RESEARCH NOTE

Sensitivity of wild-type and mutant *Trichoderma harzianum* strains to fungicides

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Abstract

R. Herrera, D. Nuñez, N. Romero, X. Besoain, L. M. Pérez, and J. Montealegre. 2012. Sensitivity of wild-type and mutant *Trichoderma harzianum* strains to fungicides. *Cien. Inv. Agr.* 39(3): 569-576. The germination of conidia in wild-type (Th11, Th12 and Th650) and mutant (Th11A80.1, Th12A10.1 and Th650-NG7) strains of *Trichoderma harzianum* that were exposed to different commercial fungicides was studied. All wild-type and mutant *Trichoderma* strains were germinated in the presence of 1,700 mg L⁻¹ of penicuron. The wild-type strains Th12 and Th650 and the corresponding mutant strains Th12A10.1 and Th650-NG7 were sensitive to all concentrations of iprodione and metalaxil + mancozeb. The EC₅₀ (Median Effective Concentration) values for the different fungicides were between 10⁻¹ and 10⁻⁴ less than the concentrations recommended for field applications; one exception was Phyto-fos on Th650-NG7, where this ratio was 0.72. These results establish whether some of these fungicides can be used in combination with the biocontrol agents evaluated.

Key words: biocontrol, biocontrol agents, control of tomato root diseases, fungicides, fungicide tolerant *Trichoderma*, integrated management.

Introduction

The *Trichoderma* species are fungi commonly found in some suppressive soils (Weller *et al.*, 2002) and are some of the most important microorganisms employed as biological control agents of plant diseases. The ability of these fungi to

parasitize and antagonize several phytopathogenic fungi is accomplished via different mechanisms: competence for space and nutrients, production of antibiotics and lytic enzymes, modification of environment conditions and promotion of the growth of plants and their defense mechanisms (Howell, 2003; Howell, 2005; Harman, 2006). Beneficial microorganisms in the soil and within the plant are also important for plant production. The maintenance of these microorganisms can be affected by conventional disease control practices

(Tilman *et al.*, 2002). In this study, we investigated alternative agricultural practices and fungicide substitutes that minimize negative effects of some agricultural practices, prevent environmental pollution and contribute to produce innocuous foods.

Although biocontrol agents (BCAs) can delay the onset of a disease or partially suppress it, they rarely are as effective as chemical fungicides. This is a challenge in the development of new disease control alternatives. The simultaneous use of BCAs and fungicides is one alternative for the control of plant diseases. To be used as complementary tools in biocontrol, fungicides must be used at concentrations lower than those used for controlling plant diseases so that BCAs and/or plant beneficial microorganisms are not negatively impacted.

The use of integrated biological and chemical control methods is an established sustainable strategy for plant disease control in the field (Elmer and McGovern, 2004). Fungal strains may differ in their sensitivity to fungicides, as previously described in fungicide-resistance studies of microorganisms (Figueras-Roca *et al.*, 1996). More efficient BCAs have improved antagonistic activity and/or the spectrum of this activity and have remained active in the presence of fungicides (Arias *et al.*, 2006).

The tomato root diseases caused by *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium solani*, *Phytophthora nicotianae*, *Pyrenochaeta lycopersici* and *Rhizoctonia solani* are controlled in Chile by several fungicides (AFIPA, 2006). Previous experiments have shown that certain wild-type and mutant *Trichoderma* strains can control some of these pathogens *in vitro* and in greenhouse conditions (Pérez *et al.*, 2002; Arias *et al.*, 2006; Besoain *et al.*, 2007). Formulations containing these strains are stable; i.e., they retain full *R. solani* biocontrol activity after six months without reductions in the CFU count of the formulation (Montealegre *et al.*, 2009). It is not known whether these *Trichoderma* strains are sensitive to the fungicides used to control those plant pathogens.

Here, we describe the *in vitro* sensitivity of the wild-type and mutant strains of *Trichoderma* previously obtained by our research group (Besoain *et al.*, 2007; Pérez *et al.*, 2007) to different fungicides commonly used to control *F. oxysporum* f. sp. *lycopersici*, *F. solani*, *P. nicotianae*, *P. lycopersici* and *R. solani*. We focused on select fungicide-tolerant *Trichoderma* strains used as part of an integrated plant management and control program that included the simultaneous use of BCAs and fungicides.

Materials and methods

Fungal strains

The fungal strains used were the *T. harzianum* mutant strains Th650-NG7, Th11A80.1 and Th12A.10.1, which had been obtained previously (Besoain *et al.*, 2007; Pérez *et al.*, 2007), and their parental strains Th650, Th11 and Th12. All isolates were cultivated in potato-dextrose-agar, and conidia were produced as described previously (Pérez *et al.*, 2002).

Fungicides

The fungicides used for evaluating the sensitivities of the *T. harzianum* strains were obtained from commercial companies and tested at the concentrations indicated in Table 1. The highest active ingredients (a.i.) concentrations tested were the highest concentrations recommended (AFIPA, 2006) for the control of root and crown rot pathogens of tomatoes in the field.

Conidial germination in fungicide containing media

Conidial germination was established by cultivating 1×10^5 conidia of each *Trichoderma* strain on sterile Petri dishes containing 2.25% of glucose-agar and increasing doses of each fungicide. For the

Table 1. The commercial fungicides used to test conidial germination sensitivity in wild-type and mutant strains of *Trichoderma harzianum* strains.

Fungicide	Concentrations tested (a.i.) ¹	Manufacturer	Recommended for ²
Thiabendazole	0 to 1,220 mg L ⁻¹	Syngenta Agribusiness S.A.	<i>Rhizoctonia</i> spp., <i>Fusarium</i> spp. and <i>Sclerotium</i> spp.
Pencycuron	0 to 1,375 mg L ⁻¹	Bayer AG	<i>R. solani</i>
Sodium tetrathiocarbamate	0 to 1,200 mg L ⁻¹	Bayer AG	Nematicide with fungicide effect
Iprodione	0 to 2,448 mg L ⁻¹	Bayer AG	<i>Rhizoctonia</i> spp., <i>Sclerotium</i> spp., <i>Sclerotinia</i> spp., <i>Penicillium</i> spp., <i>Botrytis cinerea</i> and <i>Alternaria</i> spp.
Phosetyl aluminum	0 to 2,400 mg kg ⁻¹	Bayer AG	<i>Phytophthora</i> spp. and Oomycotas
Iprodione plus captan	0 to 1,695 mg kg ⁻¹	Bayer AG	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp. and <i>Sclerotinia</i> spp.
Potassium phosphite	0 to 1,725 mg L ⁻¹	Químicas ASSEL S.A	<i>Phytophthora</i> spp. and Oomycotas
Metalaxil plus mancozeb	0 to 1,930 mg L ⁻¹	Zhejiang Chemicals Exp. Imp. Co.	<i>Phytophthora</i> spp., <i>Phyium</i> spp. and <i>Alternaria</i> spp.
Hymexazol	0 to 700 mg L ⁻¹	Sankyo Co.	<i>F. oxysporum</i> f. sp. <i>lycopersici</i> , <i>Pythium</i> spp., <i>Aphanomyces</i> spp.

¹a.i. Active ingredient. The highest concentrations correspond to those used at the field level.²AFIPA (2006).

nematicide-fungicide sodium tetrathiocarbamate, *Trichoderma* conidia were cultivated in liquid dextrose (18 g L⁻¹) and boiling potato extract (200 g L⁻¹); sealed Petri dishes were used to prevent loss during incubation and to provide an adequate availability of the chemical product. The fungicide concentration was increased as required in a second test to calculate the corresponding EC₅₀ (50% effective concentration) value.

Plates were incubated at 22 °C for 24 h, and the percentages of germinated conidia were determined using a microscope. Germinated conidia had a germinating tube length five times the conidia diameter or greater. The results for each *Trichoderma* strain are the mean of four replications of two independent experiments and are expressed as an EC₅₀ (effective mean concentration) value. The EC₅₀ values were calculated using the Probit regression of

the germination inhibition percentage versus the logarithm of the fungicide concentration (Figueras-Roca *et al.*, 1996). Conidia were considered sensitive if the EC₅₀ value of a given fungicide was lower than the highest recommended concentration used in the field. Conidia were considered resistant if the EC₅₀ value of a given fungicide was higher than the recommended field concentration. Ratios of the EC₅₀ values of different fungicides against mutant and wild-type strains were calculated; these ratios were used to determine whether the mutant strain had improved resistance compared to their corresponding wild-type strain.

Results and discussion

The germination rates of all strains in the presence of different agrochemicals are discussed in

relation to each specific fungicide. Variability is based on the specific characteristics of each strain, which agrees with the results obtained by Figueras-Roca *et al.* (1996).

Conidial germination in the presence of fungicides

The inhibitory effect of the different fungicides were evaluated on conidial germination of wild-type and mutant *Trichoderma* strains and are described as EC₅₀ values, as shown in Table 2.

Pencycuron [N-((4-chlorophenyl)-methyl)-N-cyclopentyl-N'-phenyl urea] was the only fungicide that did not inhibit the conidial germination of any *Trichoderma* wild-type or mutant strains. No effect on *Trichoderma* wild-type or mutant strains was observed *in vitro* with this fungicide at a.i. concentrations of 1,375 mg L⁻¹ and 1,700 mg L⁻¹ (higher than the commercial doses). Pencycuron has been described as a non-systemic phenyl urea fungicide with specific action against diseases caused by *R. solani* and *Pellicularia* spp. in several horticulture plants. The mode of action of pencycuron on *R. solani* is not understood, although the destruction of microtubules in the hyphal tips of the fungus

has been previously described (Ueyama and Kurahashi, 2007). The non-inhibitory effect on the fungal β -tubulin assembly and the change in the osmotic stability and fluidity of the plasma membrane in pencycuron-sensitive *R. solani* isolates has been reported. The inhibition of giant protoplast formation and late membrane lipid peroxidation are secondary effects of pencycuron (Kim *et al.*, 2001).

Conidial germination in the wild-type strains Th11, Th12, Th650 and their corresponding derived mutants Th11A80.1, Th12A10.1 and Th650-NG7 was inhibited by all other fungicides (Table 2) at concentrations lower than those recommended for use at the field level (AFIPA, 2006), with one exception. The strain Th11 was not inhibited by Phyto-fos at any of the tested concentrations. Phyto-fos is a fertilizer containing neutralized phosphorous acid, a known fungicidal compound (Guest and Grant, 1991), and is recommended for the control of *Phytophthora* spp. and *Oomycotas fungi* (AFIPA, 2006). The mechanisms of growth inhibition in *Phytophthora* spp. are related to key phosphorylation reactions and the inhibition of adenylate synthesis, as well as the alteration of nucleotide pools and the metabolism pentose phosphate (Varadarajan *et al.*, 2002).

Table 2. Median effective concentration (EC₅₀) values of different fungicides against the conidial germination of wild-type and mutant *Trichoderma* strains.

Fungicide	EC ₅₀ (mg kg ⁻¹ or mg L ⁻¹ of active ingredient)					
	Th11	Th11A80.1	Th12	Th12A10.1	Th650	Th650-NG7
Pencycuron	n.i. ¹	n.i.	n.i.	n.i.	n.i.	n.i.
Thiabendazole	1.80	1.80	0.63	1.70	2.94	6.00
Iprodione	522.40	2.00	S ²	2.30	S ²	17.30
Sodium tetrathiocarbamate	125.60	33.00	97.10	47.80	35.30	9.20
Phosetyl aluminum	75.80	1,043.20	90.30	826.70	87.30	458.80
Potassium phosphite	n.i.*	342.90	24.60	369.60	43.40	12,503.10
Metalaxil plus mancozeb	2.47	S ²	2.90	S	2.96	S
Hymexazol	509.60	135.00	506.20	195.70	365.20	4,793.70
Iprodione plus captan	7.90	1.40	6.90	4.70	7.80	15.10

¹n.i. No inhibition. 100% conidial germination was obtained at all fungicide concentrations tested.

²S: sensitive strains. Conidial germination was inhibited in all strains and at all fungicide concentrations tested.

The dicarboximide fungicide iprodione [3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioximidazolidine-1-carboxamide] controls *Rhizoctonia* spp., *Sclerotium* spp., *Sclerotinia* spp., *Botrytis cinerea*, *Penicillium* spp. and *Alternaria* spp. (AFIPA, 2006). It is described as an inhibitor of lipid and membrane synthesis. Iprodione inhibited conidial germination in the wild-type *Trichoderma* strains Th12 and Th650 at all concentrations tested. The derived mutant strains were less sensitive to this fungicide. The EC_{50} of iprodione against the wild-type *T. harzianum* strain Th11 was 522.4 mg L^{-1} , which is significantly lower than the $2,448 \text{ mg L}^{-1}$ that is recommended for field use. This strain was classified as sensitive to the fungicide. The strain Th11 could be combined with non-inhibitory concentrations of iprodione in a complementary biocontrol strategy. The EC_{50} value of iprodione against Th11 correlates with concentrations used by other authors in combined treatments with *T. harzianum* for the control of cucumber fruit and stem mold. Other authors have identified some *Trichoderma* strains resistant to iprodione, with EC_{50} values even lower than those obtained in our study (Kay and Stewart, 1994).

All wild-type and mutant *Trichoderma* strains were sensitive to the mixture of metalaxil [N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate] and mancozeb [Mn Ethylene-bis- dithiocarbamate complexed to Zn salts]. Metalaxil is an inhibitor of RNA synthesis (Papavizas and Lewis, 1983; Wollgiehn *et al.*, 1984). Mancozeb causes DNA damage and induces apoptosis (Calviello *et al.*, 2006). These results suggest that the simultaneous use of any of these wild-type or mutant strains with this mixture of agrochemicals is unfeasible. The concentration of metalaxil utilized for the control of *Phytophthora* spp. in the field (AFIPA, 2006) is approximately 10^3 -fold greater than the EC_{50} values obtained for the wild-type *Trichoderma* strains.

The EC_{50} values obtained for thiabendazole [(2-(4-thiazolyl)-1H-benzimidazole)] against *Trichoderma* strains were *ca.* 10^3 -fold lower than the $1,220 \text{ mg L}^{-1}$ concentration recommended

for the control of *Rhizoctonia* spp., *Sclerotium* spp. and *Fusarium* spp. in the field (AFIPA, 2006). Thiabendazole is a mitotic and meiotic inhibitor that prevents β -tubulin assembly. This fungicide can induce mutations in the β -tubulin gene in phytopathogens when used intensively, producing resistant strains in species such as *F. sambucinum* (González *et al.*, 2002). The low thiabendazole concentration necessary to reduce conidial germination by 50% in all *Trichoderma* strains tested suggests that its use in combination with any of the BCAs evaluated is unfeasible.

Hymexazol [5-methyl-1,2-oxazol-3-ol] is a fungicide with a DNA/RNA synthesis inhibitor activity (Kato *et al.*, 1990). It is recommended for the control of *Fusarium oxysporum* f. sp. *lycopersici*, *Pythium* and other fungi that cause damping-off (AFIPA, 2006). Hymexazol has an inhibitory effect similar to thiabendazole on the conidial germination of *T. harzianum* wild-types Th11 and Th12. The EC_{50} values of hymexazol against strains Th11 and Th12 were 509 and 506 mg L^{-1} , respectively. These fungicide concentrations are close to the 700 mg L^{-1} required to control plant pathogens in the field; thus, there is potential use of these strains in combination with non-inhibitory concentrations of the fungicide. The EC_{50} values for hymexazol against the corresponding mutant strains were lower, which would make their use in combined strategies difficult. The EC_{50} value of hymexazol against the mutant strain Th650-NG7 was more than 10-fold greater than the EC_{50} value obtained against the wild-type strain Th650 (Table 2). Thus, Th650-NG7 appears to be a good candidate for combined use with hymexazol.

The fungicides sodium tetrathiocarbonate, aluminum tris-o-ethyl-fosfonate and [N-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide] plus iprodione all inhibited conidial germination of wild and mutant *Trichoderma* at concentrations lower than those recommended for use in the field. While all mutants were sensitive to sodium tetrathionate, they were less sensitive to aluminum tris-o-ethyl-fosfonate compared to the wild-type strains. Th11

showed a ca. 66-fold increase in sensitivity towards [N-(trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide] plus iprodione compared to iprodione alone. This increase in sensitivity was not observed in the mutant Th11A80.1, where the EC_{50} value of the mixture was only 1.4-fold greater than that of iprodione alone. The EC_{50} values of the mixture against Th12 and Th650 were similar; conidia germination in both strains was completely inhibited in the presence of iprodione. The EC_{50} values of the mixture or iprodione alone against their corresponding mutants had similar orders of magnitude.

EC_{50} ratios

The ratio of EC_{50} values between mutant and wild-type strains are shown in Table 3. Values greater than 1 indicate that the conidial germination was inhibited less in the mutant strain than in the corresponding wild-type strain at the same fungicide concentration; *i.e.*, the mutants are less sensitive than their corresponding parental strains.

The EC_{50} ratios demonstrate that mutations produced by UV light in Th11 (Besoain *et al.*,

2007) did not affect the sensitivity of Th11A80.1 towards thiabendazol. The mutations induced in Th12 (Besoain *et al.*, 2007) and in Th650 (Pérez *et al.*, 2007) decreased sensitivity; this is similar to the decreased sensitivity of all mutants towards aluminum tris-o-ethyl-fosfonate. Each wild-type strain and mutant strain, independent of the genus or the mutagenic agent used, must be tested for fungicide sensitivity before any recommendations for their use in combined practices with chemical fungicides can be made.

The above results lead to the following conclusions: All wild-type and mutant *Trichoderma* strains analyzed could be used in combination with pencycuron as a potential strategy to control *R. solani*, based on their resistance to this fungicide.

The wild-type and/or mutant *Trichoderma* strains could be used in combination with thiabendazol or iprodione to control *R. solani*, but only fungicide concentrations that do not inhibit conidial germination can be used.

The combined use of different fungicide molecules with any of these BCAs is feasible only if the fungicide concentration is not inhibitory

Table 3. Mediam effective concentration (EC_{50}) ratios of different fungicides tested against the conidial germination of mutant *Trichoderma* strains related to wild-types *Trichoderma* strains.

Fungicide	Ratio of EC_{50} (mg kg ⁻¹ or mg L ⁻¹ of active ingredient)		
	Th11A80.1/Th11	Th12A10.1/Th12	Th650-NG7/Th650
Pencycuron	n.d. ¹	n.d.	n.d.
Thiabendazole	1.00	2.70	2.04
Iprodione	0.004	n.d. ²	n.c.
Sodium tetrathiocarbamate	0.26	2.03	0.26
Fosetil aluminum	13.76	9.16	5.26
Phyto-fos	<<< 1	15.00	288.80
Metalaxil plus mancozeb	n.d. ³	n.d.	n.d.
Hymexazol	0.26	0.39	13.12
Iprodione plus captan	0.18	0.68	1.94

¹n.c. No calculation. 100% conidial germination was obtained at all fungicide concentrations tested both for the wild-type and mutant strains.

²n.c. No calculation. Values approached infinity due to the sensitivity of wild-type strains at all concentrations tested.

³n.d. No determined. Values approached zero due to the sensitivity of mutant strains at all concentrations tested.

to conidial germination. If positive biocontrol is obtained, a wider spectrum of phytopathogens could be controlled by a combination of *Trichoderma* strains and low-concentration fungicides.

Further studies of the complementary use of fungicide resistant BCAs must be performed to reduce fungicide concentrations or the number of applications needed to control phytopathogens. This strategy, in addition to the persistence of

these BCAs in the soil (Montealegre *et al.*, 2009), should have a significant and positive impact on the environment.

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Resumen

R. Herrera, D. Nuñez, N. Romero, X. Besoain, L. M. Pérez y J. Montealegre. 2012. Sensibilidad de cepas silvestres y mutantes de *Trichoderma harzianum* a fungicidas. Cien. Inv. Agr. 39(3): 569-576. Se estudió la germinación de conidias de cepas silvestres de *Trichoderma harzianum* (Th11, Th12 y Th650) y de sus correspondientes cepas mutantes (Th11A80.1, Th12A10.1 y Th650-NG7) sometidas a dosis crecientes de distintos fungicidas comerciales. Tanto las cepas silvestres de *Trichoderma* como las mutantes germinaron a 1.700 mgL⁻¹ de pencycuron, mientras que las cepas silvestres Th12 y Th650 y sus correspondientes mutantes Th12A10.1 y Th650-NG7 fueron sensibles a todas las concentraciones de iprodione y metalaxil+mancozeb. La mayoría de los valores de CE₅₀ (Concentración Efectiva Media) para los diferentes fungicidas variaron desde 10⁻¹ a 10⁻⁴ veces la concentración recomendada para uso a nivel de campo, excepto para Phyto-fos sobre la cepa mutante Th650-NG7, donde este valor fue de 0,72. Los resultados permiten establecer que algunos de estos fungicidas podrían utilizarse en combinación con agentes biocontroladores.

Palabras clave: Biocontrol, fungicidas, control de enfermedades de la raíz del tomate, manejo integrado, *Trichoderma* tolerante a fungicidas.

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