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Development of a molecular approach to describe the composition of *Trichoderma* communities

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ABSTRACT

Trichoderma and its teleomorphic stage *Hypocrea* play a key role for ecosystem functioning in terrestrial habitats. However, little is known about the ecology of the fungus. In this study we developed a novel *Trichoderma*-specific primer pair for diversity analysis. Based on a broad range master alignment, specific *Trichoderma* primers (ITS*Tr*F/ITS*Tr*R) were designed that comprise an approximate 650 bp fragment of the internal transcribed spacer region from all taxonomic clades of the genus *Trichoderma*. This amplicon is suitable for identification with *Tricho*Key and *Tricho*BLAST. Moreover, this primer system was successfully applied to study the *Trichoderma* communities in the rhizosphere of different potato genotypes grown at two field sites in Germany. Cloning and sequencing confirmed the specificity of the primer and revealed a site-dependent *Trichoderma* composition. Based on the new primer system a semi-nested approach was used to generate amplicons suitable for denaturing gradient gel electrophoresis (DGGE) analysis and applied to analyse *Trichoderma* communities in the rhizosphere of potatoes. High field heterogeneity of *Trichoderma* communities was revealed by both DGGE. Furthermore, qPCR showed significantly different *Trichoderma* copy numbers between the sites.

1. Introduction

Fungi belonging to the genus *Trichoderma*/*Hypocrea* (*Ascomycetes*) can be isolated from nearly every soil, decaying wood, compost or other organic matter (Harman et al., 2004; Berg et al., 2005). Because of their aggressive lytic capacity, *Trichoderma* strains are involved in the degradation of complex organic compounds in soil (Kubicek et al., 2008). Furthermore, many *Trichoderma* isolates are known for their ability to suppress different fungal plant pathogens, e.g. *Botrytis cinerea*, *Fusarium* spp., *Phytophthora cactorum*, *Pythium* spp., *Rhizoctonia solani*, and *Verticillium dahliae* (Weinling, 1932; Chet, 1987; Grosch et al., 2006), and to promote plant growth (Inbar et al., 1994). Therefore, some strains have been used as active ingredients of biofungicides (Monte, 2001; Hermosa et al., 2004). Different mechanisms, including competition for nutrients, antibiosis, production of cell wall lytic enzymes, induction of systemic resistance, and mycoparasitism have been described as the basis for the antagonistic activity (Harman et al., 2004). In addition, the important role of auxin signalling for plant growth promotion was shown for *Trichoderma* spp. in *Arabidopsis* (Contreras-Cornejo et al., 2009). In contrast to the reported beneficial interactions with plants, several *Trichoderma* species are also known as pathogens for diverse eukaryotic hosts, e.g. *Trichoderma longibrachiatum*, an opportunistic pathogen of humans (De Miguel et al., 2005) or *Trichoderma aggressivum*, the causal agent of "green mould disease" in fungi like *Agaricus* and *Pleurotus* (Hatvani et al., 2007).

Due to the importance of *Trichoderma* in terrestrial ecosystems, in the last decade several studies on the ecology and biogeography of *Trichoderma* were published, which were based on the cultivation of selected isolates (Zhang et al., 2005; Migheli et al., 2009; Sadfi-Zouaoui et al., 2009). However, to describe the overall diversity of *Trichoderma* in soil molecular tools are needed to avoid the bias, which is caused generally by cultivation-based approaches. Therefore, Hagn et al. (2007) developed primers targeting a 540 bp fragment comprising the internal transcribed spacer region 1 (ITS 1), 5.8S rRNA gene and internal the transcribed spacer region 2 (ITS 2) of all taxonomic clades of the genus *Trichoderma*. Although this sequence was useful to analyse *Trichoderma* abundance in ecosystems (Zachow et al., 2009), it was not suitable to unambiguously identify *Trichoderma* at the species level using *Trichoderma* identification systems like *Tricho*Key 2.0 (Druzhinina et al., 2005) and *Tricho*BLAST (Kopchinskiy et al., 2005).

Therefore, the objective of our study was to develop a novel primer system that would allow the sequence-based identification of the amplicon with both *Tricho*Key 2.0 and *Tricho*BLAST. In order to evaluate the newly developed primers, amplicons obtained from total community DNA from the rhizospheres of potatoes grown at two field

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sites were cloned and sequenced. In addition, this primer system was adopted for analysis of the *Trichoderma* community composition by denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Field design and sampling of potato rhizospheres

In this study the potato cultivars 'Baltica', 'Désirée', 'Ditta' and 'Selma' were used. Furthermore two genetically modified (GM), zeaxanthin-accumulating, potato lines (SR47, SR48) which are based on the parental line 'Baltica' were included in this study. The construction of the two GM lines SR47/00#18 (co-suppression) and SR48/00#17 (anti-sense) was described in detail by Römer et al. (2002). The accumulation of zeaxanthin in the tuber reached up to 40 μg/g dry weight (dw) (SR47/00#18) and 17 μg/g dw (SR48/00#17) compared to 0.2 μg/g dw of the wildtype.

The field design has been described by Weinert et al. (2009) in detail. The two sites used are located in Southern Germany (Bavaria). The soil of the site Roggenstein was characterised by 26.1% sand, 44.0% silt, 28.1% clay, Corg: 1.1%, Nt: 0.1% and pH 6.6. The soil of the site Oberviehhausen contained 54.6% sand, 31.3% silt, 14.1% clay, Corg: 1.9%, Nt: 0.2%, and had a pH of 6.5. Plant protection management at both sites was performed according to agricultural practice.

The experimental setup was designed as a randomised field trial: each plot had a size of 9×3 m, resulting in 4 rows with a total of 40 potatoes. The cultivars and GM lines were grown in six replicated plots, of which four plots were sampled. Sampling of rhizosphere soil was performed from individual plants during the flowering period. Samples were shock frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Extraction of DNA from fungal isolates and from soil samples

Extraction of DNA from fungal isolates (plated on Sabouraud-Dextrose-Agar (Difco, Detroit, USA) for 7 to 14 days at room temperature) was performed using a modified protocol published by John J. Weiland on the FGSC-site (http://www.fgsc.net/fgn44/weiland.html). In brief: tubes were filled with 200 μg glass beads and wetted with Phenol/Chloroform/Isoamylalcohol (24:24:1, ROTH, approximately 200 μl) before adding the fresh mycelia harvested from agar plates; cell lysis was performed with the FastPrep system (Qbiogene, BIO101® Systems, Carlsbad, USA) for 20 s and 5.5 ms^{-1}.

DNA extraction was done using 0.5 g of the microbial pellet obtained from 10 g of root material with adhering soil as recently described by Weinert et al. (2009) by means of the FastDNA® Spin for Soil Kit (Qbiogene, BIO101® Systems, Carlsbad, USA). The DNA yield was checked by agarose gel electrophoresis (0.8%, 1× TBE) using 5 μl of the DNA suspension. To remove PCR inhibiting substances, the rhizosphere community DNA was purified using the GeneClean® Spin Kit (Qbiogene, BIO101® Systems, Carlsbad, USA).

2.3. In silico primer development

A total of 577 *Trichoderma*-ITS-sequences were obtained from different databases (NCBI and ISTH) and screened for appropriate size (>400 bp). For the screening of possible consensus regions, the sequences were aligned and integrated in the ARB database (Ludwig et al., 2004). The *Trichoderma* assignment was confirmed by BLAST and the probe match tool of the ARB package was used for primer design. The forward and reverse primers were selected based on a medium GC-content, a melting temperature of >50 °C/<60 °C, and an amplicon size of >300 bp that allows their identification by means of *Tricho*Key 2.0 or the ITS2 Database: forward primer ITS1*Tr*F (5′-ACTCCCAAACCCAATGTGAA-3′, Tm: 53.9 °C) and reverse primer ITS4*Tr*R (5′-TGTGCAAACTACTGCGCA-3′, Tm: 54.6 °C).

2.4. PCR conditions

For PCR amplification of genomic DNA from isolates, the following conditions were used: 5 U of *Taq* polymerase (Stoffel fragment, Applied Biosystems, CA, USA), 10× Stoffel buffer, 0.2 mM dNTPs, 3.75 mM MgCl2, 2.5% DMSO, 0.5 μM of each primer, and about 20 ng template. After an initial step for 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 35 s at 53 °C and 2 min at 72 °C, followed by a final extension step at 72 °C for 10 min were performed. The PCR products were analysed by agarose gel electrophoresis (1%, 1× TBE). For cloning experiments the PCR products were purified with the GeneClean® Spin Kit.

For amplification of soil DNA a semi-nested strategy was chosen. The first PCR reaction was performed using the fungal specific ITS forward primer ITS1F (Gardes and Bruns, 1996) and the *Trichoderma*-specific ITS reverse primer ITS4*Tr*R. For the second reaction the *Trichoderma*-specific primer system ITS*Tr*F/ITS*Tr*R developed in this study was used. To establish a *Trichoderma*-specific DGGE system a GC clamp attached to the 5′end of ITS*Tr*F was employed. The conditions for the first amplification were the same as described above except that the annealing temperature was raised to 54 °C and the number of cycles was reduced from 35 to 30. For the second amplification the amplicons of the first PCR were diluted 1:20. The conditions were the same as described above, except that the number of cycles was 25.

For the quantification of *Trichoderma* the qPCR protocol published by Hagn et al.(2007) was used.

2.5. Cloning of PCR products and sequence alignments

Cloning of PCR amplicons was done with the pGEM-T Easy vector system (Promega, Madison, USA). The ligation reaction was performed with 3 μl of the obtained PCR products. Positive clones were picked and cultivated on fresh agar plates containing 85 μg/ml ampicillin. Clones were checked for correct insert size by PCR with the SP6/T7 primer system using cell lysates as template and subsequent agarose gel electrophoresis (1%, 1× TBE). Clones with the correct insert size were sent to *IIT* Biotech (Bielefeld, Germany) for one shot sequencing.

After removing vector sequences using VecScreen (www.ncbi.nlm.nih.gov) the sequences were identified by BLASTn (Basic Local Alignment and Search Tool) as well as *Tricho*Key 2.0 and *Tricho*BLAST (Druzhinina et al., 2005; Kopchinskiy et al., 2005). To compare a set of sequences for similarities, they were first truncated to the same length using the binding sites for the *Trichoderma*-specific primer as a marker.

2.6. Denaturing gradient gel electrophoresis (DGGE) of Trichoderma-specific gene fragments

DGGE analysis was performed in a Biorad apparatus (DCode, Biorad, Germany) with a double gradient composed of 26–58% denaturants (100% denaturants defined as 7 M urea and 40% formamide) and 6.2–9% acrylamide. Approximately 3 μl aliquots of PCR products were loaded on the gel. The electrophoresis run was performed in 1× Tris-acetate-EDTA buffer at a constant voltage of 220 V for 7 h at 58 °C and gels were silver-stained according to Heuer et al. (2001). Stained gels were air-dried and scanned transmissively (Epson 1680 Pro, Seiko-Epson, Japan).

3. Results

3.1. Primer development

The designed primers were first tested *in silico* against the constructed database of 577 sequences obtained from ISTH and NCBI databases. The forward primer ITS*Tr*F had a perfect match with the ITS1 of 378 *Trichoderma* strains mainly belonging to clade I