with mock-inoculated plates using a 5-mm disk of PDA on the sterile dialysis membrane, and after removing the membrane, the plates were inoculated with one of the *R. solani* strains. The growth diameter of the pathogen in the controls was measured with a ruler and compared to that of the pathogen on the PDA where the corresponding fusant *Trichoderma* strain was previously cultured after the membrane was removed. Percent IRG was established as IRG (%) = (1 – R2/ R1) 100, where R1 is the distance covered by the pathogen in the controls, and R2 is the distance covered by the pathogen in the plates where the antagonist was previously cultured. Each experiment was run in five replicates and was repeated twice. The results (as percentages) are the mean of each treatment and were first transformed using the Bliss Angular Transformation before testing with an ANOVA. The Tukey's test was used if significant differences were obtained ($P \leq 0.05$).

In vivo effect of biocontrol strains on the development of crown and root canker caused by R. solani

The *in vivo* experiments considered five *T. harzianum* strains that were selected based on the results obtained in *in vitro* conditions. Their innocuousness on tomato plants cvs. 92.95 and Gondola was previously checked (Besoain *et al*., 2007) to test their effectiveness for the biocontrol of tomato crown canker caused by *R. solani* 618 (AG 4). The selection of this pathogen strain was based on its aggressiveness, pathogenicity and ability to infect different crops (Anderson, 1982; Sneh *et al.*, 1991).

Glasshouse assays were run using *Trichoderma* strains contained in alginate pellets (Montealegre and Larenas, 1995). The parental strains (ThV, Th291, Th11, and Th12) were used for comparison. Once fungal innocuousness was established, tomato seedlings of cvs. 92.95 and Gondola were transplanted to 2.3 L pots containing sterile soil inoculated with *R. solani* 618 (21.0 g inoculum/pot; 8.0×10^5 colony forming units (cfu) g^{-1} inoculum) grown on sterile oat seeds (Santander *et al*., 2003). The inoculum was introduced close to where the root pan and the crown of the plant were to be

placed. The concentration of the inoculum was established after grounding the oat seeds and further serial dilutions in selective culture medium. All of the experiments used 1.7 g pellets/plant [1.75 x 10^5 – 8.7 x 10^5 cfu g^{-1} pellet] of the different *Trichoderma* strains that were placed inside the hole before transplantation. Each treatment had five replicates and was repeated five times. Controls were considered to be the inoculated soil a) in the absence of any *Trichoderma* strain and commercial fungicide and b) in the presence of 0.15 mL pot^{-1} of commercial fungicide, pencycuron (liquid suspension, 250 g active compound L^{-1}). Each pot contained one tomato plant of cv. 92.95 or cv. Gondola. Plants were randomly arranged and maintained under glasshouse conditions until flowering. They were then analyzed for crown canker levels as in Montealegre *et al*. (2010) and for % mortality of the tomato plants induced by *R. solani*. The results were analyzed by ANOVA (95% confidence) followed by the Tukey's test if significant differences were detected. Canker levels were analyzed by the Kruskal and Wallis non-parametric test and the Mann-Whitney test for comparing pairs (95% confidence).

Results

In vitro assays for R. solani development inhibition

Dual cultures. The percent inhibition of *R. solani* strains 509 and 618 in dual cultures by the *Trichoderma* strains resulting from different protoplast fusions is shown in Table 1.

Fusant strains from the ThF2 series (ThVxTh291) showed improved ability to inhibit *R. solani* 509 (ThF2-1, ThF2-10 and ThF2-18) or *R. solani* 618 (ThF2-1 and ThF2-18) growth in relation to one of their parental strains. ThF2-1 and ThF2-18 were those that were significantly improved in their biocontrol of *R. solani* 509 over ThV as was the biocontrol activity of Th291 for *R. solani* 618.

Replacing Th291 in the protoplast fusion of (ThVxTh291) with Th11 or Th12 to obtain series ThF3 (ThVxTh11) and ThF4 (ThVxTh12) fusants resulted in the ThF3-3, ThF3-2 and ThF4-15 strains, which were better inhibitors than both

Table 1. Inhibitory effect of the ThF2, ThF3, ThF4, ThF5 and ThF6 series of *Trichoderma* strains from protoplast fusion on *R. solani* 509 (AG 2-1) and *R. solani* 618 (AG 4) in dual cultures.

	% Growth inhibition			% Growth inhibition	
A. Strains from series ThF2 ThV x Th291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	B. Strains from series ThF3 ThV x Th11	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
ThF2-1	89.79 a	60.19 a	ThF3-3	90.55 a	55.81 a
ThF2-10	89.02 a	53.46 ab	ThF3-2	86.61 a	51.42 a
ThF2-18	84.50 ab	54.68 a	ThF3-4	74.79 ab	49.16 a
T. h 291[1]	82.96 a	44.69 b	ThF3-6	69.13 ab	50.43 a
ThF2-12	53.77 bc	54.02 ab	ThF3-7	68.39 ab	52.58 a
T. h V [1]	47.47 c	53.98 ab	*T. h* V [1]	47.47 b	53.98 a
			T. h 11[1]	47.41 b	55.09 a
C. Strains from series ThF4 ThV x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	D. Strains from series ThF5 Th11 x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
ThF4-15	91.75 a	49.90 a	ThF5-8	77.67 a	44.46 b
T. h 12[1]	49.05 b	53.42 a	*T. h* 12[1]	49.05 b	53.42 a
ThF4-13	48.98 b	30.62 b	*T. h* 11[1]	47.41 b	55.09 a
T. h V [1]	47.47 b	53.98 a			
E. Strains from series ThF6 Th12 xTh291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)			
T. h 291[1]	44.39 a	13.17 a			
ThF6-2	28.29 b	2.44 b			
T. h 12[1]	27.80 b	8.78 a			

[1]Parental strain. Different letters in columns indicate significant differences after Tukey's test at P≤0.05.

parental strains but only towards *R. solani* 509. In contrast, the replacement of ThV with Th12 in the protoplast fusion of (ThVxTh291) resulted in ThF6-2 (Th12xTh291), which showed a decreased ability to control *R. solani* 509 or *R. solani* 618 when compared to Th291 or both parental strains, respectively. Finally, the inhibitory activity of ThF5-8 (Th11xTh12) towards *R. solani* 509 was significantly improved, but it decreased toward *R. solani* 618.

Volatile metabolites

The percent inhibition of *R. solani* 509 and *R. solani* 618 by the volatile metabolites secreted by the different *Trichoderma* strains resulting from protoplast fusion is shown in Table 2. In general, no improvement was observed in the inhibitory effect of volatiles on *R. solani* 509 or *R. solani* 618 in the cultures with the exception of ThF2-12 on *R. solani* 509 when compared to the parental strain ThV. In addition, most of these strains showed no inhibitory effect on *R. solani* 618 (Table 2).

Diffusible metabolites

The inhibition of *R. solani* 509 and *R. solani* 618 growth by diffusible metabolites secreted by the different *Trichoderma* strains resulting from protoplast fusion was higher than 80% for most of the strains, as shown in Table 3. In general, the improvement of the inhibitory effect of the different *Trichoderma* strains on *R. solani* 509 and *R. solani* 618 was due to one of the parental strains with the exception of three strains from the ThF2 series, ThF2-1, ThF2-12 and ThF2-18 (Table 3), whose results were better than both parents for the control of *R. solani* 509.

In vivo effect of biocontrol strains on the development of crown and root canker caused by R. solani

The fusants with the best biocontrol activity against *R. solani* 509 and/or 618 in the diffusible metabolite assays, ThF2-1, ThF2-18, ThF3-3, ThF4-15 and ThF5-8 (Table 3), were innocuous to tomato plant cvs. 92.95 and Gondola (data not shown) and were used in these trials.

Table 2. Inhibitory effect of the volatiles secreted by the ThF2, and ThF3, ThF4, ThF5, and ThF6 series of the *Trichoderma* strains from protoplast fusion on *R. solani* 509 (AG 2-1) and on *R. solani* 618 (AG 4).

	% Growth inhibition			% Growth inhibition	
A. Strains from series ThF2 ThV x Th291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	B. Strains from series ThF3 ThV x Th11	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
ThF2-12	31.88 a	14.15 a	ThF3-2	37.00 a	1.46 b
T. h 291[1]	28.75 a	13.17 a	ThF3-6	30.24 a	0.00 b
ThF2-10	16.88 ab	0.00 b	ThF3-3	29.76 a	0.00 b
ThF2-1	16.25 ab	0.00 b	ThF3-7	27.81 a	0.00 b
ThF2-18	8.13 b	0.00 b	*T. h* V [1]	26.83 a	7.32 a
T. h V [1]	6.25 b	7.32 a	*T. h* 11[1]	26.34 a	1.95 b
			ThF3-4	25.00 a	0.00 b
C. Strains from series ThF4 ThV x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	D. Strains from series ThF5 Th11 x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
ThF4-15	28.78 a	0.00 b	*T. h* 12[1]	27.80 a	8.78 a
T. h 12 [1]	27.80 a	8.78 a	ThF5-8	26.83 a	0.00 c
T. h V [1]	26.83 a	7.32 a	*T. h* 11[1]	26.34 a	1.95 b
ThF4-13	23.90 a	2.44 b			
E. Strains from series ThF6 Th12xTh291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)			
T. h 291[1]	44.39 a	13.17 a			
ThF6-2	28.29 b	2.44 b			
T. h 12[1]	27.80 b	8.78 a			

[1]Parental strain. Different letters in columns and rows indicate significant differences after Tukey's test at $P \leq 0.05$.

The canker level in control tomato plants inoculated with *R. solani* 618 was higher in cv. 92.95 than in cv. Gondola; no significant decrease in the disease level of any cv. was achieved with the use of the chemical fungicide (Table 4). ThF2-1 and ThF5-8 were the only fusants that significantly reduced the damage produced by *R. solani* 618 in control and pencycuron-treated cv. 92.95 tomato plants, but they showed no significant differences with their corresponding parental strains. However, all fusants showed the ability to suppress plant mortality which differed from their parental strains and the chemical fungicide (Table 4 A). On the other hand, ThF2-18, ThF4-15 and ThF5-8 significantly decreased the level of canker produced by *R. solani* 618 in the control and pencycuron-treated cv. Gondola tomato plants (Table 4B). In addition, all fusants suppressed mortality in this tomato cv. (Table 4B). ThF5-8 was the only fusant that decreased the canker level in both cvs. as its biocontrol activity was not significantly different from its parental strains.

Discussion

The growth inhibition observed in *R. solani* strains 509 (AG 2-1) and 618 (AG 4) in dual cultures by the wild *T. harzianum* strains Th291, ThV, Th11 and Th12 (Table 1) is in agreement with the findings of Küçük and Kivanç (2003), and the same result occurred for all the *Trichoderma* fusants tested. However, only ThF2-1 and ThF2-18 (Th291xThV) showed improved biocontrol activity when compared to one or both parental strains. Thus, the protoplast fusion between Th291 and ThV resulted in two fusant strains with the ability to biocontrol both *R. solani* anastomosis groups in dual cultures. The replacement of Th291 by Th11 or Th12 produced only two and one fusants, respectively, with improved biocontrol activity towards *R. solani* 509 in dual cultures (Table 2, B and C). This suggests that protoplast fusion between ThV and Th11 or Th12 resulted in the loss of the ability to biocontrol *R. solani* 618.

Table 3. Inhibitory effect of the diffusible metabolites secreted by the ThF2, and ThF3, ThF4, ThF5, and ThF6 series of the *Trichoderma* strains from protoplast fusion on *R. solani* 509 (AG 2-1) and on *R. solani* 618 (AG 4).

	% Growth inhibition			% Growth inhibition	
A. Strains from series ThF2 ThV x Th291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	B. Strains from series ThF3 ThV x Th11	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
ThF2-12	89.60 a	77.38 b	*T. h* 11[1]	98.48 a	89.82 a
ThF2-1	88.53 a	88.36 a	ThF3-7	97.55 a	81.07 b
ThF2-18	82.62 a	87.29 a	ThF3-2	88.40 b	85.60 ab
T. h 291[1]	77.24 b	75.24 b	ThF3-4	78.93 b	88.27 ab
ThF2-10	65.38 c	75.11 b	ThF3-3	78.84 b	91.73 a
T. h V[1]	64.13 c	89.38 a	ThF3-6	77.82 b	53.29 c
			T. h V[1]	64.13 c	89.38 a
C. Strains from series ThF4 ThV x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)	D. Strains from series ThF5 Th11 x Th12	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)
T. h 12[1]	91.73 a	99.11 a	ThF5-8	100.00 b	96.40 b
ThF4-13	91.64 a	97.07 a	*T. h* 11[1]	98.48 b	89.82 c
ThF4-15	70.26 b	83.78 b	*T. h* 12[1]	91.73 a	99.11 a
T. h V[1]	64.13 b	89.38 b			
E. Strains from series ThF6 Th12 xTh291	*R. solani* 509 (AG 2-1)	*R. solani* 618 (AG 4)			
T. h 12[1]	91.73 a	99.11 a			
ThF6-2	90.04 a	85.82 b			
T. h 291[1]	77.24 b	75.24 c			

[1]Parental strain. Different letters in columns and rows indicate significant differences after Tukey's test at P≤0.05.

Table 4. Effect of different mutants on canker level and mortality of tomato plants, cv. 92.95 (A) and Gondola (B), inoculated with *R. solani* 618 under glasshouse conditions.

A. cv. 92.95	Canker level[1,2]		Mortality, %	B. cv. Gondola	Canker level[1,2]		Mortality, %
Control	3	c	100	Control	5	e	80
Pencycuron	2.8	bc	80	Pencycuron	3	de	80
Th12[3]	1	ab	20	Th12[3]	2.7	cde	20
Th291[3]	0.8	ab	20	Th291[3]	1.5	abcd	10
ThV[3]	2.4	abc	10	ThV[3]	1.8	abcd	10
Th11[3]	2.3	abc	10	Th11[3]	2.5	bcd	20
ThF2-18	2	abc	0	ThF2-18	1	abc	0
ThF3-3	1.8	abc	0	ThF3-3	2.2	abcd	0
ThF4-15	1.2	abc	0	ThF4-15	0.4	a	0
ThF2-1	0.6	a	0	ThF2-1	1.8	abcd	0
ThF5-8	0.6	a	0	ThF5-8	0.6	abc	0

[1]Crown canker (size of area showing lesions in crown related to stem perimeter) was assessed using the following scale to establish the degree of the disease: 0: 0% area affected, healthy plant; 1: < 1% area affected, slight disease; 2: 5-30% affected area, moderate disease; 3: 30-60% area affected, significant disease; 4: 60-90% area affected, severe disease; and 5: > 90% area affected, dead plant.
[2]Identical letters in columns indicate that no significant differences were found after analysis by ANOVA (95% confidence). ANOVA was followed by the Tukey's test only when significant differences were detected. Canker parameters were analyzed by the Kruskal-Wallis non-parametric test with pair comparison by the Mann-Whitney test (95% confidence).
[3]Parental strains.

Protoplast fusion, whether interspecific (Mohamed and Haggag, 2010) or intraspecific (Besoain *et al*., 2007; Fahmi *et al*., 2012), is universally applied as a useful tool for improving fungal strains. Nevertheless, as gene recombination occurs randomly, fusants can improve, maintain or lose the properties of their parental strains, which supports our findings and those of other groups.

The number of fusant *Trichoderma* strains that improved their inhibitory effect on the growth of *R. solani* 509 (AG-2-1) was always higher than those inhibiting *R. solani* 618 (AG-4). Controlling the AG-4 strains has been described as more difficult because of their pathogenesis and aggressiveness (Anderson, 1982; Sneh *et al*., 1991) as has been previously shown (Anderson, 1977). Thus, results from *in vitro* experiments could reflect this difficulty. Additionally, it is important to mention that most of the fusants were better inhibitors than at least one of their corresponding parents in dual cultures, which supports the goal of obtaining improved strains from wild ones (Rey *et al*., 2000). Similar results have been reported by Sivan and Harman (1991) for *T. harzianum* 1295-22, a product of protoplast fusion (T12×T95) that was more effective for rhizosphere competition, by Besoain *et al.* (2007), who reported that several protoplast fusion strains improved their inhibitory effect on *P. lycopersici* and also showed a higher growth rates than their corresponding parents, and by Ahmed *et al.* (2007), who reported that several strains showed improved chitinase activity and reduced *R. solani* growth, among other pathogens tested, *in vitro*,.

The inhibitory activity of the *Trichoderma* strains tested in dual cultures on *R. solani* 509 and on *R. solani* 618 may be explained as competition for nutrients and space among other resources, without modifying pH, according to results obtained *in vitro* by other authors (Howell, 2003; Benítez *et al*., 2004).

The inhibition of *R. solani* 509 and 618 growth due to volatile metabolites was negligible except for fusant ThF2-12 on *R. solani* 509 (Table 3), which suggests that, in the latter case, these types

of compounds might be involved in the biocontrol of this pathogen. In opposition to our results are those of Küçük and Kivanç (2003), which showed that the *Trichoderma* T9 strain controlled the development of *R. solani,* or those of Besoain *et al.* (2007), in which a protoplast fusion product inhibited *P. lycopersici* development; volatile metabolites were involved in both cases. Nevertheless, it is important to consider that strain improvement by protoplast fusion could result in significant differences among the new strains as gene recombination could increase or decrease the secretion of volatile metabolites (Hanson, 2002).

On the other hand, most of the *Trichoderma* strains tested in this work caused growth inhibition of more than 80% in *R. solani* 509 and *R. solani* 618 through diffusible metabolites (Table 3), which is supported by previous reports (Küçük and Kivanç, 2003) of some *T. harzianum* isolates completely inhibiting *R. solani* development. The high degree of growth inhibition of *R. solani* 509 and *R. solani* 618 by all of the strains (Table 3) suggests that the inhibitory effect observed in dual cultures (Table 1) could mostly be attributed to diffusible metabolites. These are low molecular weight antibiotics (Benítez *et al*., 2004) that, in combination with hydrolytic enzymes, result in antagonistic levels (Rey *et al*., 2000) higher than could be reached separately. Moreover, low molecular weight enzymes could diffuse through dialysis membranes in the same way as antibiotics and thus produce inhibitory effects higher than those observed in dual cultures.

The effect of diffusible metabolites on pathogen development has been reported by several authors. In fact, Howell (2003) established that the biocontrol of *Pythium* spp. by *T. harzianum* T-12 and *T. koningii* T-8 was not due to mycoparasitism but to toxic metabolites that inhibited the pathogen's growth. In addition, the author also established that a *T. virens* mutant (deficient in mycoparasitism and in gliotoxin synthesis) was as efficient as the parental strain (G-6) in the control of *P. ultimum* and *R. solani*. The use of fungal culture filtrates as an alternative for testing the effect of diffusible metabolites from inter-specific protoplast mutants has also been reported (Mohamed and Haggag, 2010), and the results support the inhibition of pathogen growth by these types of metabolites.

A comparison of the results from the dual cultures and diffusible metabolites experiments (Tables 1 and 3) allowed us to see a higher biocontrol effect on *R. solani* 509 (AG 2-1) than on *R. solani* 618 (AG-4) by most of the *Trichoderma* strains tested in dual cultures (Table 1). These results were not observed in the strongest biocontrol results for diffusible metabolites (Table 3) where no significant differences were observed between the growth inhibition of *R. solani* 509 and 618 for most of the fusant strains. Therefore, and based in the diffusible metabolites results, ThF2-1, ThF2-18, ThF3-3, ThF4-15 and ThF5-8 were selected to develop the glasshouse, *R. solani* 618 control experiments. The pathogen was chosen because of its importance in terms of pathogenesis, aggressiveness, and number of target plant species (Anderson, 1982; Sneh *et al*., 1991), and the two tomato cvs., 92.95 and Gondola, differ in their susceptibility to the pathogen.

Two of the selected fusants, ThF2-1 (ThV×Th291) and ThF5-8 (Th11×Th12), were able to significantly reduce the damage produced by *R. solani* 618 in both tomato cvs. It is obvious that the susceptibility of the plant to the pathogen has no relation to the activity of the biocontrol agent. In addition, the fact that two of the five selected fusants were able to control *R. solani* 618 agrees with results obtained by Fahmi *et al*. (2012), who tested for the ability of protoplast fusion mutants of *T. harzianum* strains tolerant to fungicides to reduce tomato damping-off in greenhouse assays. They showed that only one of the seven selected mutants, which showed improved biocontrol activity *in vitro* compared to their parental strains, was capable of reducing the disease in plants. Additionally, the chemical treatment of *T. harzianum* resulted in few mutants with improved ability to control

the stem blight of soybean in glasshouse assays (Singh and Upadhyay, 2009) or tomato crown canker (Montealegre *et al*., 2010). Thus, methods involving random gene recombination, such as protoplast fusion or unspecific gene alteration as occurs in chemical mutation, will result in a high number of mutants, of which only a few will show improved biocontrol characteristics in *in vitro* experiments and even fewer in glasshouse experiments when compared to their parental strains.

Mortality suppression by all the fusants tested in the two tomato cvs. did not correlate with their ability to reduce canker level, which suggests that the suppression of plant mortality is not necessarily associated with biocontrol. Further studies will be necessary to elucidate this point.

In summary, several fusant *Trichoderma* strains obtained through protoplast fusion improved the biocontrol ability of at least one of the wild *T. harzianum* parents in the *in vitro* experiments. However, only some fusants repeated this behavior once the assays were run in glasshouse conditions. Thus, specific characteristics of the fusants would most likely be interacting with *R. solani* and/or with a specific tomato cv., a fact that must be taken into account before recommending a general use of a BCM.

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Resumen

J.R. Montealegre, F. Ochoa, X. Besoain, R. Herrera y L.M. Pérez. 2014. Biocontrol de *Rhizoctonia solani in vivo* y en invernadero, con cepas mejoradas de *Trichoderma spp.* Cien. Inv. Agr. 41(2):197-206. Se comparó la capacidad de cepas de *Trichoderma* spp., obtenidas previamente por fusión de protoplastos, con la de sus correspondientes cepas parentales, para biocontrolar a *Rhizoctonia solani*. El efecto biocontrolador se analizó sobre dos cepas de *R. solani*: 509 (GA 2-1) y 618 (GA 4). Las cepas producto de fusión de protoplastos de *Trichoderma* spp. fueron más efectivas que al menos una de las correspondientes cepas parentales. La inhibición más alta en experimentos de cultivos duales, se observó con las cepas ThF2-1 (89,79%), ThF3-3 (90,55%), ThF4-15 (91,75%) y ThF5-8 (77,67%) sobre *R. solani* 509; mientras que el efecto biocontrolador sobre *R. solani* 618, sólo logró un 60,19% de inhibición con la cepa ThF2-1. El efecto inhibidor del desarrollo se debió principalmente a la secreción de metabolitos difusibles. El porcentaje de mortalidad y nivel de cancro en plantas de tomate se evaluó en experimentos de invernadero, en los que todas las cepas producto de la fusión de protoplastos de *Trichoderma* spp. suprimieron la mortalidad de plantas de tomate, y solamente ThF2-1 y ThF5-8 disminuyeron significativamente el nivel de cancro.

Palabras clave: Bromuro de metilo, control biológico, *Solanum lycopersicum,* tomate.

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