

Production of a monoclonal antibody specific to the genus *Trichoderma* and closely related fungi, and its use to detect *Trichoderma* spp. in naturally infested composts

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Studies of the interactions between hyperparasitic fungi and their hosts are severely hampered by the absence of methods that allow the unambiguous identification of individual genera in complex environments that contain mixed populations of fungi, such as soil or compost. This study details the development of a monoclonal antibody (MF2) that allows the detection and recovery of *Trichoderma* spp. in naturally infested composts, and the visualization of hyperparasitic strains of *Trichoderma* during antagonistic interactions with their hosts. Murine monoclonal antibody MF2, of immunoglobulin class M (IgM), was raised against a protein epitope of a glycoprotein antigen(s) specific for species of the genus *Trichoderma* and for the closely related fungi *Gliocladium viride*, *Hypomyces chrysospermus*, *Sphaerostilbella* spp. and *Hypocrea* spp. MF2 did not react with antigens from *Gliocladium catenulatum*, *Gliocladium roseum*, *Nectria ochroleuca* and *Clonostachys* spp., nor with a range of unrelated soil- and compost-borne fungi. Extracellular production of the MF2 antigen was constitutive. Western-blotting analysis showed that MF2 bound to a ladder of proteins with apparent molecular masses in the range 35–200 kDa. Immunofluorescence studies showed that MF2 bound strongly to the cell walls of hyphae and phialides and the intercalary and terminal chlamydo-spores of *Trichoderma* spp., whereas immunogold electron microscopy revealed strong binding of MF2 to the cell walls and septa of hyphae and to the cell walls of phialoconidia. In immunofluorescence studies of dual cultures of *Trichoderma* and *Rhizoctonia solani*, only the cell walls of the hyperparasite, which coiled around the host, were stained by MF2. The specificity of MF2 enabled the development of a combined baiting–ELISA technique for the detection of *Trichoderma* spp. in naturally infested composts. The specificity of this technique was confirmed by phylogenetic analysis based on sequences of the ITS1–5.8S–ITS2 rRNA-encoding regions of the isolates.

Keywords: *Gliocladium*, β -1,3-glucanase, glucoamylase, ITS region

INTRODUCTION

Species of the genus *Trichoderma* Persoon are ubiquitous soil- and compost-borne saprotrophs, which have been exploited in the commercial production of enzymes

Abbreviations: IF, immunofluorescence; ITS, internally transcribed spacer; PAS, periodic acid Schiff.

(Cullen & Kersten, 1992) and in the biological control of plant diseases caused by economically important plant pathogens such as *Rhizoctonia solani* Kuhn and *Pythium ultimum* Trow (Papavizas, 1985; Whipps, 1997). *Trichoderma* spp. are the causative agent of disease in commercially produced mushrooms (Seaby, 1987), and have been identified as causal agents of disease in immunosuppressed and transplant patients (Jacobs et

al., 1992; Loepke *et al.*, 1983; Richter *et al.*, 1999; Tanis *et al.*, 1995).

Despite the enormous potential of *Trichoderma* spp. to control soil-borne plant pathogens, their widespread use as biological control agents has not been fully realized. One reason for this lack of utilization can be attributed to the absence, to date, of accurate and sensitive methods for the rapid detection and visualization of *Trichoderma* spp. in complex environments that contain mixed populations of fungi, such as soil or compost. Conventional methods for detecting *Trichoderma* propagules, which employ plate-enrichment techniques, do not always satisfactorily differentiate between *Trichoderma* spp. and contaminant fungi in mixed populations. Furthermore, these assays are labour intensive, cumbersome and require considerable taxonomic expertise. The mutation of *Trichoderma* strains to benomyl tolerance and the 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 are restricted to individual fungicide-tolerant or recombinant isolates (Ahmad & Baker, 1988; Pe'er *et al.*, 1991; Bae & Knudsen, 2000; Green & Jensen, 1995; Thrane *et al.*, 1995).

The development of a specific and sensitive assay for the detection and visualization of naturally occurring *Trichoderma* populations would enable a more accurate determination to be made of the presence of this important fungal genus in naturally infested soil and compost samples, and would facilitate studies of the spread and survival of *Trichoderma* populations artificially introduced into soils or composts. Furthermore, a procedure that also allowed the subsequent recovery of strains would aid the identification of isolates with potentially exploitable hyperparasitic activities.

Hybridoma technology allows the production of monoclonal antibodies (mAbs) that are specific to individual genera, species or even isolates of fungi (Dewey & Thornton, 1995). mAbs have been used successfully to detect, quantify and visualize the saprotrophic growth of pathogens, such as *Rhizoctonia solani*, in artificially and naturally infested soils (Dewey *et al.*, 1996; Otten *et al.*, 1997; Thornton *et al.*, 1993, 1999), and have been used to quantify the effects of the hyperparasite *Trichoderma harzianum* on the saprotrophic growth dynamics of *Rhizoctonia solani* in compost-based systems (Thornton & Gilligan, 1999). These studies showed that the most appropriate antigens for the detection of fungi in soils and composts were those that were extracellular and that were constitutively expressed or could be induced. Studies of extracellular antigen (enzyme) production in *Trichoderma* spp. have shown that the enzyme β -1,3-glucanase presents an ideal candidate for the production of mAbs specific to this genus. The enzyme is extracellular and its production is constitutive or semi-constitutive (Bull & Chesters, 1966; Elad *et al.*, 1982; Ramot *et al.*, 2000).

In this study, we used a commercial β -1,3-glucanase

preparation to develop a murine hybridoma cell line secreting mAbs specific for *Trichoderma* spp. and for fungi closely related to them. Using the mAb MF2, we show how the antigen can be used as a molecular marker for the detection and recovery of *Trichoderma* spp. in naturally infested composts.

METHODS

Fungal culture. Unless indicated otherwise, fungi were grown on Vogel's glucose agar (VGA) (Sandhu & Kalra, 1982) either in Petri dishes or on slants at 23 °C, with the exceptions of *Phytophthora fragariae* (grown on bean agar at 4 °C), *Armillaria* spp. and *Phytophthora erythroseptica* (grown on 2%, w/v, malt extract agar at 23 °C) and *Phytophthora cactorum* (grown on V8 agar, pH 7.0, at 23 °C).

Development of the mAb, preparation of the immunogen, and the immunization regime. Preparations of *Trichoderma* β -1,3-glucanase were obtained commercially. These included a chromatographically purified enzyme preparation from ICN (198904), a crude preparation from Sigma (L5272) and Glucanex from Novo Nordisk Fermentation. Preliminary examination of these preparations by PAGE and staining with Coomassie brilliant blue or the periodic acid Schiff (PAS) reaction (Carlsson, 1993) showed the chromatographically purified preparation from ICN to be composed of glycoprotein(s) of high molecular mass, consistent with the constitutive 200 kDa β -1,3-glucanase (G β -1,3-200) recently described by Ramot *et al.* (2000). Consequently, ICN 198904 was chosen for hybridoma production in an attempt to raise mAbs specific to a constitutive *Trichoderma* antigen. It is hereafter referred to as the immunogen.

Twenty-five units of lyophilized immunogen were reconstituted in 1 ml of PBS (0.8% NaCl; 0.02% KCl; 0.115% Na₂HPO₄; 0.02% KH₂PO₄; pH 7.2). Three 6-week-old BALB/c female white mice were each given four intraperitoneal injections (300 μ l per injection) of fresh immunogen at 2 week intervals. A single booster injection was administered 5 days before fusion.

Production and screening of hybridomas and the determination of antibody specificities. Hybridoma cells were produced by the method described by Dewey (1992) and the supernatants were screened by ELISA. Primary screening was performed against the immunogen. Twenty-five units of lyophilized protein were reconstituted in 30 ml PBS. Secondary screening for specificity was performed against surface washings from slant cultures of fungal isolates grown on VGA at 23 °C. Cultures were washed with 1 ml of PBS and the surface washings were centrifuged in 1.5 ml microcentrifuge tubes at 12000 *g* for 10 min to remove cell debris; the resulting supernatants were diluted 10-fold in fresh PBS. Microtitre wells arranged in strips (Labsystems Oy) were coated overnight at 4 °C with 50 μ l volumes of diluted immunogen or with 1/10 surface washings. The wells were washed four times with PBST (PBS + 0.05% v/v, Tween 20; Sigma) and once each with PBS and dH₂O. They were then air-dried at 23 °C under a laminar-flow hood. The strips were stored in sealed plastic bags at 4 °C in preparation for screening of the hybridoma supernatants by ELISA, as described below.

ELISA. Wells containing immobilized surface antigens were incubated with hybridoma supernatant for 1 h, followed by incubation with either a goat-anti-mouse polyvalent (IgG, IgA and IgM) peroxidase conjugate (diluted 1 in 1000; Sigma) or a goat-anti-mouse IgM (μ -chain specific) peroxidase conjugate

(diluted 1 in 5000; Sigma) for a further 1 h. Bound antibody was visualized by incubating the wells with tetramethyl benzidine substrate solution for 30 min; reactions were stopped by the addition of 3 M H₂SO₄. Absorbance values were determined at 450 nm with an MRX automated microplate reader (Dynerx Technologies). Wells were given four 5 min rinses with PBST between incubations. Working volumes were 50 µl per well, and control wells were incubated with tissue culture medium containing 10% (v/v) fetal calf serum. All incubation steps were performed at 23 °C in sealed plastic bags.

Determination of the immunoglobulin subclass and the cloning procedure. The immunoglobulin subclass of the mAbs was determined with a commercial mouse mAb isotyping kit, according to the manufacturer's instructions (Sigma). Hybridoma cell lines were cloned by limiting dilution and were grown in bulk in a non-selective medium. The cells lines were preserved by slowly freezing them in fetal bovine serum/dimethyl sulfoxide (92:8, v/v) and storing them in liquid nitrogen.

Epitope characterization by protease digestion. Microtitre wells containing immobilized immunogen (50 µl) were incubated with 0.25 U Pronase well⁻¹ (Protease XIV) (Sigma) or with trypsin (Sigma) solution (1 mg ml⁻¹ in PBS) at 37 °C or 4 °C for 5 h. The wells were then washed three times with PBS. Wells incubated with trypsin were treated for 10 min with a 0.1 mg ml⁻¹ solution of trypsin inhibitor (Sigma) and were given three more washes with PBS. Controls received PBS without Pronase or trypsin and inhibitor, but were otherwise treated similar to the test samples. Six replicates were performed for each treatment. The wells were assayed by ELISA with MF2, as described above.

Epitope characterization by periodate oxidation. Immobilized immunogen was treated with 50 µl of sodium *m*-periodate (20 mM NaIO₄ in 50 mM sodium acetate buffer, pH 4.5) (Sigma), whereas control wells received only buffer. After incubation for the appropriate time period in darkness at 4 °C, wells were washed three times with PBS and assayed by ELISA with MF2, as described. Four replicates were performed for each treatment.

Growth of mycelium and antigen production on laminarin and glucose. Glucose or laminarin (Sigma) were added (0.5 g) to 50 ml of Vogel's medium (VM) (Sandhu & Kalra, 1982), which had been adjusted to pH 5.5 with 1 M HCl in separate 250 ml flasks. The media were then sterilized by autoclaving at 121 °C for 15 min. The flasks were inoculated with three plugs (3 mm diameter) of mycelium taken from the leading edge of 4-day-old VGA cultures of *Trichoderma koningii* isolate TLS grown at 25 °C. The flasks were incubated as shake cultures at 25 °C, and at 3 day intervals 100 µl of culture fluid was removed from each flask. Culture fluids were centrifuged for 5 min at 12000 g and 50 µl samples were transferred to microtitre wells. There were three replicates for each treatment and the experiment was performed twice. Absorbance values in ELISA were converted to units of protein equivalents by using standard calibration curves of immunogen, prepared from doubling dilutions of a PBS solution of the immunogen in microtitre wells. After immobilization and washing of the samples, wells were assayed by ELISA with MF2 or with tissue culture medium only, as described above. An ELISA of calibration curves was performed on the same day as the assay of the culture extracts, to eliminate day-to-day variability in results (Otten *et al.*, 1997). The dry weights of mycelial mats were obtained by filtering the contents of each flask through weighed Whatman filter paper no. 1 and drying the collected material to constant weight at 80 °C.

In a separate experiment, glucose and laminarin cultures were prepared as described above and were incubated for 5 days under the same conditions. Mycelia were removed by filtration and the culture filtrates were immediately precipitated with 80% acetone. The precipitate was recovered by centrifugation at 27000 g for 45 min at 4 °C. The concentrated samples were resuspended in dH₂O, snap-frozen in liquid N₂ and lyophilized. Lyophilized material was used as a source of β-1,3-glucanase. There were two replicates for each treatment and the experiment was performed twice. Samples were assayed for β-1,3-glucanase and amylase activities, as described below. Protein concentrations were determined using the Bio-Rad Bradford protein assay, with BSA (Sigma) as the standard.

Assay of β-1,3-glucanase activity and amylase activity. The standard assay mixture (vol. 500 µl) contained protein concentrate reconstituted in 50 mM sodium acetate buffer (pH 5.0) and 5 mg of laminarin or soluble potato starch (Sigma) per ml. Reaction mixtures were incubated for 1 h at 50 °C. The reactions were stopped by boiling for 5 min, and the production of reducing sugars (glucose) was determined by using a commercial glucose oxidase assay (Sigma). An enzymic unit was defined as the amount of enzyme that catalysed the release of 1 mg glucose (mg protein)⁻¹ min⁻¹. Standards for glucose, as well as for enzyme and substrate blanks, were also included.

PAGE and Western blotting. PAGE was carried out by using the system of Laemmli (1970), with 4–20% gradient polyacrylamide gels (Bio-Rad) under denaturing conditions. Denatured samples were prepared by heating at 95 °C for 10 min in the presence of β-mercaptoethanol, prior to gel loading. Proteins were separated for 1.5 h at room temperature (165 V). Pre-stained, broad-range markers (Bio-Rad) were used for molecular mass determinations. Gels were stained for total protein with Coomassie brilliant blue or by the carbohydrate-specific PAS reaction.

For Western blots, separated proteins were electrophoretically transferred to a PVDF membrane (Immuno-Blot PVDF; Bio-Rad). Membranes were washed three times with PBS and then blocked for 16 h at 4 °C with PBS containing 1% (w/v) BSA. Blocked membranes were incubated with mAb supernatant diluted 1 in 2 with PBS containing 0.5% BSA (PBSA) for 2 h at 23 °C. After washing three times with PBS, membranes were incubated for 1 h with a goat-anti-mouse IgM (µ-chain specific) alkaline phosphatase conjugate (Sigma) diluted 1 in 15000 in PBSA. Membranes were washed twice with PBS and once with PBST. Bound antibody was visualized by incubation in substrate solution (Thornton *et al.*, 1993). Reactions were stopped by immersing the membranes in dH₂O; the membranes were then air-dried between sheets of Whatman filter paper. Labelling of the glycosylated proteins immobilized on the PVDF membranes was performed using a commercial glycoprotein detection kit, according to the manufacturer's instructions (Bio-Rad).

N-terminal sequence analysis. N-terminal sequence analysis of the antigen was carried out by Alta Bioscience with a PE Biosystems 473A automatic protein sequencer. The gapped BLAST program (version 2.0; Altschul *et al.*, 1997) was used to compare the amino acid sequences we generated with those contained within databases held at the NCBI website (<http://www.ncbi.nlm.nih.gov>).

Indirect immunofluorescence (IF). Cultures of *Trichoderma* isolate CST1, *Trichoderma* isolate S-B2 and *Trichoderma virens* isolate J13-A8 were grown for 4 days at 25 °C on slides embedded in VGA, either in pure culture or in dual culture with *Rhizoctonia solani* isolate AB1. The slides were air-dried and fixed according to Dewey (1992). Wells were incubated

Table 1. Details of ELISA results for fungal isolates screened in this study

The results presented here are representative of three replicate experiments.

Organism	Isolate no.*	Source†	A ₄₅₀ ‡
<i>Armillaria mellea</i>	AM1	D. Farley	0.023
<i>Aspergillus flavus</i>	91856ii	IMI	0.002
<i>Aspergillus fumigatus</i>	181	J. Webster	0.000
<i>Aspergillus oryzae</i>	29	J. Webster	0.010
<i>Botryotrichum piluliferum</i>	43	J. Webster	0.000
<i>Botrytis cinerea</i>	R2/239	J. Webster	0.005
<i>Botrytis cinerea</i>	WCC-A8	C. R. Thornton	0.001
<i>Ceratobasidium cornigerum</i>	34054	ATCC	0.000
<i>Chaetomium indicum</i>	182	J. Webster	0.000
<i>Clonostachys rosea</i>	710.86	CBS	0.007
<i>Clonostachys solani</i>	102418	CBS	0.008
<i>Curvularia clavata</i>	239	J. Webster	0.001
<i>Curvularia leonensis</i>	236	J. Webster	0.000
<i>Fusarium culmorum</i>	HBC-A3	C. R. Thornton	0.022
<i>Fusarium oxysporum</i> f.sp. <i>pisi</i>	260.50	CBS	0.008
<i>Fusarium solani</i> f.sp. <i>pisi</i>	231.34	CBS	0.002
<i>Gaeumannomyces graminis</i> var. <i>avenae</i>	GP36	G. L. Bateman	0.000
<i>Gaeumannomyces graminis</i> var. <i>graminis</i>	D1	C. R. Thornton	0.000
<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	16/8	G. L. Bateman	0.001
<i>Gliocladium catenulatum</i>	529.80	CBS	0.044
<i>Gliocladium roseum</i>	710.86	CBS	0.021
<i>Gliocladium viride</i>	228.48	CBS	1.189
<i>Hypocrea gelatinosa</i>	254.62	CBS	1.350
<i>Hypocrea pallida</i>	668.75	CBS	0.395
<i>Hypocrea lutea</i>	658.70	CBS	1.316
<i>Hypocrea rufa</i>	435.95	CBS	1.272
<i>Hypomyces chrysospermus</i>	140.23	CBS	1.466
<i>Mucor plumbeus</i>	96	J. Webster	0.000
<i>Mucor racemosus</i>	93	J. Webster	0.003
<i>Nectria ochroleuca</i>	245.78	CBS	0.011
<i>Penicillium expansum</i>	106	J. Webster	0.005
<i>Penicillium</i> sp.	HBC-A9	C. R. Thornton	0.006
<i>Phytophthora cactorum</i>	–	D. Pitt	0.003
<i>Phytophthora erythroseptica</i>	–	D. Pitt	0.002
<i>Phytophthora fragariae</i>	–	D. Pitt	0.000
<i>Pythium aphanidermatum</i>	WQ4	C. R. Thornton	0.008
<i>Pythium debaryanum</i>	–	D. Pitt	0.002
<i>Pythium irregulare</i>	287.31	CBS	0.011
<i>Pythium oligandrum</i>	PO1	C. R. Thornton	0.003
<i>Pythium spinosum</i>	FHS12	C. R. Thornton	0.004
<i>Pythium sylvaticum</i>	227.68	CBS	0.014
<i>Pythium ultimum</i>	PU132	D. Pitt	0.001
<i>Rhizoctonia cerealis</i>	SL4/2	P. Nicholson	0.003
<i>Rhizoctonia oryzae</i>	376335	IMI	0.004
<i>Rhizoctonia oryzae-sativae</i>	62599	IMI	0.005
<i>Rhizoctonia solani</i> anastomosis group			
1	01R01	IPO	0.001
2.1	21R01	IPO	0.000
2.2	22R11	IPO	0.002
3	03R01	IPO	0.000
4	AB1	C. R. Thornton	0.003
5	303159	IMI	0.000
7	303161	IMI	0.000
<i>Rhizopus sexualis</i>	–	C. R. Thornton	0.000
<i>Rhizopus stolonifer</i>	–	C. R. Thornton	0.001
<i>Rhizopus</i> sp.	S-A4	C. R. Thornton	0.005
<i>Sordaria fimicola</i>	152	J. Webster	0.002
<i>Sphaerostilbella aureonitens</i>	93.2121	CBS	1.214
<i>Sphaerostilbella lutea</i>	225.85	CBS	0.005

Table 1 (cont.)

Organism	Isolate no.*	Source†	$A_{450}‡$
<i>Sphaerostilbella novaezelandiae</i>	93.0907	CBS	1.093
<i>Syncephalastrum racemosum</i>	155	J. Webster	0.001
<i>Thamnidium elegans</i>	161	J. Webster	0.000
<i>Trichoderma aureoviride</i>	–	D. Pitt	0.645
<i>Trichoderma hamatum</i>	091	D. Pitt	0.903
<i>Trichoderma hamatum</i>	GOD-B4§	C. R. Thornton	0.920
<i>Trichoderma hamatum</i>	GOD-B7§	C. R. Thornton	0.934
<i>Trichoderma harzianum</i>	LEV-A8§	C. R. Thornton	1.132
<i>Trichoderma harzianum</i>	LEV-B7§	C. R. Thornton	1.067
<i>Trichoderma harzianum</i>	–	P. Mills	1.251
<i>Trichoderma harzianum</i>	–	D. Pitt	1.347
<i>Trichoderma harzianum</i>	134	D. Pitt	1.340
<i>Trichoderma harzianum</i>	136	D. Pitt	1.117
<i>Trichoderma harzianum</i>	T95	R. Baker	1.380
<i>Trichoderma koningii</i>	TLS§	C. R. Thornton	1.261
<i>Trichoderma koningii</i>	F5	D. Pitt	1.305
<i>Trichoderma koningii</i>	F10	D. Pitt	1.281
<i>Trichoderma koningii</i>	F14	D. Pitt	1.097
<i>Trichoderma koningii</i>	F16	D. Pitt	1.099
<i>Trichoderma koningii</i>	F17	D. Pitt	1.358
<i>Trichoderma koningii</i>	F62	D. Pitt	1.490
<i>Trichoderma longibrachiatum</i>	–	P. Mills	1.501
<i>Trichoderma longibrachiatum</i>	399.92	CBS	1.106
<i>Trichoderma longibrachiatum</i> (variant)	–	P. Mills	1.515
<i>Trichoderma piluliferum</i>	185209	IMI	1.270
<i>Trichoderma reesei</i>	–	P. Mills	1.446
<i>Trichoderma virens</i>	625.76	CBS	1.090
<i>Trichoderma virens</i>	J13-A8§	C. R. Thornton	1.103
<i>Trichoderma virens</i>	J13-B7§	C. R. Thornton	1.175
<i>Trichoderma viride</i>	089	D. Pitt	1.523
<i>Trichoderma viride</i>	–	P. Mills	1.334
<i>Trichoderma viride</i>	BUL-A7§	C. R. Thornton	1.283
<i>Trichoderma viride</i>	HBC-B4§	C. R. Thornton	1.308
<i>Trichoderma viride</i>	S-A8§	C. R. Thornton	0.861
<i>Trichoderma viride</i>	S-B3§	C. R. Thornton	0.952
<i>Trichoderma viride</i>	S-B9§	C. R. Thornton	1.085
<i>Trichoderma</i> sp.	HBC-A2	C. R. Thornton	0.557
<i>Trichoderma</i> sp.	S-B2	C. R. Thornton	0.504
<i>Trichoderma</i> sp.	CST1	C. R. Thornton	1.213
<i>Trichothecium roseum</i>	168	J. Webster	0.049
<i>Verticillium lecanil</i>	167	J. Webster	0.043
<i>Verticillium</i> sp.	HBC-A7	C. R. Thornton	0.009
<i>Zygorrhynchus moelleri</i>	165	J. Webster	0.000
<i>Zygorrhynchus moelleri</i>	HBC-A1	C. R. Thornton	0.005

* Isolates from commercial compost preparations: WCC, Westcountry; HBC, Homebase; S, Shamrock; GOD, Godwin; LEV, Levington; BUL, Bulrush; J13, John Innes no. 3. Contaminating fungi recovered from baits of naturally infested composts are shown in bold. *Trichoderma* isolates recovered from baits of naturally infested composts are shown in italic.

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‡ Values represent the means of replicated values corrected by subtraction of control values.

§ Species identified in this study by sequencing of the ITS region of the rRNA-encoding genes.

for 30 min with 50 µl of MF2 or with 50 µl of tissue culture medium only. Slides were washed three times with PBS with gentle agitation and were incubated for a further 30 min with a goat-anti-mouse polyvalent FITC conjugate (Sigma) diluted 1 in 40 in PBS. Slides were given three 5 min rinses with PBS, and the wells were overlaid with coverslips mounted with PBS/glycerol mounting medium (Sigma). Slides were examined with a Nikon microscope fitted with epifluorescence, using a UV excitation filter (365 nm) and an absorption filter (420 nm). All incubation steps were performed at 23 °C in a moist environment and the slides were stored at 4 °C in the dark in Petri dishes containing moistened Whatman filter paper no. 1.

In a separate experiment, suspensions of washed phialoconidia from *Trichoderma* isolate CST1 and *Trichoderma koningii* isolate TLS were prepared from 4-week-old V8 agar Petri dish cultures. The washed cells were suspended in VM and transferred to the wells of multiwell slides. After incubation at 25 °C for 16 h, the slides were air-dried and fixed as described above. Antigen production by germinated and ungerminated spores was detected by IF as described.

Immunogold electron microscopy. Immunogold labelling was performed with mycelium and phialoconidia from *Trichoderma koningii* isolate TLS. Samples of mycelium were removed from 5-day-old liquid cultures of *Trichoderma koningii* isolate TLS grown in VM containing glucose. The mycelium was homogenized in dH₂O and washed twice by repeated centrifugation and resuspension in dH₂O. Washed conidia were prepared as described. Mycelium and conidia were pelleted by centrifugation. The pellet was embedded at low temperature as described in Cole *et al.* (1998), except that the resin polymerization steps were carried out at 23 °C. Immunolabelling was carried out as described in Cole *et al.* (1998), with a goat-anti-mouse 20 nm gold conjugate (British Biocell International) as the secondary reporter molecule. Control grids were incubated with tissue culture medium instead of mAb supernatant, but were otherwise treated the same as the test grids.

Detection of *Trichoderma* spp. in naturally infested composts by baiting-ELISA, and setting of immunoassay thresholds. Commercial compost preparations were obtained from horticultural suppliers. Bulrush, Homebase, Levington and Shamrock composts consisted of sphagnum-moss peat, whereas Godwin compost was a mixture of sedge and sphagnum-moss peats. Westcountry compost was a greenwaste compost and composts John Innes No. 1, No. 2 and No. 3 consisted of peat compost and loam soil in varying proportions.

Detection of *Trichoderma* spp. in naturally infested composts using MF2 was performed by using a combined baiting-ELISA method, using the baiting technique described by Thornton *et al.* (1999). After incubation at 25 °C for 72 h, quinoa seed baits were removed from replicated baiting modules and placed individually into microtitre wells containing 50 µl of VM containing 1 mg glucose ml⁻¹. The seeds were incubated for 7 h at 25 °C to allow the production and immobilization of extracellular antigens. They were then transferred to Petri dishes containing medium for the recovery of *Trichoderma* isolates and other compost fungi (see below). Microtitre wells were washed and then assayed by ELISA using MF2 and a µ-chain-specific peroxidase conjugate, as described above. Because the specific aim of this study was to examine the efficacy of the assay for detecting *Trichoderma* spp. in the presence of contaminating fungi, no assay of baits from sterilized composts was performed.

Thresholds for the detection of *Trichoderma* spp. by ELISA were established by plotting the frequencies of the absorbance

values obtained in ELISAs for each compost against stepwise increments (0-100) in absorbance (Thornton *et al.*, 1999).

Plate culture. Quinoa seed baits were transferred to 9 cm Petri dishes containing *Trichoderma* TME medium (Papavizas, 1982). The initial identification of putative isolates of *Trichoderma* spp. was based on the presence of phialides and phialoconidia. Putative *Trichoderma* isolates were further subcultured onto V8 agar and were classified to the species level by sequencing of their rRNA genes. Isolates of other fungi that commonly grew from the baits were also subcultured and identified on the basis of gross morphological characteristics and sporulation, using standard mycological texts (Domsch *et al.*, 1980).

Confirmation of the isolation of *Trichoderma* spp. by analysis of the internally transcribed spacer (ITS) regions of the rRNA-encoding gene unit. Representative isolates of fungi, detected and recovered from each of the naturally infested composts using MF2, were selected for further study on the basis of gross morphology, and their rRNA-encoding genes were amplified using a modification of the method described by Sreenivasaprasad *et al.* (1996). Primers ITS1 (5'-TCCGTA-GGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTAT-TGATATGC-3') were used to amplify the ITS1-5.8S-ITS2 region of the rRNA-encoding gene unit of each of the representative isolates. These primers were provided by Genosys and their sequences are based on those described by White *et al.* (1990). PCR of the the ITS1-5.8S-ITS2 region was performed as follows. Approximately 10-25 ng genomic DNA was added to a 0.2 ml Eppendorf tube that had been pre-chilled to -20 °C and which contained 0.05 µM each primer, 2 mM (each) dATP, dCTP, dGTP and dTTP (Pharmacia), 0.25 U *Taq* polymerase (Promega), 1 × *Taq* polymerase thermophilic buffer (Promega) and 2 mM MgCl₂ (Promega). The final reaction volume was 25 µl. PCR amplification was performed in a thermal cycler (Hybaid Omn-E) with the following cycling conditions: 2 min at 94 °C, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with a final extension of 5 min at 72 °C. PCR products were separated using a 1.6% agarose gel. The DNA fragments obtained were purified from gels using a commercial kit (GENECLEAN; BIO 101), according to the manufacturer's instructions. Both strands of the entire ITS1-5.8S-ITS2 rRNA-encoding region were sequenced. Dye-labelled terminator cycle sequencing was performed by MWG Biotech AG (Ebersberg, Germany) and the results were processed using a Macintosh Power PC (7500/100). Double-stranded DNA sequences were aligned by using DNA Strider 1.1 (Marck, 1989) and Sequence Navigator 1.0.1 (Perkin Elmer). The gapped BLAST program (version 2.0; Altschul *et al.*, 1997) was used to compare the nucleic acid sequences we generated with those contained within databases held at the NCBI website. Multiple-sequence alignments were performed using the MULTALIGN program (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>) (Thompson *et al.*, 1994). Phylogenetic analysis was performed using the neighbour-joining method, as contained within the PAUP* (version 4.0; Swofford, 2000) package. The significance of each of the nodes within the phylogenetic tree was determined by bootstrap analysis, with 1000 replications. The ascomycete *Echinodothis tuberiformis* was used as the out-group species for the phylogenetic tree shown in Fig. 7.

RESULTS

Specificity of the hybridoma cell lines and isotyping

A single fusion was performed and from this 434 hybridoma cell lines were screened for mAb produc-

Table 2. Absorbance values from ELISAs (with MF2) for protease-treated and periodate-treated *Trichoderma harzianum* immunogen

Each value represents the mean of replicated values \pm 95% confidence intervals corrected by subtraction of control values. The results are based on six replicated experiments.

Temperature (°C)	A_{450}			
	Pronase	Control	Trypsin	Control
4	0.533 \pm 0.108	1.546 \pm 0.057	0.861 \pm 0.103	1.525 \pm 0.051
37	0.375 \pm 0.075	1.517 \pm 0.057	0.669 \pm 0.098	1.519 \pm 0.044

Time (h)	A_{450}	
	Periodate	Control
20	0.529 \pm 0.076	1.531 \pm 0.069
4	1.167 \pm 0.034	1.556 \pm 0.054
3	1.283 \pm 0.057	1.457 \pm 0.169
2	1.421 \pm 0.039	1.581 \pm 0.071
1	1.454 \pm 0.106	1.485 \pm 0.153

tion; all tested positive for recognition of the immunogen (β -1,3-glucanase; ICN 198904). One of the mAbs produced (MF2) was selected for further testing on the basis of its high absorbance value ($A_{450} > 1.500$). In secondary specificity screening tests, MF2 reacted strongly in an ELISA against surface antigens from all of the *Trichoderma* isolates tested and with antigens from the closely related fungi *Gliocladium viride*, *Hypocrea rufa*, *Hypocrea gelatinosa*, *Hypocrea lutea*, *Hypomyces chrysospermus*, *Sphaerostilbella aureonitens* and *Sphaerostilbella novaezelandiae* (Table 1). MF2 reacted weakly with surface antigens from *Hypocrea pallida* and did not react with surface antigens from the related fungi *Clonostachys rosea*, *Clonostachys solani*, *Gliocladium roseum*, *Gliocladium catenulatum* (*Gliocladium roseum* var. *viride*), *Nectria ochroleuca* and *Sphaerostilbella lutea*. MF2 did not react with surface antigens from a broad range of soil- and compost-borne fungi unrelated to *Trichoderma* spp. In all cases the absorbance value at 450 nm in ELISAs was < 0.100 . The MF2 cell line was subcloned twice.

Isotyping of MF2 showed that it belonged to immunoglobulin class M (IgM).

Characterization of the antigen and the effect of protease and periodate

Reductions in MF2 binding following digestion of the immobilized immunogen with protease showed that the antibody binds to a protein epitope (Table 2). More specifically, the sensitivity of the epitope to trypsin indicated that MF2 binds to an antigenic determinant that contains residues of the amino acids serine and

threonine. The sensitivity of the immunogen to periodate (Table 2) showed that the epitope forms part of a larger glycoprotein molecule.

Growth of mycelium and antigen production on glucose and laminarin

MF2 exhibited strong recognition of the immunogen in ELISAs. There was a typical sigmoidal relationship between the \log_{10} protein concentration and the absorbance at 450 nm, with a limit of detection of approximately 0.5 ng protein (ml buffer) $^{-1}$ (Fig. 1a). Growth of *Trichoderma koningii* TLS mycelium in liquid medium containing glucose rose steadily for 6 days after inoculation, until a plateau in growth was reached between days 6 and 9. Thereafter, growth declined up to day 12 (Fig. 1b). Growth in the presence of laminarin was similar, albeit less over the sampling period; however, following a peak in biomass at day 6, there was a steady decline in mycelium production up to day 12. Temporal production of the MF2 antigen by *Trichoderma koningii* TLS grown in liquid medium containing either glucose or laminarin was similar (Fig. 1c). In both cases, antigen production increased up to 3 days post-inoculation, after which time production declined up to day 6 on laminarin and up to day 9 on glucose. Thereafter, the antigen concentrations present in cultures grown in both media increased up to 12 days post-inoculation.

Enzyme activity assays

The chromatographically purified β -1,3-glucanase preparation from ICN (immunogen) contained 3.1 ± 0.0 and

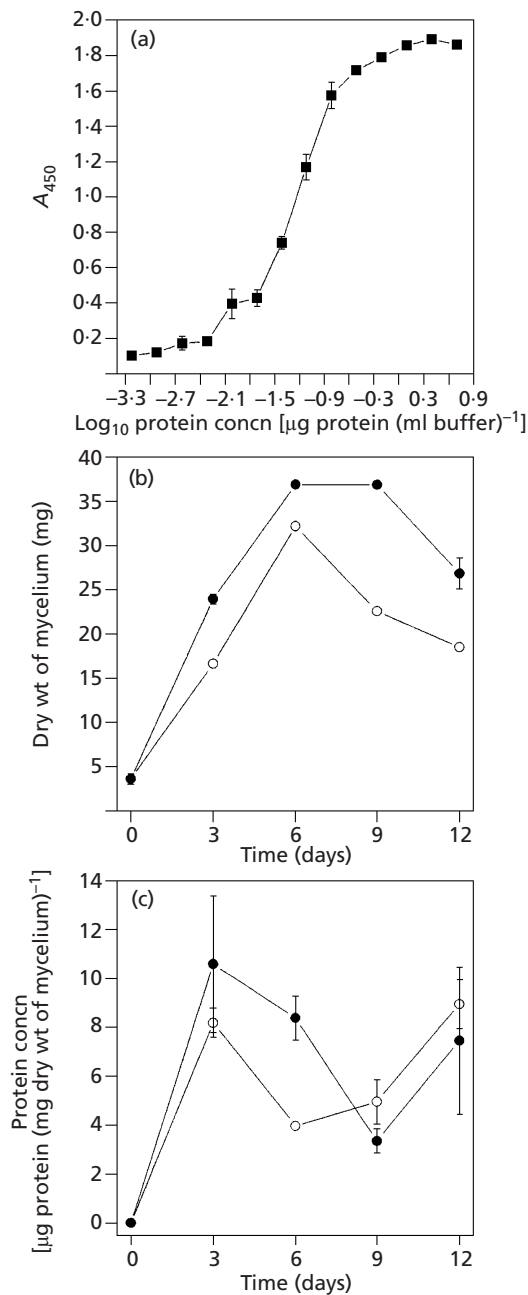


Fig. 1. (a) Standard calibration curve for the immunogen. (b) Growth of *Trichoderma koningii* isolate TLS in liquid cultures containing glucose (●) and laminarin (○). (c) Antigen production by *Trichoderma koningii* isolate TLS in liquid cultures containing glucose (●) and laminarin (○). All points are the mean of three replicate samples \pm SE. The antigen contents of the filtrates were determined by converting the absorbance values obtained from ELISAs (with MF2) to equivalents of protein concentration using the standard calibration curve.

3.2 ± 0.1 units of β -1,3-glucanase and amylase activity, respectively. Protein samples concentrated from 5-day-old laminarin culture filtrates of *Trichoderma koningii* TLS contained 15.1 ± 0.2 and 17.6 ± 0.3 units of β -1,3-glucanase and amylase activity, respectively. Protein samples concentrated from glucose cultures contained

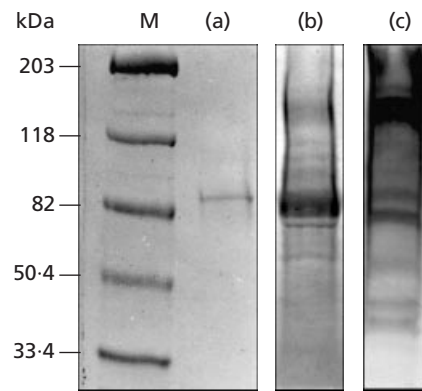


Fig. 2. Analysis of chromatographically purified β -1,3-glucanase preparation by PAGE and Western-blotting analyses. M represents the molecular mass marker. (a) Denaturing SDS-PAGE followed by staining for protein with Coomassie blue. (b) Denaturing SDS-PAGE followed by staining with the carbohydrate-specific PAS reaction, for the detection of glycoproteins. (c) Western immunoblot (with MF2) following separation of the immunogen by SDS-PAGE under reducing conditions. All wells were loaded with $1.25 \mu\text{g}$ protein.

Immunogen	1	A V D D F I N T Q - P I A L N N L L	17
<i>H. grisea</i>	32	<u>A</u> V D T F I N T E K P I A W N K L L	49
<i>N. crassa</i>	36	S <u>V</u> D S Y I Q T E T P I A Q K <u>N</u> L L	53

Fig. 3. Comparison of the N-terminal-amino-acid sequence of the purified β -1,3-glucanase preparation (immunogen) with amino acid sequences held in the NCBI databases. The sequences from *Humicola grisea* and *Neurospora crassa* shared 72% and 55% identity, respectively, with the immunogen. Identical amino acids are underlined.

3.6 ± 0.1 and 6.6 ± 0.3 units of β -1,3-glucanase and amylase activity, respectively.

PAGE and Western blotting

Denaturing PAGE of the purified immunogen followed by staining with Coomassie blue revealed a single protein with a molecular mass of approximately 90 kDa (Fig. 2a). Staining of replica gels with the PAS reaction, for the detection of carbohydrate residues in glycoproteins, revealed an intensely stained, diffuse band in the range 80–90 kDa, a diffuse band at approximately 150 kDa and additional weaker bands in the ranges 90–120 and 30–50 kDa (Fig. 2b). All bands were red, indicating the presence of highly glycosylated proteins. No other bands were detected using this technique over a range of protein concentrations, and silver staining did not improve the sensitivity of detection (results not shown). Western-blotting analysis of the immunogen under denaturing conditions showed that MF2 bound to two bands with molecular masses in the range 80–90 kDa, a diffuse band at approximately 150 kDa and two weaker bands in the range 30–50 kDa (Fig. 2c).

N-terminal sequence analysis

Comparison of the N-terminal amino acid sequence of the immunogen with amino acid sequences held in the NCBI databases revealed close identity with glucoamylases (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) from *Humicola grisea* and *Neurospora crassa* (Fig. 3). Sequence alignment of the immunogen with the glucoamylases of *Humicola grisea* and *Neurospora crassa* showed 72% and 55% amino acid identity, respectively.

IF and immunogold electron microscopy

In IF studies, there was strong recognition by MF2 of the cell walls of the mycelium and phialides of *Trichoderma* sp. CST1 (Fig. 4a, b) and *Trichoderma virens* JI3-A8 (Fig. 4c, d). There was no binding by MF2 to the mature phialoconidia of either fungus, but binding to the cell walls of developing conidia was apparent (Fig. 4d). Strong binding by MF2 to the intercalary and terminal chlamydospores of *Trichoderma* sp. CST1 was also observed (Fig. 4e, f). There was intense staining of the cell walls of germ tubes of *Trichoderma* sp. CST1 by MF2, but no staining of the conidium was observed (Fig. 4g, h). Strong binding of MF2 to the germ tubes and conidia of *Trichoderma koningii* isolate TLS was observed (Fig. 4i, j).

In dual culture studies with *Trichoderma* sp. S-B2 and *Rhizoctonia solani* isolate AB1, there was intense staining of the *Trichoderma* sp. S-B2 hyphae that coiled around the *Rhizoctonia solani* mycelium (Fig. 5a, b). The *Rhizoctonia solani* mycelium was not stained by MF2 (Fig. 5b).

In immunogold electron microscopy studies with MF2, intense staining of the cell walls and septa of hyphae of the mycelium of *Trichoderma koningii* isolate TLS was observed when an immunogold conjugate was used as a secondary reporter (Fig. 6a). Strong staining was also exhibited by the cell walls of *Trichoderma koningii* TLS phialoconidia (Fig. 6c). There was no staining of control samples by the immunogold conjugate (Fig. 6b, d).

Setting of thresholds for the baiting-ELISA method

A threshold absorbance value was determined for each of the composts tested, above which individual baits in ELISAs were assigned as positive for the presence of *Trichoderma* spp. Threshold values were determined by plotting the frequencies of absorbance values obtained from each compost against stepwise increments (0-100) in absorbance (Thornton *et al.*, 1999). The histograms generated were examined for discontinuity in the distribution of absorbance, with a tight clustering of samples expected at low absorbance, indicative of background values for samples with no *Trichoderma* spp., and higher values for absorbance indicative of the presence of a variable amount of *Trichoderma* spp. All baits that were colonized with fungi other than *Trichoderma* spp., or those that were uncolonized, consistently gave absorbance values <0.100. These were identified

as background values. Quinoa seed baits that were colonized with fungi positively identified as *Trichoderma* spp. gave absorbance values in ELISAs that were consistently >0.100. Consequently, the threshold value of 0.100 was set, above which baits were considered to be positive for the presence of *Trichoderma* spp. Formal statistical analysis of thresholds, by fitting probability distributions and setting cut-off values, was precluded because of the absence of independent data for populations of test composts with and without *Trichoderma* spp. present.

Immunoassay of the baits from naturally infested composts

For each of the nine composts, the number of baits positive for *Trichoderma* spp., fixed by a threshold absorbance value of 0.100, was determined in a total of 18 baits (distributed between two replicate dishes, A and B, for each compost). In all cases, results from the ELISAs were in close agreement with those from the plate-culture assays (Table 3). Differences in absorbance values were most likely due to variations in the fungal biomass present on individual baits.

Plate culture of baits

Following assay by ELISA, baits were removed from the microtitre wells and were transferred to TME medium. The number of baits colonized by fungal contaminant(s), either in the presence or absence of *Trichoderma* spp., after 3 days exposure to the compost surfaces varied (Table 3). The predominant contaminating fungi in all of the composts examined were identified as species belonging to the genera *Botrytis*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Verticillium* and *Zygorrhynchus*. The number of quinoa seed baits colonized by *Trichoderma* isolates was in close agreement with the results from the ELISA (Table 3). The greater number of positive identifications by the ELISA compared to the plate-culture assay was due to exclusion of *Trichoderma* isolates by faster-growing fungal contaminants. It was for this reason that not all *Trichoderma* isolates could be recovered from baits that tested positive in ELISA (Table 3). Surface antigens were prepared from all of the *Trichoderma* isolates and from representative isolates of contaminating fungi recovered from the baits. These were tested by ELISA to confirm the recognition of recovered *Trichoderma* isolates by MF2 and to corroborate the lack of recognition of contaminating fungi by MF2. All of the recovered *Trichoderma* isolates tested positive in an ELISA with MF2, with no detection of contaminating fungi by the mAb (Table 1).

Confirmation of identity of isolates as *Trichoderma* spp. by ITS region sequencing

Representative isolates of fungi, detected and recovered from naturally infested composts using MF2, were

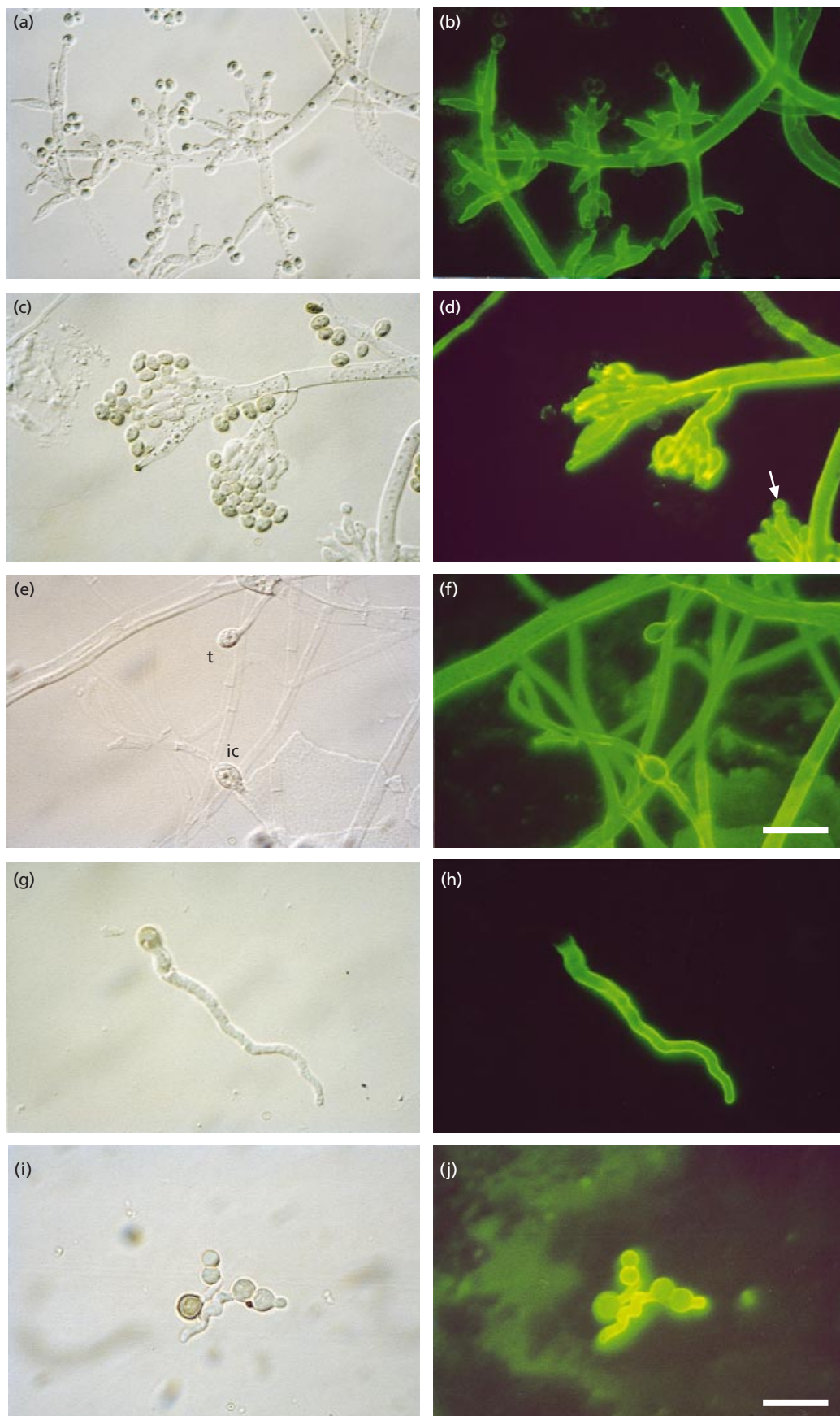


Fig. 4. For legend see facing page.

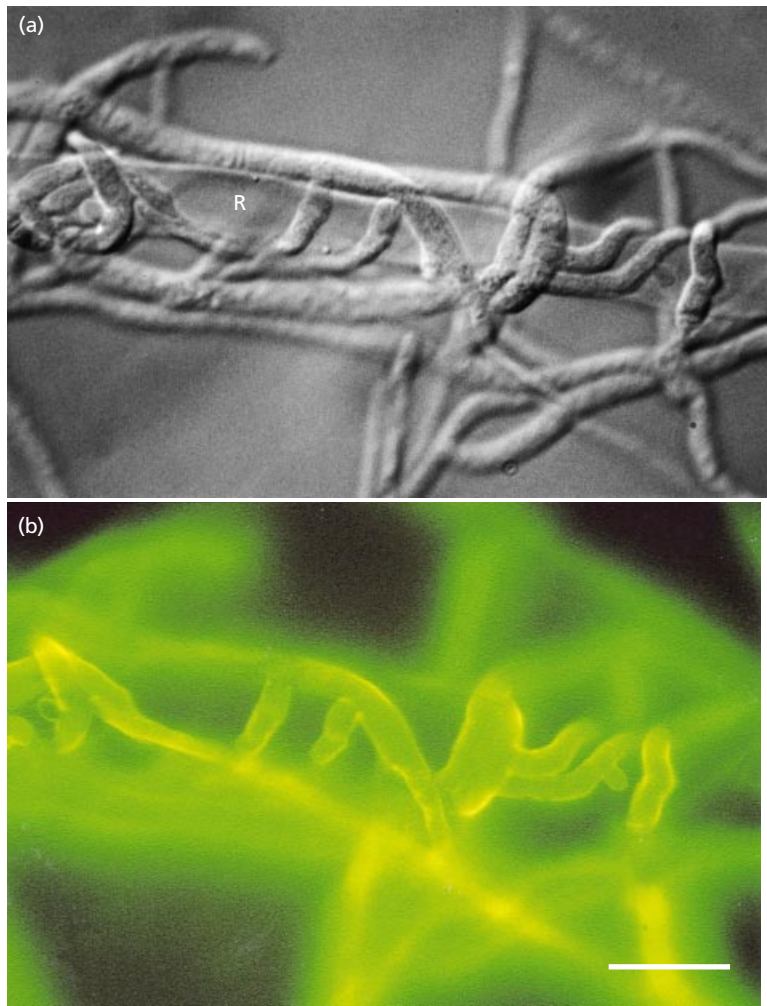


Fig. 5. Photomicrographs of *Trichoderma* isolate S-B2 and *Rhizoctonia solani* isolate AB1 grown on glass slides in dual culture, and immunostained with MF2 and anti-mouse polyvalent immunoglobulin FITC. (a) Mycelium examined under bright-field microscopy, showing coiling of the hyperparasite *Trichoderma harzianum* around hypha of its host, *Rhizoctonia solani* (R). (b) Same slide as in (a), but examined under epifluorescence. The intense staining of the cell walls of the *Trichoderma* mycelium and the unstained hypha of *Rhizoctonia solani* can be seen. Bar, 10 μ M.

confirmed as *Trichoderma* spp. by comparison of their nucleic acid sequences (ITS1–5.8S–ITS2 rRNA-encoding regions) with those contained within databases held at the NCBI website (Table 4). The phylogeny of the *Trichoderma* isolates tested is shown in Fig. 7. The *Trichoderma* isolates were distributed amongst five clades that equated to five species, namely *Trichoderma asperellum*, *Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma virens* and *Trichoderma viride*. Isolates CST1 and TLS were *Trichoderma* spp. used in this study, but whose affiliation with the *Trichoderma* fungi had not previously been determined. Isolate TLS (accession no. AF483581) fell within a clade

consisting of a single isolate of *Trichoderma koningii* and was therefore referred to as *Trichoderma koningii* isolate TLS. Isolate CST1 (accession no. AF483580) fell within a clade consisting of *Hypocrea vinosa*, *Trichoderma asperellum*, *Trichoderma harzianum* and *Trichoderma viride* and *Trichoderma* isolate S-B2, recovered in this study from naturally infested Shamrock compost. Consequently, CST1 and S-B2 are referred to in the text as *Trichoderma* isolates CST1 and S-B2 or in tables as *Trichoderma* sp. Ambiguity also exists with respect to the identification of HBC-A2, isolated from Homebase compost, and it is therefore referred to in Tables 1 and 4 as *Trichoderma* sp. HBC-A2.

Fig. 4. Photomicrographs of *Trichoderma* sp. CST1 cells and *Trichoderma virens* cells immunostained with MF2 and anti-mouse polyvalent immunoglobulin FITC. (a) Hyphae, phialides and phialoconidia of *Trichoderma* CST1 examined under bright-field microscopy; (b) same slide as in (a), but examined under epifluorescence. (c) Hyphae, phialides and phialoconidia of *Trichoderma virens* isolate J13-A8 examined under bright-field microscopy; (d) same slide as in (c), but examined under epifluorescence. The mature conidia were not stained, but the cell wall of an immature conidium exuded from the tip of a phialide was (shown by an arrow). (e) Intercalary (ic) and terminal (t) chlamydospores of *Trichoderma* sp. CST1 examined under bright-field microscopy; (f) same slide as in (e), but examined under epifluorescence. (g) Germinated conidium of *Trichoderma* sp. CST1 examined under bright-field microscopy; (h) same slide as in (g), but examined under epifluorescence. The cell wall of the germ tube was intensely stained, but no staining of the conidium was seen. (i) Germinated and ungerminated conidia of *Trichoderma koningii* isolate TLS examined under bright-field microscopy; (j) same slide as in (i), but examined under epifluorescence. The cell walls of the germ tubes and conidia were intensely stained. Bar, 10 μ M.

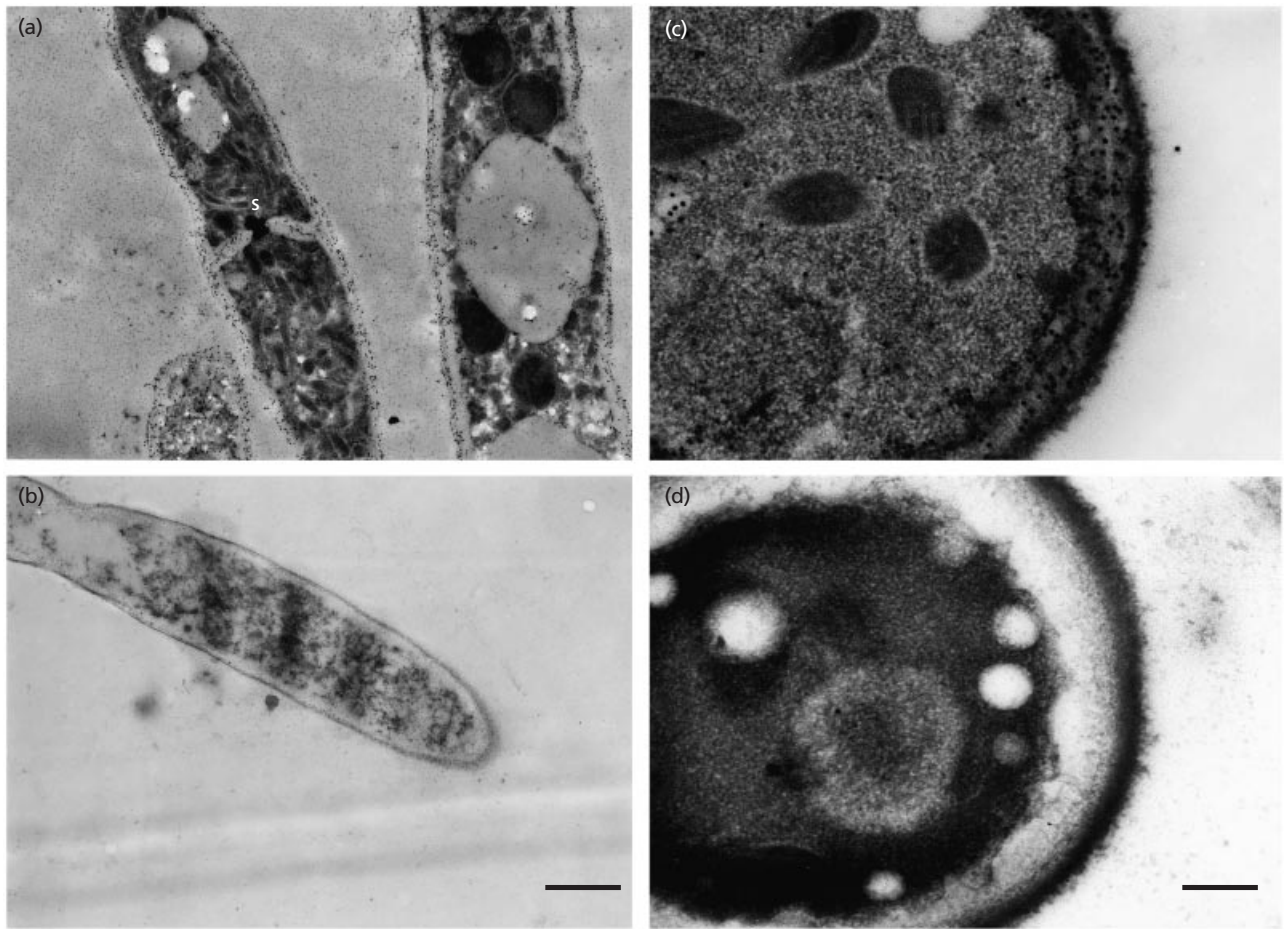


Fig. 6. Immunogold labelling of sections of cells of *Trichoderma koningii* isolate TLS. (a) Section of mycelium incubated with MF2 and anti-mouse immunoglobulin 20 nm gold particles, showing intense staining of the cell walls and septum (s) by the secondary reporter. (b) Control section of mycelium incubated with tissue culture medium and the immunogold conjugate. There was a lack of staining by the secondary reporter. Bar, 1.0 µm. (c) Section of conidium incubated with MF2 and the immunogold conjugate, showing intense staining of the cell wall by the secondary reporter. (d) Control section of conidium incubated with tissue culture medium and the immunogold conjugate. There was a lack of staining by the secondary reporter. Bar, 220 nm.

DISCUSSION

The development and testing of immunoassays in previous investigations (Thornton *et al.*, 1993, 1997, 1999; Thornton & Gilligan, 1999) has shown that the most appropriate antigens for the detection of fungi in soils and composts are those that are extracellular and that are constitutively expressed or can be induced by specific substrates. Studies of extracellular antigen production in *Trichoderma* spp. have shown that the enzyme β -1,3-glucanase represents an ideal candidate for the production of mAbs specific to this genus, since the enzyme is extracellular and is produced constitutively or semi-constitutively (Bull & Chesters, 1966; Elad *et al.*, 1982; Reese & Mandels, 1959).

Using a chromatographically purified *Trichoderma* β -1,3-glucanase preparation, a hybridoma cell line was raised which secreted an IgM class mAb, MF2, that

recognized a protein epitope specific to the genus *Trichoderma* and to a group of closely related fungi. The members of this group included species identified as part of a monophyletic clade by Rehner & Samuels (1994), in a study using parsimony analysis of partial sequences from nuclear large subunit rDNA (28S rDNA). This clade contained elements of the genus *Hypocrea* (*Hypocrea gelatinosa*, *Hypocrea lutea*) plus *Trichoderma* spp., *Hypocrea pallida*, *Hypomyces* spp. and *Sphaerostilbella* spp. *Gliocladium* anamorphs in this clade included *Gliocladium penicilliodes*, the type species of *Gliocladium* and anamorph of *Sphaerostilbella aureonitens*, and *Trichoderma virens* (= *Gliocladium virens*), which was a member of the clade containing *Hypocrea* and *Trichoderma*. Rehner & Samuels (1994) also identified a second clade grouped around *Nectria ochroleuca*, whose anamorph is *Gliocladium roseum* – neither *Nectria ochroleuca* nor

Table 3. Results of ELISA and plate culture tests for naturally infested composts

Each sample was baited with nine quinoa seeds. Contaminating fungi were identified as species belonging to the genera *Botrytis*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Verticillium* and *Zygorrhynchus*.

Compost	No. of baits:		
	With fungal contaminants	From which <i>Trichoderma</i> spp. were recovered	Positive for <i>Trichoderma</i> spp. in baiting-ELISA
Bulrush			
Sample A	8	1	1
Sample B	8	0	1*
Godwin			
Sample A	8	0	0
Sample B	7	2	2
Homebase			
Sample A	4	1	1
Sample B	6	1	1
John Innes No. 1			
Sample A	4	0	0
Sample B	5	0	0
John Innes No. 2			
Sample A	8	0	0
Sample B	5	0	0
John Innes No. 3			
Sample A	6	1	1
Sample B	2	1	1
Levington			
Sample A	5	3	5*
Sample B	8	1	2*
Shamrock			
Sample A	6	3	6*
Sample B	5	4	4
Westcountry			
Sample A	4	0	0
Sample B	6	0	0

* Not all isolates from these samples could be recovered for further testing, due to exclusion by faster-growing contaminating fungi.

Gliocladium roseum was recognized by MF2. *Gliocladium roseum* is distinguishable from other fungi by the formation of two distinct types of conidiophores, one *Gliocladium*-like and producing chains of conidia, and the other *Verticillium*-like and producing conidia in colourless drops of liquid (Domsch *et al.*, 1980). However, the *Verticillium*-like synanamorph is not unique to *Gliocladium roseum*. *Sphaerostilbella lutea* also produces a *Verticillium*-like state and while the *Verticillium*-like synanamorphs produced by *Gliocladium roseum* and *Sphaerostilbella lutea* are not strictly equivalent, their close taxonomic proximity may explain the lack of recognition of *Sphaerostilbella lutea* antigens by MF2. MF2 also did not recognize species of the genus *Clonostachys*, which was proposed as the

appropriate genus for the *Gliocladium roseum* anamorph by Domsch *et al.* (1980).

ELISA studies showed that production of the MF2 antigen, by actively growing mycelium of *Trichoderma koningii*, was constitutive and occurred in the absence of laminarin, a substrate shown to induce the production of β -1,3-glucanases in *Trichoderma* spp. (De La Cruz *et al.*, 1993, 1995; Noronha & Ulhoa, 1996; Vasquez-Garciduenas *et al.*, 1998). Furthermore, extracellular antigen production by *Trichoderma koningii* was as pronounced in the presence of glucose as in the presence of laminarin. This suggested that MF2 had been raised against an antigen other than β -1,3-glucanase or against additional antigens that shared a common epitope with

Table 4. Absorbance values obtained in ELISA from baits of naturally infested composts and species designation of recovered isolates as determined by ITS sequence analysis

Compost	Isolate no.	Absorbance value in baiting-ELISA*	Species	Accession no.
Bulrush	BUL-A7	0.130	<i>Trichoderma viride</i>	AF483589
Godwin	GOD-B4	0.253	<i>Trichoderma hamatum</i>	AF483582
	GOD-B7	0.153	<i>Trichoderma hamatum</i>	AF483594
Homebase	HBC-A2†	0.488	<i>Trichoderma</i> sp.	AF483584
	HBC-B4	0.439	<i>Trichoderma viride</i>	AF483583
John Innes No. 3	J13-A8	0.937	<i>Trichoderma virens</i>	AF483585
	J13-B7	0.235	<i>Trichoderma virens</i>	AF483586
Levington	LEV-A2	0.753	<i>Trichoderma</i> sp.	
	LEV-A8	0.606	<i>Trichoderma harzianum</i>	AF483587
	LEV-A9	0.948	<i>Trichoderma</i> sp.	
	LEV-B7	1.062	<i>Trichoderma harzianum</i>	AF483588
Shamrock	S-A1	0.168	<i>Trichoderma</i> sp.	
	S-A3	0.146	<i>Trichoderma</i> sp.	
	S-A8	0.417	<i>Trichoderma viride</i>	AF483590
	S-B2†	0.363	<i>Trichoderma</i> sp.	AF483591
	S-B3	0.249	<i>Trichoderma viride</i>	AF483592
	S-B5	0.144	<i>Trichoderma</i> sp.	
	S-B9	0.548	<i>Trichoderma viride</i>	AF483593

* Threshold absorbance value for detection of *Trichoderma* spp. in baiting-ELISA was set at $A_{450} = 0.100$.

† Species affiliation could not be assigned due to ambiguity in phylogeny when using ITS sequences.

the enzyme. Furthermore, these antigen(s) must have been present as contaminants in the enzyme preparation.

Analysis of the chromatographically purified enzyme preparation by gel electrophoresis followed by staining with Coomassie blue revealed a single 90 kDa protein. However, staining of replica gels for carbohydrate, using the PAS reaction, revealed highly glycosylated proteins with masses in the range 80–200 kDa. N-terminal sequence analysis of the preparation revealed a protein sequence (Ala-Val-Asp-Asp-Phe-Ile-Asn-Thr-Gln-Pro-Ile-Ala-Leu-Asn-Asn-Leu-Leu-Asn) with strong identity, in database searches, to glucoamylases from *Humicola grisea* and *Neurospora crassa* and to a previously reported N-terminal sequence for *Trichoderma reesei* glucoamylase (Val-Asp-Asp-Phe-Ile-Ser-Thr-Glu-Thr/Asn-Pro-Ile-Ala-Leu-Asn) (Fagerstrom, 1994). An assay of the purified preparation for enzyme activities revealed both β -1,3-glucanase and amylase activities. Similarly, both activities were also found in acetone precipitates from 5-day-old cultures of *Trichoderma koningii* grown in the presence of glucose or laminarin. The constitutive nature of secretion of both enzymes in the absence of their natural substrates would account for the strong reactivity of MF2 with culture filtrates and surface washings from slope cultures of *Trichoderma* spp.

Western-blotting analysis of the immunogen preparation showed that MF2 bound to a ladder of proteins whose molecular masses corresponded to those of proteins visualized by Coomassie blue and PAS staining.

The molecular masses of these bands were in keeping with those described elsewhere for *Trichoderma* glucoamylases and β -1,3-glucanases. For example, Fagerstrom & Kalkkinen (1995) reported a 66 kDa glucoamylase in *Trichoderma reesei*, while Okada (1977) described a 75 kDa glucoamylase in *Trichoderma viride*. Studies of *Trichoderma* β -1,3-glucanases have revealed single, inducible enzymes with molecular masses of 78 kDa (De La Cruz *et al.*, 1995) and 36 kDa (Noronha & Ulhoa, 1996), at least seven inducible enzymes with masses in the range 35–80 kDa (Vazquez-Garciduenas *et al.*, 1998) and a complex system of constitutive and inducible enzymes with molecular masses in the range 30–200 kDa (Ramat *et al.*, 2000).

From the results of this study, and those described elsewhere, we conclude that MF2 binds either to a protein epitope specific to glucoamylases produced by *Trichoderma* spp. and closely related fungi or to a protein epitope that is shared by glucoamylases and by β -1,3-glucanases, but which is specific to the enzymes secreted by *Trichoderma* spp. and closely related fungi.

Localization of the MF2 antigen(s) by immunogold electron microscopy showed that it was expressed in hyphal cell walls and septa and in the cell walls of phialoconidia; IF studies further illustrated its extra-cellular nature. This pattern of staining is consistent not only with the cellular localization of constitutive β -1,3-glucanases in *Trichoderma harzianum* (Gohl *et al.*, 1998), but also with the distribution of glucoamylase secretion in *Aspergillus niger* (Gordon *et al.*, 2000).

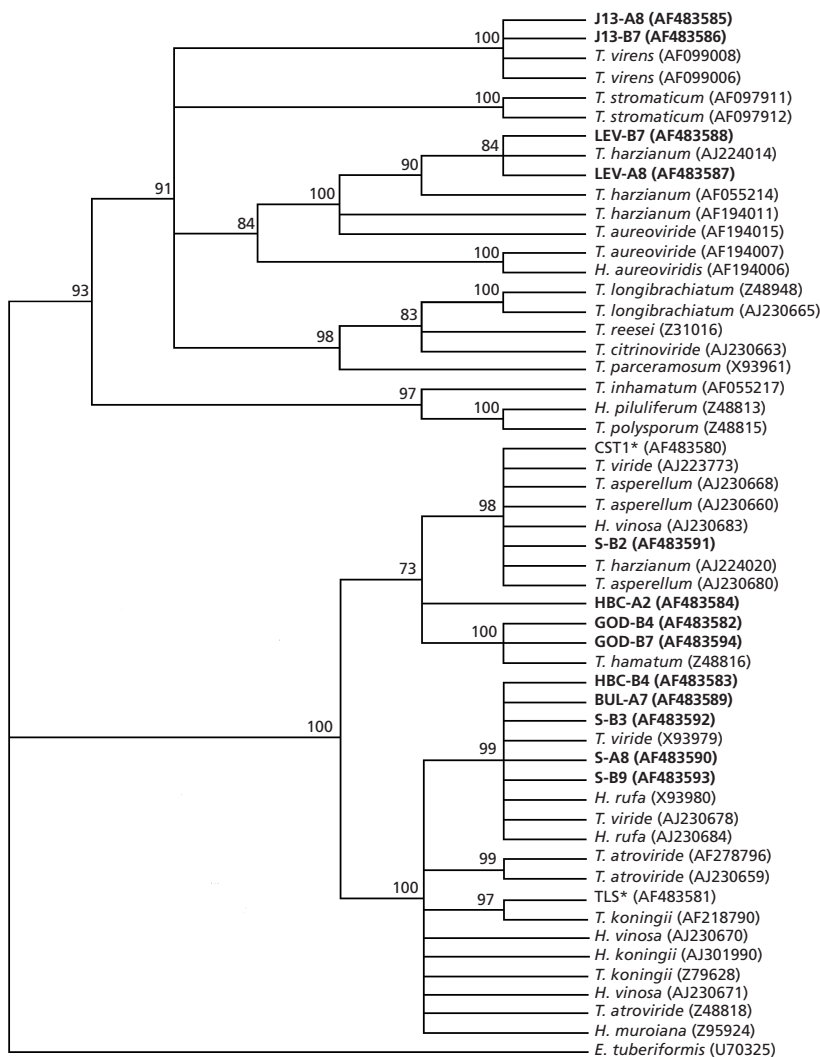


Fig. 7. Fifty percent majority-rule consensus tree based on ITS1-5.8S-ITS2 sequences of *Trichoderma* isolates detected and recovered from naturally infested composts using MF2 (bold type) compared with sequences of fungi contained in GenBank. Relationships were determined using the neighbour-joining method in PAUP* (version 4.0). Bootstrap values, shown as a percentage of 1000 replications, are given at branches; only those associations that were present in > 50% of the replicate trees are shown at the nodes. *Echinodothis tuberiformis* was used as the outgroup. Accession numbers of GenBank isolates are shown in parenthesis after species names. Isolates followed by an asterisk are *Trichoderma* spp. that were used in this study, but whose species affiliation to the genus has not previously been determined.

Production of a mAb to a constitutive, extracellular, antigen enabled the development of an immunoassay for the detection of *Trichoderma* spp. in complex environments such as composts. By combining a baiting procedure with ELISA, we were able to detect and recover isolates of *Trichoderma* spp. present in naturally infested commercial compost preparations. The specificity of the mAb was illustrated *in vivo* by its ability to differentiate *Trichoderma* spp. from contaminant fungi of unrelated genera, whereas its ability to detect isolates from a range of closely related species was shown by the recovery and characterization of the isolates by analysis of the nuclear ITS regions of the rRNA-encoding gene unit. The widespread occurrence of different *Trichoderma* spp. in the compost preparations was most likely due to their composition. All of the samples tested, with the exception of a single greenwaste compost where no *Trichoderma* spp. were detected, were composed of sphagnum or sedge peats. Natural populations of *Trichoderma* and *Gliocladium* spp. occur in high numbers or predominate in peat deposits (Dickinson & Dooley, 1969; Domsch *et al.*, 1980).

The ability to recover fungal isolates using plate-enrichment techniques after immunodetection allowed us to examine whether any of the strains exhibited potential biological control properties. [These control properties have been defined as the production of antibiotics and lytic enzymes, and physical interactions with host hyphae (Chet *et al.*, 1981; Elad *et al.*, 1983; Sivan & Chet, 1989; Benhamou & Chet, 1993)]. A *Trichoderma* isolate (S-B2), detected and recovered in this study using the combined baiting-ELISA method, emitted a strong coconut odour, characteristic of *Trichoderma* strains that produce the volatile antibiotic alkyl pyrone (Claydon *et al.*, 1987; Ghisalberti & Sivasithamparam, 1991; Maplestone *et al.*, 1991). The production of this antibiotic by *Trichoderma* spp. has been shown to significantly affect the saprotrophic growth of *Rhizoctonia solani* in compost-based systems (Thornton & Gilligan, 1999). Isolate S-B2 also exhibited directed growth toward – and coiling around – hyphae of the plant pathogen *Rhizoctonia solani* *in vitro*, which was visualized by IF microscopy using MF2. MF2 is currently being used to monitor the growth and survival of *Trichoderma* S-B2 and *Trichoderma virens* JI3-A8

during their antagonistic interactions with *Rhizoctonia solani* and *Pythium ultimum* in plant rhizosphere and bulk soil studies, under controlled laboratory conditions.

We believe that the identification of MF2 represents a significant advance in the specific immunological detection and monitoring of *Trichoderma* spp. in complex environments such as composts and soils. This mAb could be used in conjunction with nucleic-acid-based diagnostic techniques (Abbasi *et al.*, 1999; Hermosa *et al.*, 2001) to aid in the rapid detection, specific monitoring, recovery and identification of *Trichoderma* spp.

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