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Quantitative detection of *Lactarius deliciosus* extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition

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Abstract

Real-Time PCR has been applied to quantify extraradical soil mycelium of the edible ectomycorrhizal fungus *Lactarius deliciosus* in an interspecific competition experiment under greenhouse conditions. Couples of *Pinus pinea* seedlings inoculated with either *L. deliciosus*, *Rhizopogon roseolus*, or non-inoculated (control) were transplanted into pots filled with two types of soil in all the possible combinations. Total DNA was extracted from soil samples at 3 and 6 months after transplantation to perform real-time PCR analysis. DNA extractions from soil mixed with known amounts of mycelium of *L. deliciosus* were used as standards. Six months after transplantation, the percentage of mycorrhizas of *L. deliciosus* and seedling growth were significantly affected by the soil type. Extraradical soil mycelium of *L. deliciosus* was positively correlated with the final percentage of mycorrhizas and significantly affected by the sampling time and soil depth. The competition effect of *R. roseolus* was not significant for any of the measured parameters, probably due to the sharp decrease of the mycorrhizal colonization by this fungus. We conclude that real-time PCR is a powerful technique for extraradical mycelium quantification in studies aimed at evaluating the persistence of introduced strains of *L. deliciosus* in field plantations.

Keywords: Competition; Ectomycorrhizal fungi; *Lactarius deliciosus*; *Pinus pinea*; Real-time TaqMan PCR; Root colonization

1. Introduction

The beneficial effects of mycorrhizas are mostly related to the fungus-mediated increased absorption of water and nutrients by plants (Smith and Read, 1997). Extraradical mycorrhizal mycelium has a key role in soil nutrient uptake and reciprocal transfer of carbon and nutrients between plants (Simard et al., 2002; Guidot et al., 2003; Landeweert et al., 2003a,b). However, it is the most poorly understood phase of the symbiosis (Read, 1992; Horton and Bruns, 2001; Leake et al., 2004). Standard methods for assessing

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mycorrhizal occurrence are generally based on identification and quantification of colonized roots by different methods (Brundrett et al., 1996; Parladé et al., 1996). On the other hand, the mycelial systems of saprotrophic and mycorrhizal fungi are difficult to detect in the soil matrix by observational techniques. Innovative methods to study structure and function of extraradical mycorrhizal mycelium such as biochemical and DNA-based markers, observation in vitro and in soil, and root-free hyphal compartmentation have been reviewed in Leake et al. (2004). Among the developed techniques, molecular identification has allowed studies on fungal diversity and spatial structure of ectomycorrhizal fungal mycelia in the soil (Chen and Cairney, 2002; Dickie et al., 2002; Landeweert et al., 2003a, 2005; Smit et al., 2003; Koide et al., 2005; Genney et al., 2006; Suz et al., 2006). Furthermore, molecular methods have been used for quantification of specific ectomycorrhizal fungi in soil to evaluate the ecological and functional impact of a given species in its natural environment. Guidot et al. (2002, 2003) quantified mycelium of *Hebeloma cylindrosporum* Romagn. in complex DNA mixtures extracted from forest soils by competitive PCR. Most of the recent DNA quantification studies apply the real-time PCR technique (Heid et al., 1996; Schild, 1996; Schena et al., 2004). This technique is based on the detection and quantification of a fluorescent signal generated by a fluorescently labelled sequence-specific probe (Lee et al., 1993; Livak et al., 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. Thus, by recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target DNA template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Landeweert et al. (2003b) compared real-time PCR to conventional quantification techniques (total hyphal length, biochemical markers) for mycelial biomass estimates. They found that soil DNA extracts gave fluctuating results in the real-time quantitative PCR whereas the standard samples with purified ITS plasmid inserts produced highly repetitive standard curves. Also, they concluded that, since copy numbers of ITS genes on the genome can vary between fungal species, it would be most reliable to use a biomarker gene with a known copy number for quantification purposes. Schubert et al. (2003) used real-time PCR with specific primers and a TaqMan® probe for absolute quantification of extraradical hyphal biomass of *Piloderma croceum* Erikss. & Hjortst in pure cultures and rhizotron samples. They pointed out that the application of the technique in environmental samples strongly depends on the availability of fungal DNA extraction protocols for different types of soil. Raidl et al. (2005) calibrated the method to obtain a correlation between mycelial biomass of *P. croceum* and isolated rDNA ITS copies for absolute quantification.

In this study we have applied the technique of real-time PCR for tracking the persistence of the edible ectomycorrhizal fungus *Lactarius deliciosus* (L. ex Fr.) S.F. Gray in nursery-inoculated seedlings. This fungal species is highly appreciated in local European markets and its trade has become an important local business in many countries (Singer, 1986; FAO, 2004). The experimental conditions to obtain pines mycorrhizal with *L. deliciosus* have been optimised by Guerin-Laguette et al. (2000) and Parladé et al. (2004b). Molecular tracking of *L. deliciosus* in experimental plantations, established with nursery-inoculated seedlings and aimed at producing edible fruitbodies, has been performed by PCR with specific primers and SSCP analysis (Hortal et al., 2006). As a further step, it is necessary to quantify *L. deliciosus* extraradical soil mycelium to determine its persistence and competitiveness against native ectomycorrhizal fungi in the experimental plantations. The objectives of this study are: (1) to design and apply specific oligonucleotides for quantitative detection of *L. deliciosus* extraradical soil mycelium by real-time PCR and (2) to determine the persistence of mycorrhizas and extraradical soil mycelium of *L. deliciosus* in an interspecific competition experiment established under greenhouse conditions.

2. Materials and methods

2.1. Seedling inoculations

Vegetative inoculum of *L. deliciosus* strain 312 (IRTA ECM Culture Collection) was prepared according to Marx and Bryan (1975) modified as described in Parladé et al. (2004b). After 10 weeks of incubation at 25 °C, the inoculum was mixed at the proportion 1:10