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Secretome analysis of *Trichoderma atroviride* T17 biocontrol of *Guignardia citricarpa*

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HIGHLIGHTS

- *Trichoderma atroviride* T17 is a biocontrol agent of *Guignardia citricarpa*.
- The control mechanisms of citrus black spot were unraveled with proteomics techniques.
- *T. atroviride* secretes proteins related to mycoparasitism and systemic resistance of the plant.
- Extracellular enzymes may be useful in the formulation of new more effective fungicides.

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ABSTRACT

The fungal species *Guignardia citricarpa* is an important pathogen in citriculture. Members of the fungal genus *Trichoderma* are recognized as biocontrol agents but studies on the interactions between both fungi are scarce. This study aimed to identify extracellular proteins secreted by *Trichoderma atroviride* T17 that are related to the control of *G. citricarpa*. Two-dimensional gel electrophoresis (2D) was used to study the patterns of proteins secreted by *T. atroviride* T17 in medium containing glucose (control) and in medium containing *G. citricarpa* GC3 inactivated mycelium. We identified 59 of the 116 spots differentially expressed (50.86%) by LC–MS/MS. Of these, we highlight the presence of glycoside hydrolases (CAZy families 3, 43, 54, 76 and 93), chitinase, mutanase, α-1,3-glucanase, α-1,2-mannosidase, carboxylic hydrolase ester, carbohydrate-binding module family 13, glucan 1,3-β-glucosidase, α-galactosidase and Neutral protease 2. These proteins are related to mycoparasitism processes, stimuli and therefore to the biological control of pathogens.

The results obtained are in agreement with reports describing an increase in the secretion of proteins related to mycoparasitism and biological control and a reduction in the secretion of proteins related to the metabolism of *Trichoderma* species grown in the presence of the pathogen. Moreover, these results are pioneer in understanding *T. atroviride* interaction with *G. citricarpa*. For the first time, we identified potential candidate proteins that may have a role in the antagonism mechanism of *G. citricarpa* by *T. atroviride* T17. Thus our results shed a light into the molecular mechanisms that *T. atroviride* use to control *G. citricarpa*.

1. Introduction

The citrus industry has a major relevance in the economy, leading to the creation of new jobs, to capital formation, to income generation and also in regional development where it operates. A worldwide importance of citrus industry is the import and export trades of these products and by-products. Neves et al. (2010) stated that the sector held a total of 230,000 direct and indirect jobs in Brazil and an annual payroll of 174 million dollars. Nonetheless, the monoculture of citrus is threatened by abiotic and biotic stressors that favor the development of diseases. Black spot disease, caused by the fungus *Guignardia citricarpa* Kiely

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(=*Phyllosticta citricarpa* (McAlpine) Aa) is of major concern causing black lesions on fruit making them unfit for sale.

Black spot disease occurrence has been reported in Argentina, Brazil, China, Philippines, Indonesia, Japan, Mozambique, New Zealand, Peru, Kenya, Taiwan, Uruguay (Sutton and Waterson, 1966), and recently in the United States (Adaskaveg et al., 2010; Schubert et al., 2012), suggesting the cosmopolitan occurrence of this pathogen, which develops in tropical regions (Paul et al., 2005). Although this disease does not damage the internal fruit quality, with symptoms restricted to the flavedo (Aguilar-Veldoso et al., 2002), it disables the *in natura* trade of fruits (Aguilar-Veldoso et al., 2002; Timmer et al., 2008). Additionally, the pathogen leads to great losses due to premature decline, reducing the productivity of plants. In severe attacks, losses up to 80% have been observed (Klotz, 1978; Spósito et al., 2004).

The control of the symptoms of this disease is mainly made via the use of chemicals that are applied four to five sprays per crop. This strategy adds increased production costs while it has severe toxicity issues associated (Bernardo and Bettiol, 2010). According to Hortifruti Brazil (2012), producers estimate that the cost of agrochemicals reaches 30% of the production cost. An additional complication of using this type of control is the selection of resistant strains to the active ingredients. Rodrigues et al. (2007) found isolates of *G. citricarpa* resistant to the fungicide carbendazim in Brazilian plantations, probably generated by the intensive use of agrochemicals and the selection pressure exerted by them. In addition to the resistance caused by the excessive use of these chemicals, there is also a growing concern about the socio/environmental impacts (Ali, 2014), which has stimulated the search for new effective methods to control this pathogen. It is the objective of these new strategies to replace or decrease fungicide applications, consequently reducing costs for the farmers/exporters as well as reducing ecological costs. One alternative is the use of antagonistic microorganisms with potential to inhibit some stages of the disease or the life cycle of pathogens (Strobel, 2006; Vinale et al., 2006; Isaias et al., 2014; Miao et al., 2015; Parmar et al., 2015).

Species of *Trichoderma* are considered effective biocontrol agents of several fungal pathogens such as *Fusarium, Rhizoctonia* and *Botrytis* (Belete et al., 2015; El-Komy et al., 2015; Kotasthane et al., 2015; Rao et al., 2015; Talla et al., 2015; Vos et al., 2015). Some species of *Trichoderma* are also known for their ability to induce systemic resistance against plant diseases (Singh et al., 2014; Lamdan et al., 2015; Rao et al., 2015; Salas-Marina et al., 2015; Vos et al., 2015). The biocontrol exercised by *Trichoderma* can occur by several mechanisms such as mycoparasitism (Gruber and Zeilinger, 2014; Troian et al., 2014). Mycoparasitism occurs due to the activity of degrading extracellular cell wall enzymes, allowing the penetration of the antagonistic fungus and the death of the pathogen (Viterbo et al., 2002; Bech et al., 2014; Troian et al., 2014). The process of mycoparasitism involves sequential events that include the reconnaissance and the penetration into the host culminating into its death. After detection and recognition of a pathogen, *Trichoderma*'s hyphae are directed towards the pathogen. The attack occurs when *Trichoderma* secretes cell wall degrading enzymes (endochitinases, β-glucosidase, mannosidases, and proteases) that act synergistically to control pathogens such as *Botritys cinerea*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* sp, among others (Monteiro et al., 2010). These enzymes are capable of hydrolyzing the cell wall of the host, releasing oligomers that activate the expression of genes involved in mycoparasitism (Vinale et al., 2008). When the host and the mycoparasite establish physical contact, the mycoparasite adheres to the host via apressorium surrounding the host hyphae (Chet et al., 1998). The mycoparasite then enters the lumen of the hyphae of the host, assimilating and metabolizing the protoplasmic content (Suárez et al., 2007). Fungi of the genus *Trichoderma* are considered excellent hyperparasites, attacking hyphae, reproduction and survival structures of plant pathogens, reducing the infective capacity of the pathogen (Benhamou and Chet, 1996). This is a broad description of the mycoparasitism mechanism and variations may occur according to the host that is involved. Monteiro et al. (2010) reported that the mycoparasitic response of pathogen *Trichoderma harzianum* ALL42 is host dependent with variations in both hyphae winding and secreted proteins.

Despite the recognized potential of *Trichoderma* as a biological control agent of plant pathogens (Inch et al., 2011; Wijesinghe et al., 2011; Martínez-Medina et al., 2014), the number of studies on *Trichoderma* action on *G. citricarpa*, or regarding the mechanisms used by *Trichoderma* in controlling this pathogen are scarce. Among the few studies involving these two fungi (antagonist/pathogen) those by Guimarães (2008) and Pandolfo (2011) stand out. These authors reported the antagonistic action of *Trichoderma koningii* towards *G. citricarpa in vitro*, suggesting *Trichoderma* potential as a biological control agent citrus disease.

The strain used in his work was selected due to its biocontrol over *Guignardia*: the Biological Control Laboratory of Plant Diseases (University of Caxias do Sul, Brazil), in partnership with producers from the Vale do Caí-RS (Brazil), showed that strain T17 of *Trichoderma atroviride* is able to reduce on the symptoms of black spot of citrus in orchards (unpublished results).

Proteomics allow unraveling proteins involved in the interaction between organisms (Yang et al., 2009; Vincent et al., 2012; Lemos et al., 2010). Therefore, this study aims to identify the molecular mechanisms of interaction between *T. atroviride* T17 and *G. citricarpa* GC3, with emphasis in the identification of extracellular proteins, using proteomics methodologies.

2. Material and methods

2.1. Guignardia citricarpa isolation and identification

Guignardia citricarpa Gc3 belongs to the in house culture collection of the Biological Control Laboratory of Plant Diseases (University of Caxias do Sul, Brazil. This strain was isolated from orange with symptoms of the citrus black spot disease and identified by morphology (microscopy).

Molecular identification of *G. citricarpa* Gc3 was carried out by sequencing the internal transcribed spacer (ITS) region, as described by Alves et al. (2004). Fungal isolate was grown in PDA (Potato Dextrose Agar), for 20 days, at 25 °C with controlled photoperiod (12/12 h) (Guimarães, 2008). Genomic DNA was isolated from fresh mycelium following the method of Santos and Phillips (2009).

After DNA isolation, the ITS region was amplified using the primers ITS1 and ITS5 (White et al., 1990) as described by Alves et al. (2004). *Taq* polymerase, nucleotides and buffers were supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves et al. (2004). The amplified PCR products were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). The PCR products were sequenced by STAB Vida Lda (Portugal). ITS sequences were checked manually, and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. The ITS sequence was then used in a BLAST search against the GenBank nucleotide sequence database.

2.2. Cultivation and deactivation of Guignardia citricarpa

The pathogen was cultured in Potato Dextrose Agar (PDA), for 30 days at 25 °C with controlled photoperiod. Afterwards, mycelium was scraped off and inoculated into 100 mL of Potato Dextrose Broth medium (PDB) for 10 days, 25 °C, 180 rpm. The