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Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent

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ABSTRACT

The selection of new isolates of *Trichoderma harzianum* with high suppressive activity against *Fusarium oxysporum* is a suitable strategy to avoid the increase of chemical pesticides. In this study, 31 isolates of *Trichoderma* sp. were analyzed by RAPD-PCR and five isolates of *T. harzianum* (T-30, T-31, T-32, T-57 and T-78) were selected. The expression of genes encoding for NAGases (*exc1* and *exc2*), chitinases (*chit42* and *chit33*), proteases (*prb1*) and β-glucanases (*bgn13.1*) activities and their respective in vitro enzymatic activities were measured. Dual plate confrontation assays of the isolates against *F. oxysporum* were also tested. Different profiles of gene expression between the different *T. harzianum* isolates were related to enzymatic activities values and dual plate confrontation. In this work, the *T. harzianum* isolates T-30 and T-78 showed the greatest mycoparasitic potential against *F. oxysporum*, which could lead to improved biocontrol of this phytopathogen.

1. Introduction

The intensive use of fungicides in agriculture results in the accumulation of toxic compounds, which is potentially dangerous for humans and the environment in general (Cook and Baker, 1983), and in the resistance of pathogens to these fungicides (Dekker and Georgopolous, 1982). The use of biological control agents (BCAs) is a promising alternative to chemical products (Alabouvette et al., 2006). *Trichoderma harzianum* is a mycoparasite used extensively as a BCA of phytopathogenic fungi (Chet, 1987). The mechanisms involved in the control of phytopathogens by *Trichoderma* species include antibiosis (Howell, 2003) and mycoparasitism (Hjeljord and Tronsmo, 1998).

The complex process of mycoparasitism involves different steps, such as recognition of the host, attack and subsequent penetration and killing. During this process *Trichoderma* secretes hydrolytic enzymes that hydrolyze the cell wall of the host fungus (Kubicek et al., 2001; Woo et al., 2006; Verma et al., 2007), consisting of chitin and β-glucan fibers embedded in a protein matrix. Therefore, the main mechanism of antagonism of *T. harzianum* against pathogenic fungi is the extracellular secretion of chitinases, β-1,3-glucanases and proteases (Elad et al., 1982; Geremia et al., 1993).

The chitinolytic enzymes are divided into *N*-acetylglucosaminidase (EC 3.2.1.52) and chitinases (EC 3.2.1.14), depending on the manner in which they hydrolyze chitin (Seidl, 2008). The chitinolytic system of *T. harzianum* contains from five to seven enzymes (Haran et al., 1996), of which the action of two endochitinases (CHIT42 and CHIT33) (Gokul et al., 2000) and two *N*-acetyl-β-D-glucosamini (EXC1 and EXC2) (Seidl et al., 2006) have been studied. The expression of these enzymes is induced in the presence of cell walls, colloidal chitin or carbon starvation, and is repressed by glucose (Silva et al., 2004). The β-1,3-glucanases are classified as exo- (EC 3.2.1.58) and endo-glucanases (EC 3.2.1.6, EC 3.2.1.39), and hydrolyze the glucose residues from the β-glucans that occur in the cell wall of pathogenic fungi (Benítez et al., 1998). The most important glucanase secreted by *T. harzianum* (BGN13.1) acts as an endoglucanase: its expression is induced by fungal cell wall and laminarin and is repressed by glucose (De la Cruz et al., 1995).

The proteolytic activity of *T. harzianum* is a prerequisite for the lysis of the protein matrix of the pathogen cell wall, and for inactivation of the hydrolytic enzymes secreted by the pathogen, which decreases its pathogenicity (Markovich and Kononova, 2003). The alkaline protease PRB1 produced by *T. harzianum* (EC 3.4.21.-) is encoded by the *prb1* gene. This gene is induced when the fungus grows in media containing cell walls of *Rhizoctonia solani*, mycelium or chitin as the sole source of carbon. Production of PRB1 is inhibited by glucose (Geremia et al., 1993).

The mycoparasitic capacity of the genus *Trichoderma* varies depending on the species or isolate, with *T. harzianum* having a high efficiency as a mycoparasite (Markovich and Kononova, 2003). Isolates of *T. harzianum* with a high potential for the

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secretion of hydrolytic enzymes can be obtained through the transformation of the fungus by multiple copies of these genes (García et al., 1994). However, such transformations techniques are not available to all laboratories. The creation of transformants is not always easy and low transformation rates of the fungi often result (Ruiz-Díez, 2002). Moreover, the insertion of genes which encode lytic enzymes can affect the production of antibiotics and other enzymes also involved in the mycoparasitism, as well as the growth rate and colonization properties of the BCA (Flores et al., 1997). One alternative to transformation is the use of *T. harzianum* isolates obtained naturally from different sources such as compost and/or agricultural soils (Rincón et al., 2008). Study of the mycoparasitic capacity of such isolates allows the selection of those which over-express the genes of interest.

The objective of this work was to study the mycoparasitic capacity of different natural isolates of *T. harzianum* which were characterized by random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland, 1990; Williams et al., 1990), which separate the different isolates in similar groups, favoring the elimination of duplicated isolates (Samson, 1995). Five isolates were selected and used for determination of their hydrolytic enzyme activities, the relative expression of the genes that encode these enzymes, by quantitative reverse transcription and polymerase chain reaction (qRT-PCR), and their effects on the phytopathogenic fungus *Fusarium oxysporum f.* sp. *melonis*, the causal agent of melon vascular *Fusarium* wilt, by dual-plate confrontation assay.

2. Materials and methods

2.1. Fungal strains

Thirty-one *Trichoderma* isolates were collected and cultivated in potato dextrose agar (PDA) (Scharlau, Spain; 39 g L^{-1}). The strains were isolated from different sources: agricultural soil (T-20, T-22, T-23, T-24, T-28, T-29, T-36, T-37, T-38, T-40, T-42, T-43, T-44, T-46, T-78), green compost (T-50, T-54, T-55, T-57), peat (T-32), commercial products (T-30, T-31) and the Spanish Culture Type Collection (CECT 2926, CECT 2930, CECT 2413, CECT 20105, CECT 20513, CECT 2424, CECT 2937, CECT 20107, CECT 20103).

The phytopathogen *F. oxysporum f* sp. *melonis* was isolated from infected melon plants from a greenhouse nursery in Murcia, Spain, and cultivated in PDA.

2.2. Preparation of fungal mycelium, DNA extraction and RAPD-PCR analysis

The isolates were grown for 48 h in PDA plates covered with cellophane. Mycelium was collected and ground in liquid nitrogen, with a mortar and pestle. The DNA extraction was carried out with phenol and chloroform, followed by isopropanol precipitation according to Hartl and Seiboth (2005). The total DNA extracted was checked in 1% agarose gel for electrophoresis with 1× Tris–acetate–EDTA (TAE) buffer, and the DNA concentration was measured with Nanodrop ND-1000 (Thermo Fisher Scientific Inc.).

The DNA extracted from isolates was amplified with the random amplified polymorphic (RAPD) technique, using the primer pr3 (Table 1). Amplification was carried out in a Takara PCR Thermal Cycler, in a volume of 25 μL containing: 200 ng of fungal DNA, 2.5 μL of reaction buffer (Biotools, Spain), 1.5 μM of $MgCl_2$ (Biotools, Spain), 0.4 μM of each primer, 0.5 U of Taq polymerase (Biotools, Spain) and sterile water (Molecular Biology Grade Water, AccuGENE). The amplification conditions were as described by Yu and Pauls (1992): an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 5 s at 94 °C, 30 s at 36 °C and 60 s at 72 °C, and a final extension step of 5 min at 72 °C.

Table 1
RAPD-PCR primers and real time PCR primers used in this study.

Technique	Primer	Sequence (5′–3′)
RAPD-PCR	pr3	TCGCCAGCCA
Real time PCR	exc1-RT-fw	CGCGGTGTTGAGGTCATTA
	exc1-RT-rv	CCTTGGTGTTGTTGATGGA
	exc2-RT-fw	AATACCTTCAAGCCTCTGAAC
	exc2-RT-rv	CTCATCGCGAATCGAAACA
	chit42-RT-fw	CTGGGAGTACCCTGCAGA
	chit42-RT-rv	CGCAGCTTGGAGTAGTTATC
	chit33-RT-fw	AATCCGGAAGCTCTGTC
	chit33-RT-rv	GGGAAGAAGCACTGAGG
	prb1-RT-fw	GTCTGAGGAACTATTGGTGG
	prb1-RT-rv	TCCCTGGAAGACCTTAACG
	b13glu-RT-fw	TCTCCAAGACTCTGAGATTCAACA
	b13glu-RT-rv	ACCAAGTTCTTAATAGCTACGG
	tef-fw	GGTACTGGTGAGTTCGAGGCTG
	tef-rv	GGGCTCGATGGAGTCGATAG

Twenty microliters of product were loaded in a 1.5% agarose gel for electrophoresis with 1× TAE buffer, followed by staining with ethidium bromide. The bands were visualized under UV light and the band sizes were estimated with the molecular size marker Eco-Ladder II (Ecogen, Spain).

Analysis of RAPD (Fig. 1A) was performed with the Quantity One program (Bio-Rad). The Dice similarity coefficient (Dice, 1945) was determined for the resulting DNA band profiles, and the clustering algorithm of Ward (1963) was used to calculate the dendogram (Fig. 1B).

One isolate of each group was selected and the internal transcribed spacers and 5.8S gene were sequenced (ITS1, 5.8S and ITS2) with ITS 1/ITS 4 primers (White et al., 1990). The sequences were analyzed with the *Trich*OKey 2 program, for the molecular identification of *Hypocrea*/*Trichoderma* at the genus and species levels (Druzhinina et al., 2005).

2.3. Fungal cultivation conditions for enzyme production

The *Trichoderma* isolates were grown in minimal medium consisting of (g/L): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $Mg_2SO_4{\cdot}7H_2O$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 0.005; $ZnSO_4{\cdot}7H_2O$, 0.002; $MnSO_4{\cdot}H_2O$, 0.002; and 0.05% peptone plus 0.1% cell walls of *F. oxysporum*, to active the expression of mycoparasitism-related genes, or 0.1% glucose, to check the normal growth of all the isolates in shaken flask cultures (control).

Each treatment consisted of 150 mL of the above-mentioned growth medium inoculated with 1 mL containing 10^6 conidia of each isolate, incubated on a rotary shaker at 220 rpm in darkness at 28 °C: 40 mL samples of each culture were taken at 24 and 48 h. These were filtered through Miracloth (Calbiochem, Germany), washed with sterile water and the mycelia were collected, frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Each treatment was performed three times.

For production of *F. oxysporum* cell walls, *F. oxysporum* was grown in potato dextrose broth (PDB, Scharlau) in a rotary shaker, for 10 days at 28 °C and 230 rpm. The mycelium was then harvested in Miracloth, washed with sterile water, lyophilized and crushed into powder in liquid nitrogen using a mortar and pestle. The powder was suspended in sterile water, sonicated four times for 1 min on ice and washed by centrifugation for 10 min at 13,000*g*, at 4 °C. The cell walls were lyophilized and stored at −80 °C.

2.4. Enzyme assays

Samples from shaken flask cultures were centrifuged at 13,000*g*, at 4 °C for 10 min, and the supernatants were used for