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Beneficial effect of *Trichoderma harzianum* strain *Ths97* in biocontrolling *Fusarium solani* causal agent of root rot disease in olive trees

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ABSTRACT

Fusarium root rot is a major cryptogamic disease in olive trees caused by the soil-borne fungus *Fusarium solani*. Controlling this disease requires the extensive use of chemicals. However, using BCAs such as some *Trichoderma* strains may be an opportune alternative to fungicides in protecting olive plantations. A new isolate (*Fso14*) was isolated from young olive trees showing severe dieback symptoms. The objective of this work was to analyze the biocontrol behavior of a Tunisian strain of *T. harzianum* (*Ths97*) on olive trees against *Fso14* by assessing both mycoparasitic activity (*in planta* and *in vitro*) and ability to locally modulate different gene-related defenses of the plant. *Ths97* was found to inhibit *Fso14* growth *in vitro*. Optical microscopic analysis at the confrontation zone between hyphae showed that *Ths97* grew alongside *Fso14* with numerous contact points suggesting parasitic activity. On olive trees, *Ths97* developed a strong protective role against root infestation by *Fso14*, whether inoculated before or after the pathogenic agent. When inoculated alone, *Fso14* and *Ths97* did not modulate (or only slightly with inhibitions or inductions, respectively) the expression of genes involved in plant immunity (oxidative stress, phenylpropanoid pathway, PR-proteins and JA/Et-SA hormonal status). However, when *Ths97* was inoculated in combination with *Fso14*, several defense-related genes were highly up-regulated, indicating probable primed-plant events. These promising results provided valuable information on using *Ths97* as a beneficial agent to control fusarium root rot disease caused by *F. solani* in olive trees.

1. Introduction

Olive trees (*Olea europaea* L.) are one of the most ancient domesticated fruit trees in the Mediterranean basin, and remain a fruit crop of interest worldwide. There is currently an increase of olive tree orchards worldwide, due to both their resilience to climate change, and also a larger demand for olive oil. However, like any plant species of agronomical interest, olive trees are subject to severe attacks from a variety of pathogens that affect their health, and ultimately their yield and oil organoleptic quality (Triki et al., 2009; Boulila, 2011; Gharbi et al., 2016). An increased incidence of drying syndromes has been observed, especially in some young olive tree orchards and in nurseries. They result in partial wilting and sudden death of young trees. In all of the countries where olive trees are grown, a number of fungi have been reported to be associated with root diseases in nurseries and new orchards. In Tunisia, preliminary studies were carried out on related mycoflora from symptomatic olive plants in different oil-producing regions. One of several potential soil-borne pathogens, a new strain of

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Fusarium solani (*Fso14*), was isolated. This strain is known to be responsible for fusarium root rot disease, and was the focus of this study.

The *Fusarium* genus is presented as a cosmopolitan soil saprophyte, but it includes some of the most aggressive phytopathogen species (Dean et al., 2012). Under certain environmental conditions, strains can become facultative biotrophic parasites, causing cortical decay, root rot, and leaf yellowing and wilting, and ultimately, the premature death of the infested plants (Coleman, 2016). Regarding olive trees, variations in pathogenicity of different *Fusarium* spp. isolates have been reported, and *F. solani* was described as one of its most aggressive pathogens (Barreto et al., 2001; Trabelsi et al., 2016). Despite substantial economic losses caused by *F. solani*, disease control is still limited to systemic fungicide treatments and prophylactic actions. Both measures seem to be very limited (Chandel and Deepika, 2010). They become rapidly ineffective in controlling phytopathogens under conditions conducive to disease and raise serious environmental and safety concerns. In addition, these measures could quickly become ineffective in controlling phytopathogens, with risks of resistant strains emerging (Saiz-Jimenez et al., 2012). The latest surveys conducted in the highest olive-producing areas portray a scenario where the disease is steadily expanding (Sergeeva, 2011). For these reasons, it is crucial to intensify efforts to develop alternative agricultural management practices that could replace chemicals to biologically control *F. solani* on diseased olive plants, thereby acting in a sustainable and environmentally friendly way. One such alternative requires biological control agents (BCAs) that reduce the amount of inoculum and/or disease-producing activity of pathogens (Cook, 1993). So far, however, few detailed scientific studies have been carried out on the efficient protection of olive trees against *Fusarium* spp. through the use of BCAs belonging *inter alia* to the *Trichoderma* genus.

Several rhizocompetent filamentous fungus *Trichoderma* spp. are now attractive biofungicide agents, able to control various plant pathogenic fungi. *Trichoderma* is synergistically beneficial by competing for space and nutrients, and inhibiting and/or parasiting off pathogens using highly fungitoxic antibiotics (Vinale et al., 2014) in combination with extracellular cell wall-degrading enzymes (CWDEs) (Qualhato et al., 2013). In addition, *Trichoderma* spp. exhibits biostimulating abilities, inducing plant resistance mechanisms and root development and plant growth (Lorito et al., 2010). Locally and systemically inducing plant defenses is currently viewed as the pivotal mechanism by which *Trichoderma* spp. diminishes plant disease symptoms at the root and foliar levels. These modulations in host plant's resistance (also called ISR, Induced System Resistance) result in a superficial and long-lasting *Trichoderma* colonization of root tissues (Shoresh et al., 2010; Hohmann et al., 2012). These plant-mediated responses may begin because elicitors originating from *Trichoderma* itself are recognized, or due to the hydrolytic activity on its prey (Djonovic et al., 2006; Monteiro and Ulhoa, 2006). They are usually regulated and relayed by jasmonic acid (JA)- and ethylene (ET)-dependent signaling pathways (Pieterse et al., 2009). However, the complex underlying molecular mechanisms in the *Trichoderma*-induced resistance have only been partially revealed and understanding them is highly difficult. It is assumed that the plant recognition of *Trichoderma* triggers the activation of a cascade signal which then in turn activates a variety of defense responses, including the secretion of antimicrobial reactive oxygen species (ROS), numerous secondary metabolites such as phytoalexins and pathogenesis-related proteins, callose depositions and the possible development of a type of programmed cell death (PCD) known as hypersensitive response (HR) (Shoresh et al., 2010). *In fine*, the completion of these physiological events leads to multiple host resistances. While a considerable body of literature demonstrates the use of antagonistic *Trichoderma* spp. as an efficient alternative to conventional approaches in controlling various soil-borne phytopathogens (Verma et al., 2007), and that includes several *F. solani* strains capable of infesting different hosts like annual plant species (Rojo et al., 2007; Tae Gwan and Knudsen, 2013), only a few preliminary reports have evaluated the effectiveness of *Trichoderma* spp. against several olive tree pathogens (including some *Fusarium* strains) on olive trees (Moussa et al., 2006). The tripartite interaction *T. harzianum*/olive tree/*F. solani* needs to be further understood.

This study was undertaken to investigate the antagonistic effect of the fungus *Trichoderma harzianum* strain *Ths97* on the phytopathogen *Fusarium solani* strain *Fso14* in olive trees. First, we examined *Ths97*'s capability to reduce fusarium dieback symptoms caused by *Fso14*, both in the root system (primary site of infection) and in the aerial plant parts that develop secondary symptoms with leaf collapses. Second, we evaluated the impact of *Ths97* and *Fso14* (alone, and in combination, either preventively in primed plants or curatively in diseased plants) on expressing defense-related genes from major defense pathways. Third, we investigated the potential mycoparasitic activity of *Ths97* on *Fso14* in *in vitro* condition at a microscopic level. Then we discussed how a better understanding of the multiple mechanisms involved when *F. solani Fso14* is inhibited by *T. harzianum* strain *Ths97* could provide valuable guidance for using *T. harzianum* strain *Ths97* to control fusarium root rot biologically.

2. Materials and methods

2.1. Plant material and fungal strains

Olea europaea cultivar *Chemlali* obtained from herbaceous cuttings of two-year-old plants were used for assays. This cultivar is highly susceptible to several soil-borne fungi such as *Fusarium* spp., including *F. solani* strain *Fso14* (Triki et al., 2009). Plants were planted in plastic bags containing autoclave-sterilized sandy clay soil, and kept in a growth chamber. Bioassays were carried out using gnotobiotic experimental conditions aimed at reducing any artifact tied to possible microorganisms that can interfere with the *Trichoderma* and *Fusarium* protagonists or the relationship between them. Plant growth parameters were 16-h photoperiod, 26/23 °C (day/night), relative humidity of around 70%, and regular irrigation. *F. solani* strain *Fso14* was previously isolated from rotted roots of young olive trees showing dieback symptoms in Tunisian orchards. *Fso14* was identified by using molecular methods based on the sequencing amplification products of the *ITS* regions by PCR with an accession number KU863548. *Trichoderma harzianum* strain *Ths97* (Triki and Priou, 1997) from Tunisian farmland was the bioagent used as an antagonist against *Fso14*. All fungi strains are recorded at the "Institut de l'Olivier" (University of Sfax, Tunisia). For experiments, fungi were grown on PDA plates (Potato Dextrose Agar: 200 g potato, 15 g dextrose, 20 g Agar-agar, *qsp* 1L distilled water), and incubated in darkness for 7–10 days, and at 25 °C and 27 °C for *Fso14* and *Ths97*, respectively, to allow abundant conidia production.

2.2. In planta antagonism assay

Antagonism tests on plants were conducted by inoculating olive tree roots with conidia suspensions prepared by mixing the contents of four agar plates (6 cm diameter) with 600 ml of sterile deionized water in a blender for 2 min at high speed. Roots were carefully dug out, cleaned with tap water, and submerged for 1 h in the inoculum suspensions (Triki et al., 2011). Control plants were handled similarly with sterile water. After inoculation, plants were replanted in plastic bags containing new autoclave-sterilized sandy clay soil. For the confrontation assays, *Ths97* and *Fso14* were inoculated successively with 6 days between each inoculation. The preventive assay corresponds to plants inoculated with *Ths97* in the first step, and the curative assay corresponds to plants inoculated with *Ths97* in the second step. The pathogenicity of *Fso14* was assessed weekly for 8 weeks by measuring the severity of aerial symptoms using the 0–4 scale developed by Sanchez-Hernandez et al. (1998). This scale examines the percentage of