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Combining the oomycete *Pythium oligandrum* with two other antagonistic fungi: Root relationships and tomato grey mold biocontrol

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ABSTRACT

To reduce *Pythium oligandrum* biocontrol variability and improve its efficacy, experiments were performed by combining the oomycete with two other antagonistic fungi, *Fusarium oxysporum* strain *Fo*47 and *Trichoderma harzianum*. In Petri dishes, *Fo*47 or *T. harzianum* hyphae destroyed *P. oligandrum* cells by antibiosis and mycoparasitism processes; in the rhizosphere of tomato plants (*Lycopersicon esculentum*), the same antagonistic features were observed. However, in the rhizosphere, hyphae are frequently separated by a certain distance; this allows the coexistence and the persistence of the three microorganisms on the root systems. When introduced in the rhizosphere, *Fo*47 and *P. oligandrum* were able to penetrate the root tissues with *Fo*47 limited to the epidermal and upper layers of cortical cells while *P. oligandrum* colonized deeper tissue at a faster rate. The two antagonists were killed in few days within roots following elicited plant-defense reactions. *T. harzianum* was not able to penetrate root tissues. Root colonization with either *P. oligandrum* alone or in combination with *Fo*47 and/or *T. harzianum* resulted in systemic plant resistance which provided plant protection against *Botrytis cinerea* infection of leaves. The level of control and the expression of pathogenesis-related proteins (PR-proteins) in leaves were similar whatever the antagonistic microbial treatment applied to roots.

1. Introduction

The worldwide-distributed inhabitant of soils, *Pythium oligandrum* Dresch., has been identified as one of the most numerous species observed in agricultural soils (Martin and Hancock, 1987; Mulligan and Deacon, 1992; Plaats-Niterink, 1981). In certain areas, it has proven its ability to induce plant disease suppression; for instance, Martin and Hancock (1987) described soil-suppression to the pathogen *Pythium ultimum* Trow concomitantly with the presence, at high densities, of *P. oligandrum* propagules in the soil. The biological control exerted by *P. oligandrum* is a complex process, which includes direct effects on pathogens in the rhizosphere and/or *P. oligandrum*-mediated effects on the plant, such as induction of resistance and growth promotion (Rey et al., 2008). Regarding the direct effect of *P. oligandrum* on other fungi, several reports have clearly stated that antagonism is a multifaceted and target fungus-dependent process characterized by several distinct traits: mycoparasitism and antibiosis, attack of fungal sclerotia and competition for nutrients (Benhamou et al., 1999; Foley and Deacon, 1986; Lewis et al., 1989; Picard et al., 2000b; Rey et al., 2005).

P. oligandrum has an intrinsic ability to quickly penetrate the root tissues of tomato plants, hyphae cannot stay alive in planta, at least because of the elicitation of plant-defense reactions (Le Floch et al., 2005; Rey et al., 1998). Subsequently to this specific relationship, various indirect effects on plants have been described, all of them proved to have a positive impact on either plant-resistance or plant-growth. For example, following the inoculation of roots with *P. oligandrum,* an array of defense-related reactions upon challenge with different plant pathogens, e.g. *Botrytis cinerea* Pers., *Fusarium oxysporum* f.sp. *radicis lycopersici, Phytophthora parasitica* Dast., *Ralstonia solanacearum* (Smith) (Benhamou et al., 1997; Brozova, 2002; Le Floch et al., 2003b; Lherminier et al., 2003; Mohamed et al., 2007; Takenaka et al., 2008) is triggered in various plants. The antagonistic oomycete secretes an elicitin-like protein that induces resistance against different phytopathogens (Benhamou et al., 2001; Picard et al., 2000a). Takenaka and co-workers (2003, 2006) isolated two types of cell wall protein fractions from *P. oligandrum* and demonstrated their elicitor activity. Root growth and yield are also enhanced by root colonization by *P. oligandrum* due to auxin-compounds such as tryptamine (Le Floch et al.,

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2003a). The direct implication of *P. oligandrum* in phosphorus uptake within cucumber plants also provided growth promotion (Krakta et al., 1994).

Beneficial effects due to this oomycete introduction in the rhizosphere, depends on cultural system and persistence of root colonization. For instance, tomato yield was increased in hydroponic system like Nutrient Film Technique (NFT) once *P. oligandrum* was present over the whole cultural season (Le Floch et al., 2003b). However, no significant yield increase was observed, when plants grown in another soilless system (coco fiber growing medium) (Le Floch et al., 2007).

According to Takenaka and co-workers (2008), this biocontrol oomycete is rhizosphere-competent, but does not actively spread along roots. Generally, root colonization by the antagonist was not associated with a significant reduction in the number of root-infecting pathogens, e.g. *Pythium* populations (Le Floch et al., 2007). Conversely to the induction of plant resistance, which seems to be prevalent, direct competition for infection sites with root pathogens in the rhizosphere is likely not the primary biocontrol mechanism.

Despite the evidence of the potential benefits of *P. oligandrum* and other biocontrol agents provided by many studies, inconsistency in performances is frequently reported (Alabouvette et al., 2006). Indeed, variability is likely increased by numerous biotic and abiotic factors, and among them, it is worthwhile citing adaptation to various environmental conditions, fluctuations in antagonist activity, inadequate colonization of the rhizosphere under field conditions. For instance, an antagonistic strain is adapted to a particular niche and it cannot be expected to perform equally well in different climatic zones: seasons and adaptation sites should also be taken into account. As even strains of the same species can exhibit significant differences, natural microbial communities are more closely mimicked through application of a mixture of several species; moreover, this control strategy may prove to be more relevant in the long term. According to the literature on suppressive soils (Weller et al., 2002), suppressiveness is based on interactions between several microorganisms which have been implicated to act together to consistently control disease despite differences in their mode of action. To enhance biocontrol-efficacy and -consistency, one can either use several strains from the same antagonistic microorganism, or combine different biocontrol species (Alabouvette and Lemanceau, 1998; Guetsky et al., 2002). Our group recently followed the first strategy and used a *P. oligandrum* inoculum containing three strains with different biological traits: persistence was proved to be greatly strain-dependent (Vallance et al., 2008). For the second strategy, according to studies by Guetsky and co-workers (2001, 2002), a combination of several different species of biocontrol agents, each of them having different mechanisms of disease suppression, can reduce the variability of biological control and improve its effectiveness. However, other authors have pointed out that this beneficial effect was not always observed (Hervas et al., 1997).

To test this second biocontrol strategy, the study reported here relied on the association, for the first time, of the antagonistic oomycete, *P. oligandrum*, with two other antagonistic fungi, i.e. *Fusarium oxysporum* Schltdl. strain *Fo*47 and/or *Trichoderma harzianum* Rif., in the rhizosphere. These latter two fungal biocontrol agents have been the subject of extensive studies (Fravel et al., 2003; Harman et al., 2004; Vinale et al., 2008; Woo et al., 2006); moreover, like *P. oligandrum*, they can protect the plants via different direct and indirect mechanisms, e.g. mycoparasitism, antibiosis and nutrient competition. The relative importance of each mechanism depends on the individual species and may differ among strains (Fravel et al., 2003; Harman et al., 2004). A preliminary step was to gain insight into the compatibility of the two fungi with *P. oligandrum* in dual culture as well as in the rhizosphere of tomato plants. The proven ability by *Fo*47, *P. oligandrum* and *T. harzianum* to induce systemic resistance in various plants (Benhamou et al., 1997, 2001; Fuchs et al., 1997; Le Floch et al., 2003b; Woo et al., 2006) led us to test whether an increase on tomato plant protection against the foliar pathogen, *Botrytis cinerea*, was enhanced or not. This pathogen can attack more than 200 plants species, and is responsible for economic losses on numerous field cultures such as vineyard or tomatoes in glasshouse (Elad, 1996). The foliar protection ensured by *P. oligandrum* alone, in association with one or two biocontrol agents and the expression of pathogenesis-related proteins (PR-proteins) as a marker of induced systemic resistance (Van Loon, 1997) were both assessed.

2. Materials and methods

2.1. Fungal cultures and inoculum productions

The strain of *P. oligandrum* isolated from pea roots in Denmark was provided by Dr. J. Hockenhull from the Royal Veterinary and Agricultural University of Copenhagen (ref# 1.01.631; Brittany Culture Collection, ESMISAB, France). *T. harzianum* was obtained from the CABI Bioscience culture collection (ref# 206040). The non-pathogenic strain, *Fo*47, was initially isolated by Alabouvette et al. (1987) from a suppressive soil located at Châteaurenard (France) and provided to us by this group. The strain of *B. cinerea* was isolated from diseased leaves of tomato (Brittany Culture Collection, ESMISAB, France). The strains were all grown on either corn meal agar (CMA, Difco), or malt yeast agar (MYA) and incubated at 24 °C in the dark and regularly subcultured.

To produce *P. oligandrum* inoculum, the oomycete was cultured at first in a liquid medium containing cane molasses as described by Le Floch and co-workers (2003b). The flasks filled with 100 ml of culture medium were inoculated with 10 agar plugs of *P. oligandrum*, and then incubated in the dark for 14 days at 25 °C. Mycelial mats were then removed and fragmented in distilled water with a blender. The production of oospores was determined three times using a Malassez cell, and the concentration was adjusted to 2×10^5 oospores ml^{-1} with sterile-distilled water. To obtain conidial suspensions of *Fo*47 and *T. harzianum*, the fungi were cultured on potato dextrose agar (PDA, Difco) for 1 week at 25 °C in the dark. After three determinations using a Malassez cell, the conidial concentration was adjusted with sterile-distilled water to 8×10^6 spores ml^{-1}. For *B. cinerea* inoculum, the fungus was also cultured on PDA for 1 week at 25 °C in the dark. Two milliliters of sterile-distilled water containing 0.05% of Tween 80 were poured in culture plate, conidia were scraped from the agar by a sterile loop and spore concentration was also determined three times using a Malassez cell to get a final concentration of 2×10^4 spores ml^{-1} in potato dextrose broth (PDB, Difco).

2.2. Dual-culture tests

The effect of strain *Fo*47 and *T. harzianum* on the growth, morphology, and ultrastructure of *P. oligandrum* was assayed in dual-culture tests using the modified method of Chérif and Benhamou (1990). Five-millimeter mycelial discs collected from the edges of actively growing colonies from each fungus were placed 3 cm apart on the surface of PDA medium and left to grow at 25 °C in the dark. Mycelial samples from the interaction region were collected 2, 3, 4 and 5 days later and processed for electron microscopy as described in Section 2.6. The experiment was repeated twice with three Petri dishes per sampling time.

2.3. Plant growth

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Prisca) were sterilized by immersion in 70% ethanol for 5 min, then soaked in