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REVIEW

Tracking fungi in soil with monoclonal antibodies

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Abstract Species of the genus *Trichoderma* are ubiquitous soil-borne fungi that exhibit antagonism towards a number of economically important plant-pathogenic fungi and oomycetes. This review discusses recent developments in the use of monoclonal antibodies to detect these fungi in their natural soil environments and to quantify their population dynamics during antagonistic interactions with saprotrophic competitors in soil-based systems. Immunological approaches to detection and quantification are examined in relation to conventional plate enrichment techniques and to nucleic acid-based procedures. An example of recent research using a mAb-based assay to quantify the effects of saprotrophic competition on the growth of *Trichoderma* isolates in mixed species, soil-based, microcosms is presented. Future technological developments in immunoassays for tracking *Trichoderma* populations in soil are discussed and results presented showing the accurate detection and visualization of a plant growth-promoting isolate of *T. hamatum* in the rhizosphere of lettuce using mAb-based immunodiagnostic assays.

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Historical perpectives

Soil fungi are a diverse group of eukaryotic organisms that encompass economically important root-infecting plant pathogens, beneficial mycorrhiza, root endophytes, nematode-trapping fungi and mycoparasites. As a consequence of their saprotrophic activities, soil fungi are the principal degraders of biomass in terrestrial ecosystems and are responsible for much of the organic re-cyling in the environment. As a group of organisms, they are arguably the most difficult to study since their natural habitat, soil, is chemically and biologically complex and remains largely uncharacterized. The close associations of fungi with other soil-borne micro-organisms such as bacteria, actinomycetes and oomycetes presents a significant challenge for detection and quantification of individual genera or species and their often copious production of asexual conidia causes significant restrictions in the accurate quantification of population dynamics.

Traditionally, detection of soil fungi has relied on plate enrichment techniques that incorporate selective or semi-selective media. Attempts have been made to replace these techniques with serological and nucleic-

acid based procedures but it is worth noting that many such procedures still require a period of biological amplification through nutrient enrichment to allow the magnification of propagule numbers available for detection (Lees et al. 2002; Thornton et al. 1993, 2004). Biological amplification is not a suitable system for quantification of soil fungi. Colonies derived from asexual conidia lead to significant bias in quantification and mask ecologically important fluctuations in active hyphal biomass. Procedures have been developed for determining population dynamics based on measurements of colony-forming units (CFUs), but they are limited to experimental systems containing individual non-sporulating organisms such as the sterile root-infecting pathogen *Rhizoctonia solani* (Dewey et al. 1997; Thornton et al. 2004). For the quantification of population dynamics of spore-producing species, alternative methods needed to be developed that allowed discrimination between active hyphal growth and quiescent spore production. This has, so far, not been achieved using DNA-based techniques since they are unable to discriminate between spores and hyphae. Monoclonal antibody-based techniques do allow discrimination of biomass components and, provided that mAbs are raised against constitutively expressed antigens that are secreted during hyphal development, they can be used to quantify changes in active growth. The aim of this review is to examine techniques that have been developed to track fungi in soil, using as a case study species of the genus *Trichoderma*. Recent developments in immunological techniques that allow the accurate quantification of population dynamics of *Trichoderma* species during antagonistic interactions with other fungi in soil-based systems will be discussed.

Trichoderma—a case study

Species of the genus *Trichoderma* are cosmopolitan soil and compost-borne saprotrophic fungi. Certain strains of *T. harzianum* are noxious compost-borne pathogens of cultivated mushrooms (Seaby 1987), while a number of thermo-tolerant species have emerged as medically important opportunistic pathogens of healthy humans and of immuno-compromised patients (Walsh and Groll 1999). However, it is the ability of certain isolates to suppress plant disease (Whipps 1997, 2001) and to promote plant growth (Harman et al. 2004) that has lead to intense and prolonged scientific interest in this important group of organisms.

The mycoparasitic activities displayed by certain *Trichoderma* strains has made them attractive candidates as biological control agents in the fight against plant diseases and considerable efforts have been made to promote their use for this purpose. Nevertheless, despite their unquestionable potential as ecologically sound alternatives to synthetic pesticides, their widespread application in the control of soil-borne pathogens has not been realized. Historically, biocontrol strains have been selected on the basis of activities exhibited under controlled laboratory conditions, conditions that are often far removed from those experienced in nature. Consequently, levels of disease control achieved in laboratory tests are rarely repeated following introduction of strains into alien soil environments. One reason for this is that artificially introduced strains are unable to compete for niches with soil saprotrophs already resident in soil ecosystems. It should be remembered that the mycoparasitic properties demonstrated by *Trichoderma* species (hyperparasitism, production of lytic enzymes, secondary metabolites and antibiotics) are also properties displayed many other species of soil fungi. Consequently, a biocontrol strain must possess both antagonistic activity and strong competitive saprotrophic ability (CSA), if it is to have a realistic chance of delivering levels of disease control comparable to conventional control methods. Determining whether a strain has a strong CSA in soil-based systems is problematic and requires techniques that allow saprotrophic activities to be accurately monitored during antagonistic interactions with other soil saprotrophs *in vivo*.

A number of procedures have been developed to track *Trichoderma* species in soil ranging from traditional techniques that employ selective media, through nucleic-acid based techniques that exploit polymerase chain reaction (PCR) to immunological techniques that incorporate specific monoclonal antibodies.

Conventional techniques

Conventional methods for detecting *Trichoderma* propagules, which employ soil dilution and plate-

enrichment techniques, have been used to recover isolates from naturally infested soils. The most specific medium for this purpose is TSM (*Trichoderma* Selective Medium)(Elad et al. 1981) which, when amended with compounds to inhibit fast growing oomycetes, is sufficient for general detection, although contamination by other fungi, particularly *Gliocladium* species, remains a problem. Assays based on selective isolation are also laborious and require taxonomic expertise to provide definitive identification of isolated fungi. The specificity of plate enrichment techniques can be improved by combining them with additional means of identification. For example, Thornton et al. (2002) used a *Trichoderma*-specific mAb-based enzyme-linked immunosorbent assay (ELISA) in combination with baiting and selective isolation to detect *Trichoderma* species in naturally infested composts. Isolates identified using the ELISA were recovered from the selective medium and their identity determined by analysis of the internally transcribed spacer (ITS) regions (ITS1-5.8S-ITS2) of the rRNA-encoding regions of the fungi.

A further limitation of plate-enrichment techniques is their inability to differentiate between colonies derived from hyphae and those derived from spores (conidia and chlamydopores). Consequently, selective media cannot be used to quantify the population dynamics of *Trichoderma* species based on active growth or measurements of CFUs.

Measurements of colony densities have also been shown to correlate poorly with other measures of biomass such as ATP, chitin, detection of respiration and ergosterol (Lumsden et al. 1990). Measures of ATP and chitin were regarded as suitable markers for estimates of *Trichoderma* biomass, but quantification base on amounts of chitin could only be used during interactions with oomycetes such as *Pythium* species that lack chitin in their cell walls (Lumsden et al. 1990), although these organisms have since been shown to have a chitinous cell wall component. In addition, ATP measurements can only be used to estimate total biomass because this technique is unable to discriminate between the metabolic activities of fungi and bacteria (Eiland 1985).

Nucleic acid-based techniques

Mutation of *Trichoderma* strains to benomyl tolerance and transformation of *Trichoderma* strains with β-glucuronidase and green-fluorescent protein-encoding genes have provided useful tools for ecological studies in the rhizosphere and bulk soil, but these studies have so far been constrained to individual fungicide-tolerant or recombinant isolates (Ahmad and Baker 1988; Bae and Knudsen 2000; Green and Jensen 1995; Hermosa et al. 2001; Pe'er et al. 1991; Thrane et al. 1995). An alternative approach adopted by Abbasi et al. (1999) employed a combination of sequence-characterized amplified region (SCAR) markers and dilution plating on a semi-selective medium to detect and enumerate propagules densities. However, the technique only permitted quantification of total propagule densities, because it was unable to differentiate between colonies derived from spores and from mycelium.

Immunological techniques

Hybridoma technology allows the production of monoclonal antibodies (mAbs) that are specific to individual genera, species or even isolates of fungi (Dewey and Thornton 1995) and are capable of discriminating between active growth and quiescent spore production (Thornton 2004; Thornton and Dewey 1996; Thornton et al. 1994). A monoclonal antibody (HD3) raised against a 20 kDa intracellular protein from *T. harzianum* was the first mAb produced using hybridoma technology that displayed a high degree of specificity to *Trichoderma* species (Thornton et al. 1994). Monoclonal antibodies have also been produced to the closely related fungus *Gliocladium roseum* using hybridoma technology (Breuil et al. 1992). The protein bound by mAb HD3 is only produced during active growth and, at the onset of sporulation, is no longer detected. The hyphal-specific nature of the antigen therefore represented a potentially useful target for the quantification of active growth of *Trichoderma* species in soil. However, subsequent studies showed that the low abundance of the antigen meant that it was not readily detected in soil extracts. Parallel studies using mAbs specific to *Rhizoctonia* species (Thornton and Gilligan 1999) showed that the most appropriate targets for the development of quantitative immunology assays were extracellular, constitutively expressed, antigens. Consequently, a mAb (MF2) was raised specific to *Trichoderma* species and the closely related fungi

Gliocladium viride, *Hypomyces chrysospermus*, *Sphaerostilbella* species and *Hypocrea* species (Thornton et al. 2002). It does not react with *Gliocladium catenulatum*, *G. roseum*, *Nectria ochroleuca* and *Clonostachys* species, or with a wide range of unrelated soil- and compost-borne fungi. Monoclonal antibody MF2 binds to a constitutive, extracellular, antigen secreted from the growing tip of hyphae and that, in certain species, is also released from conidia. In combination with PCR, Thornton et al. (2002) used mAb MF2 to develop a highly specific diagnostic procedure for the detection and identification of *Trichoderma* populations in naturally-infested peat-based composts (Thornton et al. 2002). Furthermore, studies showed that the mAb could be used to quantify the saprotrophic activity of biocontrol strains during antagonistic interactions with the plant pathogen *R. solani* in peat and soil (Thornton 2004) and to visualize plant-growth-promoting *Trichoderma* species in the plant rhizosphere (Thornton and Talbot 2006).

The specificity of the quantitative assay also allows the accurate monitoring of the saprotrophic competences of *Trichoderma* species and enables the competitive saprotrophic abilities of potential biocontrol strains to be determined during competitive interactions with other saprotrophs in soil-based systems. An example is shown in Fig. 1. Here, the population dynamics of *Trichoderma longibrachiatum* is quantified during antagonistic interactions with the abundant and widely distributed soil saprotroph *Aspergillus fumigatus*. Saprotrophic activity of *T. longibrachiatum* was quantified using the *Trichoderma*-specific ELISA and standard calibration curves of biomass equivalents as described in Thornton (2004). Population dynamics of *T. longibrachiatum* were determined in single (*T. longibrachiatum* only) and mixed species (*T. longibrachiatum* and *A. fumigatus*) soil-based microcosms.

In microcosms containing *T. longibrachiatum* only (•), there was a rapid increase in active biomass of the fungus between days 1 and 2 post-inoculation. Thereafter, active biomass fluctuated, but there was an overall decline up to day 21 (see Fig. 1). Active growth dynamics of the fungus followed a similar trend in mixed species microcosms (○), but was significantly reduced by *A. fumigatus*. The aggressive saprotrophism exhibited by *A. fumigatus* was likely

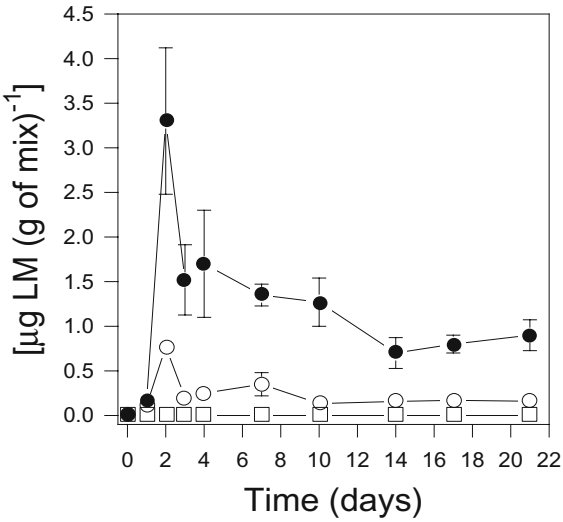

Fig. 1 Population dynamics of *T. longibrachiatum* in the presence (*empty circle*) and absence (*filled circle*) of *A. fumigatus*, quantified using the *Trichoderma*-specific mAb-based assay described in Thornton (2004). Absorbance values were converted to biomass equivalents {expressed as [µg LM (g of mix)$^{-1}$]} using a standard calibration curve. The mean ± SE was then calculated for each set of samples from the populations on each day of sampling. The specificity of the *Trichoderma*-specific mAb MF2 was shown using extracts from microcosms containing *A. fumigatus* only (*empty square*)

facilitated by the production of inhibitory secondary metabolites and antibiotics such as gliotoxin (Bok et al. 2006; Cramer et al. 2006) and the production of fungal cell wall-degrading enzymes such as chitinase (Escott et al. 1998), mechanisms that are similarly attributed to the antagonistic properties of *Trichoderma* species (Harman and Kubicek 1998). Consequently, while *T. longibrachiatum* has been identified as a potential biocontrol agent for the control of oomycete plant pathogens (Sreenivasaprasad and Manibhushanrao 1990, 1993; Migheli *et al.* 1998), its efficacy might be significantly impaired in soil systems where aggressive soil saprotrophic fungi also reside. These types of considerations need to addressed if the biocontrol properties of *Trichoderma* species are to be fully exploited. In contrast to nucleic-based techniques that have been developed to detect individual *Trichoderma* strains, immunological techniques that incorporate genus-specific mAbs such as MF2 can be used to quantify the saprotrophic competence of multiple potential biocontrol strains.

Future technological developments

The use of immunological techniques for the detection of *Trichoderma* species is restricted to those laboratories that possess *Trichoderma*-specific mAbs. In order to expand the access of hybridoma technology to other workers interested in the biocontrol and plant-growth-promoting properties of these fungi, Thornton and co-workers have developed a 'user-friendly' mAb-based lateral flow device (LFD) for the specific detection of *Trichoderma* species in naturally infested soils and in the plant rhizosphere by adapting techniques previously developed for the detection of the soil-borne pathogen *R. solani* (Thornton et al. 2004). Briefly, the *Trichoderma*-specific mAb MF2 is immobilized to a defined capture zone on a porous nitrocellulose membrane, while the same mAb conjugated to colloidal gold particles serves as the detection reagent. Samples of solubilised antigens are added to a release pad containing the antibody-

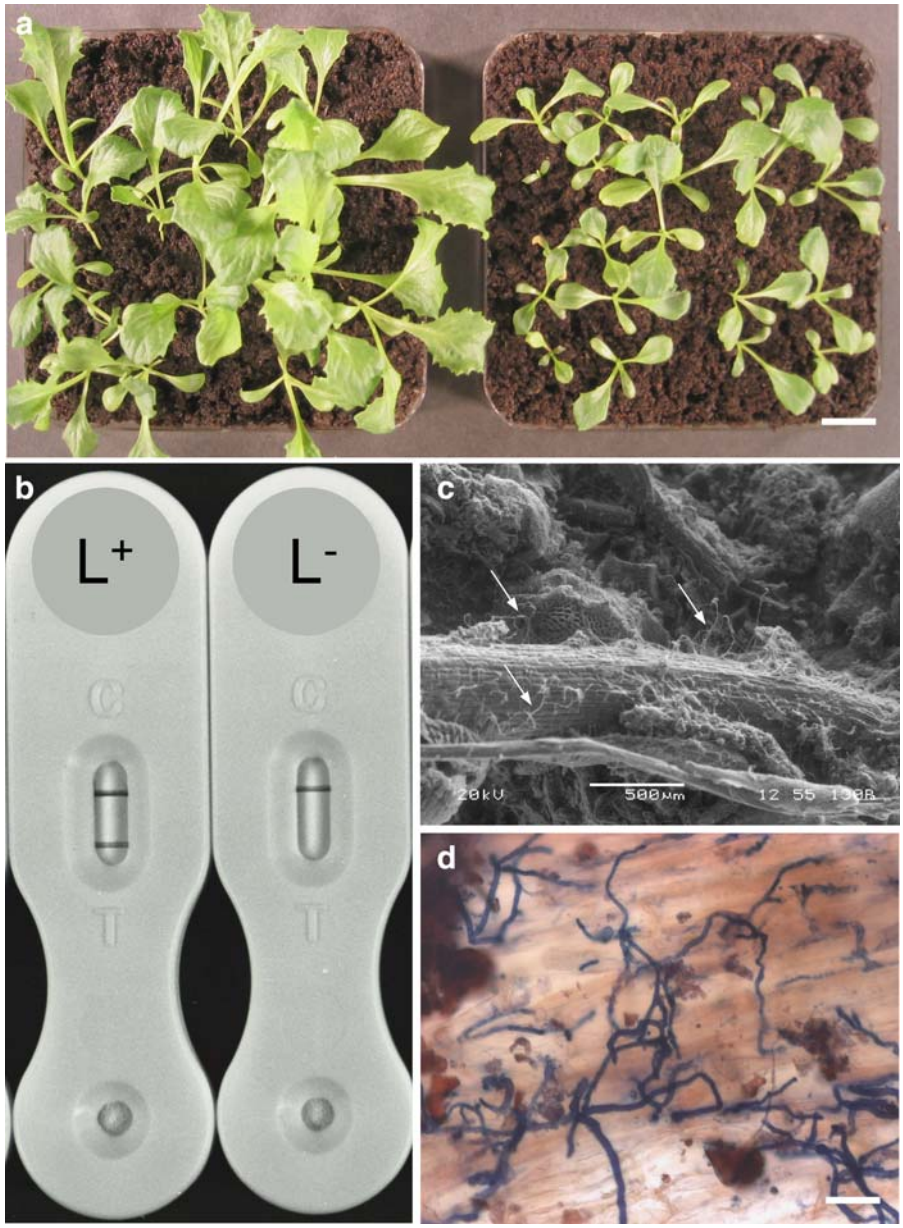

Fig. 2 Detection of *T. hamatum* in the rhizosphere of lettuce. **a** Growth promotion of lettuce plants by the root-colonising *T. hamatum* strain GD12. The left hand microocosm shows plants grown in the presence of the fungus. Control plants grown in the absence of the fungus are shown on the right. Scale bar=1.7 cm. **b** Results of LFD tests using extracts prepared from root sections of lettuce plants grown in the presence (L^+) and absence (L^-) of GD12. **c** Scanning electron micrographs of a lettuce root showing colonisation of the root surface with mycelium of GD12. Scale bar=500 µM. **d** Immunoenzymatic staining of the root surface of a lettuce plant grown in the presence of GD12, showing extensive colonisation by the fungus. Scale bar=20 µM

gold conjugate. The antibody-gold conjugate binds to the target antigen, passes along the porous membrane by capillary action, and binds to the mAb immobilized in the capture zone. Once an antigen extract is prepared and applied to the LFD, the test result is recorded within 10 min. Bound antigen-antibody-gold complex is seen as a red line with an intensity that is proportional to the antigen concentration. Anti-mouse immunoglobulin immobilized to the membrane in a separate zone acts as an internal control. In the absence of the *Trichoderma* antigen, no complex is formed in the zone containing solid-phase antibody, and a single internal control line is seen. In the presence of *Trichoderma* antigen, two lines are clearly visible. Antigens are extracted from soil samples using a simple 1-h extraction procedure, making the assay appropriate for use by personnel with limited access to microbiological facilities. The test can be used to monitor the presence of *Trichoderma* strains derived from commercial biocontrol and plant-growth-promoting formulations.

An example of the use of the LFD to track *Trichoderma* in the plant rhizosphere is shown in Fig. 2. Lettuce (*Lactuca sativa* cv. Webb's Wonderful) seeds were planted in peat containing inoculum of a plant growth-promoting strain of *T. hamatum* (strain GD12). After 2 weeks growth, seedlings were carefully removed at random from each tray and excess peat removed by shaking. Sections of root tissue 0–1.5 cm below the hypocotyl were excised, transferred to antigen extraction buffer and the roots crushed with a hand-held micro-pestle. Suspensions were centrifuged to pellet insoluble debris and supernatants tested for the presence of *Trichoderma* antigens using the LFD. Roots of plants were also examined under compound and scanning electron microscopes for colonisation by *T. hamatum*. Visualization of *T. hamatum* under the compound microscope was aided by immuno-localization of the fungus on the root surface using the *Trichoderma*-specific mAb MF2, according to the immuno-enzymatic staining procedure described in Thornton and Talbot (2006). Electron microscopy was carried out using a JEOL 6360LV scanning electron microscope.

Growth promotion of lettuce plants as a consequence of *T. hamatum* GD12 root colonization is shown in Fig. 2a. Positive results in LFD tests of extracts from lettuce roots were obtained with all plants grown in the presence of GD12 only. A typical reaction of a positive sample is shown in Fig. 2b, where two lines in the LFD test shows the presence of *Trichoderma* antigens in root extracts. No antigens were detected in extracts from the roots of control plants grown in the absence of the fungus, indicated by a single line in the LFD test (Fig. 2b). Representative root sections were examined for the presence of the fungus using scanning electron microscopy and a *Trichoderma*-specific monoclonal antibody-based immuno-enzymatic staining procedure (Thornton and Talbot 2006). Representative sections from control plants all showed the absence of fungus in both SEM and IES tests. Sections of roots from plants grown in the presence of the fungus all showed the presence of *Trichoderma* mycelium both in SEM and IES tests (Fig. 2c and d respectively).

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