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Real-time PCR for detection and quantification of the biocontrol agent *Trichoderma atroviride* strain SC1 in soil

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Abstract

Trichoderma (*Hypocreales*, *Ascomycota*) is a widespread genus in nature and several *Trichoderma* species are used in industrial processes and as biocontrol agents against crop diseases. It is very important that the persistence and spread of microorganisms released on purpose into the environment are accurately monitored. Real-time PCR methods for genus/species/strain identification of microorganisms are currently being developed to overcome the difficulties of classical microbiological and enzymatic methods for monitoring these populations. The aim of the present study was to develop and validate a specific real-time PCR-based method for detecting *Trichoderma atroviride* SC1 in soil. We developed a primer and TaqMan probe set constructed on base mutations in an endochitinase gene. This tool is highly specific for the detection and quantification of the SC1 strain. The limits of detection and quantification calculated from the relative standard deviation were 6000 and 20,000 haploid genome copies per gram of soil. Together with the low throughput time associated with this procedure, which allows the evaluation of many soil samples within a short time period, these results suggest that this method could be successfully used to trace the fate of *T. atroviride* SC1 applied as an open-field biocontrol agent.

Keywords: Monitoring; Risk assessment; Real-time PCR; *Trichoderma atroviride*

1. Introduction

The genus *Trichoderma* (*Hypocreales*, *Ascomycota*) is widespread in nature and has a wide range of characteristics which makes it useful for several practical applications. For example, *Trichoderma* species are used in the production of enzymes for the pulp and paper industry, and as biocontrol agents against plant pathogens (Harman and Kubicek, 1998). For biocontrol applications, *Trichoderma* spp. are generally mass produced and applied directly to the crop or soil (O'Neill et al., 1996; Elad, 2000).

Monitoring the fate and behavior of a released microorganism in the environment is of utmost importance. Registration of a specific biocontrol agent (BCA) as a pesticide in Europe (Regulation EC 414/1991) requires a risk assessment focused on the persistence and multiplication of the BCA in the environment, in addition to an evaluation of any possible contamination (viable and non-viable BCA residues) of foodstuffs. These legal requirements require the use of monitoring methods that can accurately identify the released strain, distinguish it from the native microbial community and track its population dynamic over time.

In the past, this was achieved through the classical microbiological method of counting the colony forming units (CFU) of the microorganism grown on a selective culture medium. This method presents several difficulties: species/strain-specific selective media are seldom available, the procedure is extremely time-consuming and the results are not immediately available. When strain-specific media are not available, the fact that agricultural soils are rich in a wide variety of native microorganisms, many of which are present in the soil at frequencies much higher than that of the introduced biocontrol agent, necessitates the counting and identification

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of large numbers of microorganisms, which can carry significant costs.

If a selective medium is not available, identification of microorganisms at the strain/species level must be achieved by other means, such as morphological analysis, immunological assays (e.g. ELISA tests) (Thornton et al., 2002), or molecular methods such as probe-hybridization of the 18S rDNA sequence (Wu et al., 2003) or examination of microsatellite markers (Naef et al., 2006). The amount of time needed to develop and perform these tests and their high costs are the major constraints of most of these techniques. Consequently, recent studies have focused on PCR-based methods, which are easier to develop, reliable and quick (Providenti et al., 2004; Gobbin et al., 2007). These methods can also be easily automated. If the detection of a microorganism can be easily achieved by conventional PCR, its precise quantification is not possible, real-time PCR has become the most popular quantitative PCR technique (Schena et al., 2004; Valasek and Repa, 2005). The popularity of RT-PCR has also been encouraged by its repeatability and reliability.

Real-time PCR markers for genus/species/strain identification of microorganisms can be developed from regions of the microorganism's genome whose functions are not known, such as the sequence-characterized amplified region (SCAR) markers (Paran and Michelmore, 1993; De Clercq et al., 2003; Pujol et al., 2005, 2006; Sarlin et al., 2006; Gayoso et al., 2007), or from genes or sequences with known functions, such as the internal transcribed spacer regions located between rDNA large subunits (ITS1 and ITS2) (Lees et al., 2002; Atkins et al., 2003; Ippolito et al., 2004; Boyle et al., 2005), the 18S rRNA gene (Zeng et al., 2004), a heat shock protein gene (Khan and Yadav, 2004) and a translation elongation factor alpha gene (Filion et al., 2003). The recent wide availability of many gene sequences has allowed the identification of a large number of single-nucleotide polymorphisms (SNPs) that, coupled with real-time PCR, can be used as high-resolution genetic markers for genotyping (Mhalanga and Malmberg, 2001; Gibson, 2006). Specific fluorogenic probes, such as Taqman, Molecular Beacons and Scorpion ARMS primers, are frequently used to discriminate between fungal and bacterial strains (Bates and Taylor, 2001; Schena and Ippolito, 2003; Ciancio et al., 2005; Massart et al., 2005).

Strain specific SCAR markers, mainly derived from RAPD analysis, were developed for a range of potential BCAs, both in conventional and real-time PCR (Hermosa et al., 2001; Dodd et al., 2004; Weaver et al., 2005; Rubio et al., 2005; Cordier et al., 2007), even if the development of a specific SCAR marker is a long and laborious process. A recent study showed the feasibility of detection and quantification of *Trichoderma* spp. in different soils by real-time PCR using a common genus-specific 600 bp ITS region (Hagn et al., 2007).

Currently, we are testing *Trichoderma atroviride* strain SC1 as a BCA against root rot pathogens, primarily *Armillaria* spp., in vineyard and orchard soils. Therefore, a specific and highly sensitive method for quantifying the BCA population in the soil following application is necessary. SCAR markers and the published primers used in previous studies both for qualitative and real-time PCR (Abbasi et al., 1999; Rubio et al., 2005; Cordier et al., 2007) were not suitable for this task because they cannot specifically amplify the DNA of this strain or discriminate SC1 from other soil microorganisms. Therefore, the aim of the present study was to develop and validate (by comparison with the CFU counting method) a real-time PCR-based method to specifically detect and quantify *T. atroviride* SC1 in soil.

2. Materials and methods

2.1. Fungal strains

T. atroviride SC1 strain was isolated from decaying hazelnut in North Italy. The *Trichoderma* isolate was identified as *atroviride* species by morphological analysis and by comparison of the Ribosomal Intergenic Spacer sequences between 18S–28SrRNA, comprehensive of ITS1-5.8SrRNA-ITS2 regions, in databanks (NCBI http://www.ncbi.nlm.nih.gov/BLAST/ and ISTH-TrichOkey at http://www.isth.info/tools/molkey/index.php). The strain showed a biocontrol activity in laboratory and field tests against *Armillaria* spp. (Pertot, *personal communication*) and it was deposited in CBS restricted collection under the regulations of the Budapest Treaty.

The isolates of *Trichoderma* spp. and all the fungal strains utilized in our study are listed in the Table 1. All fungal isolates were grown on potato dextrose agar (PDA, Oxoid, Basingstoke, United Kingdom) at 20 °C and stored in tubes at 10 °C or in microbanks by cryopresevation at −80 °C.

2.2. Soil and grape samples

Ten soil samples, four from Trentino-Sud Tirol and six from other Italian regions (Marche, Valle d'Aosta, Emilia-Romagna and Calabria) and the DNA from *Vitis vinifera* cv. Cabernet were included in the specificity test to control for amplification of DNA from root material present in *T. atroviride* SC1-treated soils.

2.3. DNA extraction

Grape DNA and fungal DNA were extracted directly from 50 to 100 mg of leaf or mycelia using the DNeasy plant Mini Kit (Qiagen, Hilden, Germany). Total DNA from soil samples, if not otherwise noted, was always extracted from 200-mg soil samples dried overnight, according to the protocol prescribed for the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, California).

2.4. Real-time PCR primers and probe

T. atroviride SC1 endochitinase gene (*ech42*) (Carsolio et al., 1994) was amplified using consensus primers designed on sequences already present in the NCBI GeneBank. The obtained complete sequence was compared and aligned with 34 sequences of the same database using the BLAST program Altschul et al., 1997 and the ClustalW program (available at the