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Development of a Quantitative Monoclonal Antibody-based Immunoassay for *Humicola lanuginosa* on Rice Grains and Comparison with Conventional Assays

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A sensitive, specific, quantitative enzyme-linked immunosorbent assay (ELISA) has been developed that can be used to determine the extent of mycelial growth of a sporulating thermophilic fungus, *Humicola lanuginosa* on the surface of rice grains. The assay employs a monoclonal antibody EC6, developed in a previous study, which does not recognize spores of the fungus. Using antigen-coated wells, a direct linear relationship was established between dilutions of extracts from freeze-dried mycelium (0.5 to 3 µg/ml) and absorbance values but to eliminate day-to-day variations it was found to be necessary to run a dilution series, prepared from stock freeze-dried mycelium, with every test sample. The ELISA method was compared with conventional quantitative methods. Estimates of total mycelial length in freeze-dried material by ELISA were found to be in the same order of magnitude as those determined by ergosterol and a theoretical calculation. The ELISA method also compared favourably with direct linear measurements (by photomicrography) of live mycelium present in aliquots from homogenates of a 1 cm² plug taken from a plate but estimates of the latter by the dilution plate count method were much lower. In assays with inoculated rice grains, the quantitative ELISA method proved more sensitive than either the ergosterol method or direct plating of surface-sterilized grains. The ELISA method also has the advantage of being highly specific and quick to conduct.

Keywords: ELISA, ergosterol, fungus *Humicola lanuginosa*, monoclonal antibody, plate counts, quantitative methods, rice grains.

INTRODUCTION

Current methods of estimating total mycelial mass have several limitations and the measurement of the mycelial mass of specific fungi growing in a mixed environment

(such as in mixed cultures, in soils, in foods and feedstuffs) and as pathogens on or within plants is particularly difficult. Direct plating or dilution plating methods generally overestimate the mycelial growth of sporulating fungi, and underestimate, or do not detect, the mycelial mass of fungi that are slow growing or only weakly viable *in vitro* (Dewey *et al.*, 1989; Dewey, 1992). To quantify fungi growing in or on plant material, particularly grains and roots, it is frequently necessary to surface-sterilize the host material before plating out to prevent overgrowth from saprophytic fungi. Thus information about the extent of growth of fungi on surfaces is inevitably lost. While plating methods do have the advantage of only measuring viable propagules, a knowledge of the total mycelial growth, whether dead or alive, is often important in foods or feeds.

Other methods of measuring fungal growth such as determination of the total ergosterol (Naewbanij *et al.*, 1984) or chitin content (Thornton *et al.*, 1991) are appropriate for assays where only one species of fungus is involved. However, they do not allow measurement of individual fungi to be made in the presence of others, or distinguish spores from mycelial mass. Methods such as the Howard Mould Count (AOAC, 1984), which rely on visualization of the fungal particles, do enable a distinction to be made between spores and mycelial fragments, but distinguishing between mycelial fragments from two or more species is clearly difficult unless there are major differences in hyphal widths. All the current methods are tedious and time-consuming, and require expertise. A rapid, user-friendly, quantitative, highly-specific assay such as an enzyme-linked immunosorbent assay (ELISA) is needed.

Banks *et al.* (1992) have raised polyclonal antibodies with wide cross-reactivities for use in double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs) for the estimation of total fungal biomass in field or storage conditions. Quantitative ELISAs employing pooled polyclonal antisera have been developed to measure the total fungal biomass in tomato paste (Lin *et al.*, 1986; Robertson & Patel, 1989). However, the difficulties in raising antisera to fungi that are species-specific have limited the development of immunoassays for specific fungi (Mohan & Ride, 1982; Musgrave, 1984; Clarke *et al.*, 1986; Mohan, 1989; Dewey, 1992). Notermans *et al.* (1985) have raised antisera to fungi that are almost genus-specific and have used these in various immunological assays for fungi in foods but they have not developed quantitative specific assays. The advent of hybridoma technology (Kohler & Milstein, 1975) has enabled highly specific monoclonal antibody (Mab)-based immunodetection assays to be developed, but relatively few attempts have been made to correlate measurements of mycelial biomass by ELISA with conventional quantitative assays or with absolute values of mycelial length.

In this study we have sought to establish that quantitative ELISA tests, using extracts from freeze-dried mycelia as standards and fungal specific MAb, can be developed to determine the mycelial content of a specific fungus in a complex environment. We have done this by comparing estimates of mycelial mass obtained by quantitative ELISA tests with those from conventional methods such as dilution plate counting, measurement of hyphal length by photo-microscopy and ergosterol determinations. These were compared with theoretical estimates. As a model system we have chosen to work with *Humicola lanuginosa* (Griffon & Moubanc) Bunce, a thermophilic, saprophytic fungus that is known to cause post-harvest deterioration of rice grains (de Padua, 1977; Phillips *et al.*, 1989) and for which quantitative information would be useful in assessing the role of this fungus in the discolouration of rice grains. We used the *Humicola*-specific monoclonal antibody EC6, raised in a previous study (Dewey *et al.*, 1989). Using the data obtained above, conventional methods were compared with immuno and chemical assays for the measurement of growth of *H. lanuginosa* in inoculated rice paddy grains.

MATERIALS AND METHODS

Culture Conditions

A culture of *H. lanuginosa* originally isolated by direct plating (Phillips *et al.*, 1989) of naturally infected rice grains from Indonesia was used throughout the study. The isolate was maintained in pure culture on slants of yeast starch extract agar (YSA) (Cooney & Emerson, 1964). For quantitative studies the fungus was either used as live mycelium or freeze-dried mycelium, prepared as follows:

(a) *Live Mycelium.* *H. lanuginosa* grown in Petri dishes containing 20 ml YSA per dish and incubated at either 45°C or 55°C for three days.

(b) *Freeze-dried Mycelium.* *H. lanuginosa* grown in shake cultures in flasks with 20 ml yeast starch broth at 45°C for three, four or six days. Mycelium from liquid cultures was harvested by centrifugation, the mycelial pellet frozen in liquid nitrogen, freeze-dried and kept sealed at -20°C.

Preparation of Samples for Quantitative Studies

Preparation of Inoculated Rice Grains. The moisture content of 300 g of autoclaved (121°C/15 min) rice paddy was adjusted to 24% with sterile distilled water and conditioned in a polyethylene container at 4°C for one week with daily shaking. A spore suspension was prepared by adding 10 ml of sterile 0.2% agar to a five-day-old culture of *H. lanuginosa* grown on a slope of YSA. Spores were released from the culture by gently stroking the surface of the culture with a sterile needle. Five millilitres of this suspension, containing 4×10^5 spores, was evenly distributed, with thorough mixing, over the conditioned rice which was then maintained at 45°C for one week. Samples of 50 g were removed daily to monitor the growth of *H. lanuginosa* by direct plating, ergosterol, and ELISA and dip-stick immunoassays.

Homogenates of Live Mycelium from YSA Plate Cultures. Live mycelium from agar plate cultures was used for all the quantitative studies because freeze-dried mycelium was not found to be viable and could therefore not be used in dilution plate counts. Homogenates of live mycelium taken from solid plate cultures were prepared as follows. Four 0.5 cm diameter plugs were taken from the leading edges of two-day-old cultures grown in petri dishes at 55°C on YSA plus 100 p.p.m. chloramphenicol. Ten replicate samples were prepared. Four mycelial plugs (giving a total of 1 cm²) were comminuted by emulsifying in a Silverson (Bucks, UK) Laboratory Mixer for three bursts of 30 s full speed, in 10 ml of reverse osmosis water (R.O.) at 4°C. To estimate mycelial mass, from the 10 ml homogenate, 5 ml was retained for dilution plating purposes and photomicrography and 5 ml was diluted into PBS for ELISAs (Figure 1). All samples were kept on ice between proceedings.

Extracts from Freeze-dried Mycelium. Extracts were prepared by resuspending 5 mg of freeze-dried material in 5 ml phosphate-buffered saline (PBS) in a 50 ml Falcon tube and emulsifying by blending at full speed in a Silverson Laboratory Mixer for three bursts of 30 s with two 2 min intervals. Samples were kept on ice between operations. After grinding, the suspension was diluted to 50 ml with further PBS, centrifuged (Econospin, Sorvall) at 200 × g for 10 min and the cell-free supernatant removed for ELISA testing.

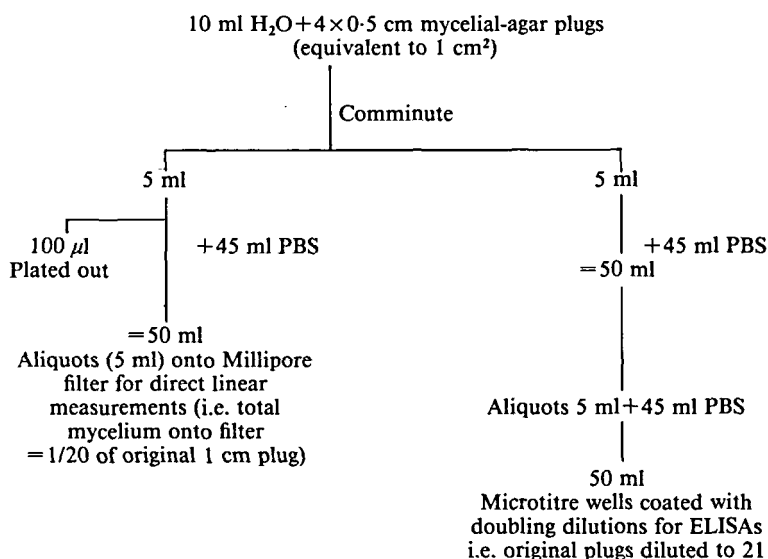


FIG. 1. Flow chart showing sequence of dilutions from the original mycelium of *H. lanuginosa* (4×0.5 cm plugs = 1 cm^2) in 10 ml. Agar plugs were taken from the leading non-sporulating edge of solid cultures of the fungus grown at 55°C .

Assay Protocols

Direct Plating. A total of 100 inoculated rice grains per sample were placed directly on the surface of YSA plates (10 grains per plate). A further 100 grains per sample were surface-sterilized in 1.5% w/v sodium hypochlorite solution for 2 min, washed in sterile distilled water, surface dried and the grains plated as for untreated samples (NSS). Treated grains were referred to as surface sterilized (SS).

Plates were incubated at 45°C for five days and the percentage of grains infected with *H. lanuginosa* was determined.

Dilution Plate counts. Samples were taken directly from the original 5 ml homogenate in R.O. water. Three 0.1 ml aliquots of the 10 replicates (i.e. total number of samples = 30) were spread onto 9 cm petri-plates of YSA containing 100 p.p.m. chloramphenicol using a bent sterile glass rod. Plates were incubated at 55°C and colonies formed were recorded after three, four and five days.

Direct Linear Measurements by Photo-micrography. For photo-micrography, mycelial plugs were prepared as described above. The original 5 ml homogenate from each of the 10 replicates was further diluted to 50 ml with R.O. water and one 5 ml sample was filtered through a $0.4 \mu\text{m}$, 2.5 cm diameter 'black' millipore filter (Cat. no. HTBP02500; filter type HTBP; Millipore Co., Watford, Herts.), with a total millipore area of 490.0 mm^2 . After air-drying, each filter was wetted with 150–200 μl of distilled water followed by an application of 100 μl ANS (1-anilino-8-naphthalene sulphonc acid).

Micrographs were taken at a magnification of $500\times$ on a black-and-white Ilford HP5 Plus film with a Zeiss Axiophot microscope fitted with an epifluorescence filter. The exposure time was 10 s. There were 10 replicates, and 10 micrographs were taken per replicate. The field of view for each frame was 0.0541 mm^2 , and thus the total

number of fields of view possible per filter was 9057. Micrographs were enlarged and total hyphal length estimated according to the method for straight or curved filaments of Olsen (1950), using a 20×20 cm grid of 1 cm units. A conversion of (1 cm print=10 µm actual size), based on direct measurement, was used.

Dip-stick Immunoassay. A dip-stick immunoassay was carried out on inoculated rice grains (25 per sample) to determine the percentage of rice grains infected with *H. lanuginosa*. Individual rice grains were soaked overnight in Eppendorf tubes to allow binding of the antigen to a Millipore membrane, Immobilon-P. The dip-sticks were processed by the assay protocol of Dewey *et al.* (1989) using the monoclonal antibody EC6. A positive result on each dip-stick indicated the presence of *H. lanuginosa* in an individual grain.

ELISA Procedures.

(a) *ELISA protocol.* The quantitative ELISA test developed in this study was based on a modification of the detection assay developed previously (Dewey *et al.*, 1989). The following method was adopted as the standard assay protocol. A reference curve using a series of standards with PBS extracts from freeze-dried mycelium was run in parallel with all quantitative ELISA samples.

Microtitre wells were coated overnight at 4°C, with 100 µl of the sample antigen solution or, for the reference series, cell free supernatants from freeze-dried mycelium (1 mg/ml), double-diluted into PBS. Wells were washed four times with PBST (PBS+0.02% Tween 20), inverted and banded dry, blocked for 30 min with 1% w/v bovine serum albumin (BSA) in PBS, and washed four times in PBST. The plates were then incubated successively with the *Humicola*-specific MAb-EC6 for 10 min, goat anti-mouse peroxidase conjugate (a 1/1000 dilution (v/v) in PBST of Sigma A-0412) for 1 hr, and the substrate, tetramethylbenzidine (TMB) in acetate-citrate buffer, pH 5.4, for 30 min. The reaction was stopped by the addition of 5 M-H₂SO₄, and absorbance at 450 nm was read on a Dynatech MR 700 Microplate Reader that had been blanked against an empty well. All working volumes were 100 µl per well.

(b) *ELISA with homogenates from live mycelium.* The original 5 ml of homogenized suspension prepared from four 0.5 cm plugs from agar plate cultures (Figure 1) were diluted in Falcon tubes to 50 ml with PBS and spun on a bench-top centrifuge for 5 min at 200×g to pellet the mycelial fragments. From the cell-free supernatant 5 ml were removed and again diluted to 50 ml with PBS. Microtitre wells were coated overnight at 4°C with 100 µl aliquots of the cell-free supernatant further diluted by doubling dilutions into PBS. These wells were then washed and processed as described above.

(c) *ELISAs with diffusates from single rice grains, batches soaked in PBS and rice grains ground in a blender.* The mycelial mass in rice grains was estimated by ELISA by soaking them individually overnight at 4°C in 150 µl PBS in microtitre wells. The rice grains were removed the following day by inverting and banging the plates, then the plates were processed as described in (a) above except that all volumes were maintained at 150 µl. In addition, 1 g batches of rice grains (approximately 35 grains) were soaked overnight in 100 ml of PBS in Duran bottles at 4°C and the cell free supernatant used to coat microtitre wells, 100 µl per well overnight at 4°C, and then the wells were processed as described in (a).

Two samples of 2.5 g of uninoculated and inoculated grains were ground separately in a coffee grinder to a fine powder. The rice powder was then suspended in 25 ml PBS alone or 25 ml PBS to which an extract from freeze-dried mycelium had been added to give a final concentration of 2 µg/ml. These suspensions were plated out onto

microtitre wells by doubling dilutions into further PBS. The wells were then processed by the standard ELISA test protocol.

Ergosterol Estimation. Ergosterol levels in freeze-dried mycelium and rice grains were determined using a modification of the procedure of Seitz *et al.* (1977). Approximately 10 mg of freeze-dried mycelium were extracted in 10 ml of 80% methanol (Fisons)/20% 'Spectrosol' hexane (BDH) by shaking for 30 min on a wrist action shaker. After filtering (Whatman 541), the extract was made up to 15 ml with further 80% methanol/20% hexane. The extract was placed into a screw-capped bottle and 1.5 g KOH was added. Rice grains were ground (2×60 s) to a fine powder in a laboratory mill (Janke and Kunkel GmbH, Germany), extracted in 20 ml of 80% methanol/20% hexane, filtered and made up to 30 ml with further 80% methanol/20% hexane. The extract was placed in a screw-capped bottle and 3 g of KOH were added.

The bottles were sealed tightly and the contents saponified at 70°C in a water bath for 20 min. Each extract was allowed to cool to near room temperature (below the boiling point of hexanes), transferred to a separating funnel and shaken with 5 ml of water for 10–15 s. The layers were allowed to separate and the hexane (non-saponified) layer collected. The extraction was repeated twice, each time with 5 ml hexane. The hexanes were evaporated to dryness using a rotary evaporator (Buchi) and the final residue redissolved in 1 ml of absolute methanol (volumetric) and filtered through a 0.5 μ m filter (Millipore). Ergosterol in the extracts was determined by high performance liquid chromatography (HPLC) analysis as follows. Sample aliquots (10 μ l) were injected onto a 25 cm Spherisorb C18 ODS 5 μ column maintained at 25°C. Solvent (95% Fisons HPLC grade methanol/5% Fisons HPLC grade water) was passed through the column at a constant flow-rate of 1.5 ml min⁻¹ using a Spectra Physics SP8700 solvent delivery system. Ergosterol was detected in column eluent using a variable wavelength UV/Vis detector (Spectra Physics SP8440) set at 282 nm. Chromatographs were integrated using Nelson Analytical PC integrator software.

RESULTS

Dilution Plate Counts

Early results with 0.1 ml samples (prepared according to Figure 1), grown at 45°C for two and five days gave very high numbers of colony forming units (c.f.u.) (80 000 and 230 000 c.f.u. per 1 cm² respectively). This was found to be due to the presence of aleuriospores which are not detected by the *Humicola*-specific monoclonal antibody, EC6. To avoid complications from aleuriospores and to make the results for all quantitative methods comparative, the inocula for plate cultures and dilution plates were subsequently grown at 55°C for two days to prevent aleuriospore formation. From these dilution plates we obtained an average of 18 fungal colonies per plate (Figure 2). Thus the total number of c.f.u. per 1 ml of homogenate was 180, and the total for the four 0.5 cm² plugs (equivalent to 1 cm²) was $180 \times 10 = 1800$. If it is assumed that one c.f.u. arises from a hyphal fragment of 100 μ m in length or more, then the estimated total length of mycelium per 1 cm² plug was $1800 \times 100 \mu\text{m} = 180\,000 \mu\text{m}$ or 0.18 m.

Direct Linear Measurements

The average number of intersections on a grid, using the method of Olsen (1950) were used to determine the average hyphal lengths (taken from 10 fields of view = 10 prints) (Table 1). The average hyphal length per field of view obtained was 1913.6 μ m (SD = 561.6 μ m). To calculate the total length of hyphae/1 cm² disc:

$$\begin{aligned}\text{Total hyphal length/1 cm}^2 &= \text{actual length/print} \times \text{fields of view (9057)} \times 100/5 \\ &\quad (\text{as one filter} = 5 \text{ ml of a } 1/1000 \text{ dilution}). \\ &= 346 \text{ m}\end{aligned}$$

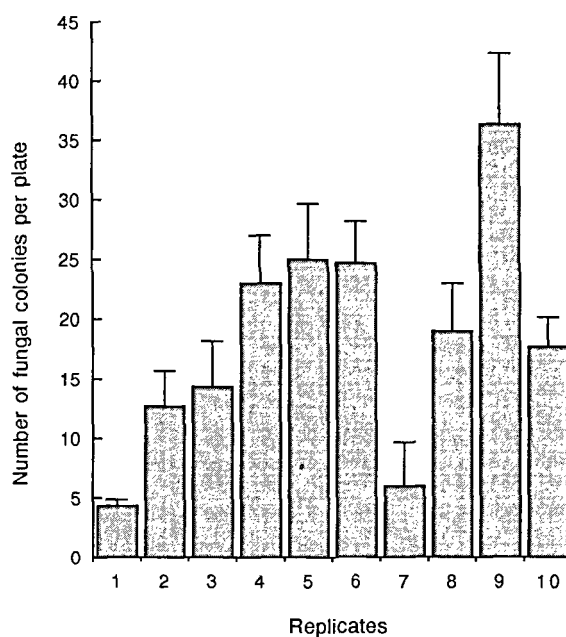


FIG. 2. Number of colony forming units per 100 µl sample from four 0.5 cm agar plug cultures homogenized in reverse osmosis water.

TABLE 1. Measurement by photomicrography and the combined intersection method of Oslen (1950) of hyphal lengths from 5 ml samples of homogenates of 1 cm² plugs of mycelial agar plugs in 100 ml, filtered through 0.2 µm membrane filters

Replicates	Number of prints	Actual hyphal length ^a (µm)	SD (µm)
A	10	2180	2150
B	11	1740	1210
C	10	1960	1050
D	10	1420	720
E	10	2660	4610
F	9	1370	1000
G	10	2200	1070
H	10	1490	1250
I	11	1030	940
J	10	2390	1400

^aThis is derived from the average combined intersections (counted intersects $\times \pi/4$), $\times 10 \mu\text{m}$, where 1 cm print = $10 \mu\text{m}$ of true hyphal length.

ELISAs

Establishment of Standard ELISA Test Procedures. The best method of releasing fungal antigens from infected grain appeared to be soaking the grain overnight in a large volume of PBS (Figure 3) and this method was adopted for all quantitative grain work. For studies involving antigens from live and freeze-dried mycelium alone a 30 min soak was adopted as the standard procedure (Figure 3).

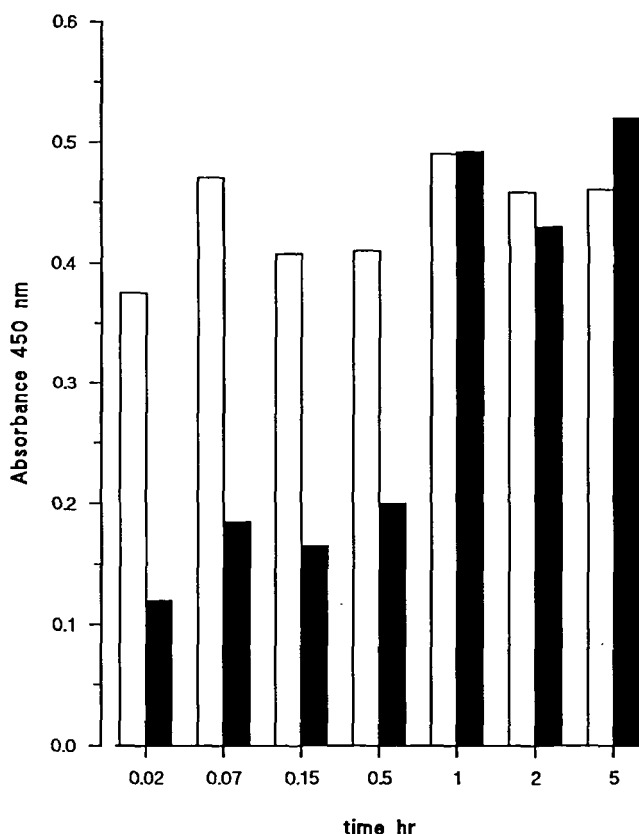


FIG. 3. Solubilization of fungal antigens by passive diffusion from freeze-dried mycelium, $0.25 \mu\text{g ml}^{-1}$ (□), and 1 g of inoculated grains soaked in 100 ml of phosphate-buffered saline diluted to 1600 ml (■) as measured by ELISAs.

The process of binding of the solubilized antigens to the microtitre wells (coating) was found to take place slowly therefore an overnight coating step was adopted for the standard ELISA procedure in order to ensure the maximum coating of microtitre wells.

Standards Using Freeze-dried Mycelia. A linear relationship was consistently obtained by ELISA, between absorbance values and concentrations of freeze-dried mycelial extracts from 0.5 to 3 $\mu\text{g mycelium/ml}$ (Figure 4). Although the same shaped linear relationship was obtained in all experiments, the absolute absorption values varied from day to day and therefore a series of standards was always run with every test sample to give a reference curve.

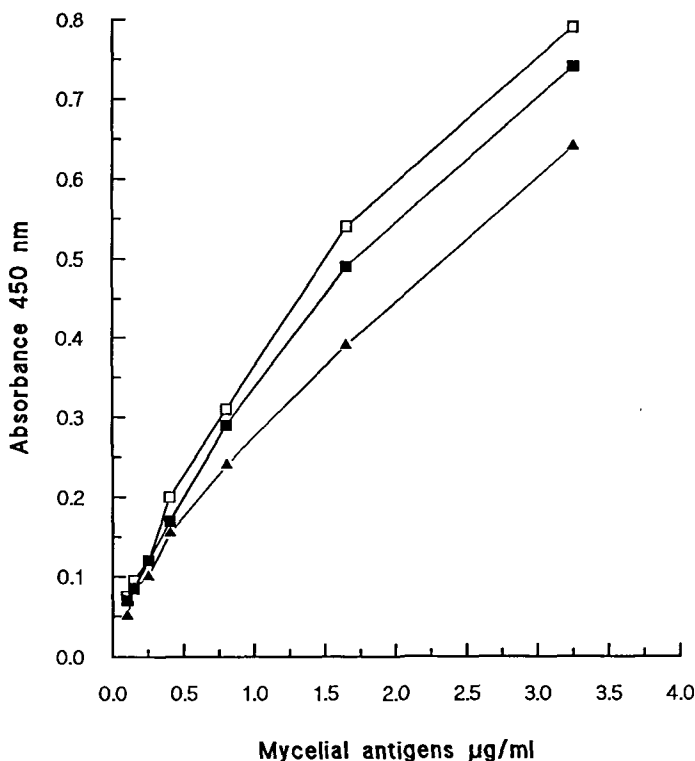


FIG. 4. Phosphate-buffered saline extracts from freeze dried mycelial antigens tested by ELISA against the specific monoclonal antibody EC6 to determine day to day variation. Dates of assay tests were (□) 20 September 91 (■) 30 September 91, and (▲) 31 October 91.

Live Mycelia. When homogenates from four 0.5 cm plugs (equivalent to 1 cm²) were homogenized, centrifuged and diluted into PBS to give a final dilution of 1 cm² equivalent disc in 2 l, the cumulative average absorbance value from standard ELISAs of the 10 replicate samples was 0.317 (see Table 2) which is equivalent to that obtained from cell-free extracts from 0.8 µg/ml freeze-dried mycelium (Figure 4). Thus the total amount of live mycelium present on a 1 cm² disc is equivalent to 0.8 µg/ml × 2000 = 1600 µg of freeze-dried mycelium. Using the theoretical conversion, explained later, of 1 µg freeze-dried mycelium = 1.31 m, then the total length of fungal hyphae determined by the ELISA method is 1.31 × 1600 = 2096 m.

Diffusates from Grains. Absorbance values for all inoculated grains soaked individually, overnight, in 150 µl PBS gave the maximum absorption values of 2.0 indicating that each well 150 µl was equivalent to 150 µl of > 5 µg/ml freeze-dried mycelium, i.e. > 0.75 µg per grain. In order to obtain a more accurate estimate of mycelium per grain, inoculated rice grains were soaked in larger volumes of PBS (1 g per 100 ml) for 16 h and the cell-free diffusates plated out by doubling dilution into further PBS in microtitre wells. The average absorbance value from a dilution equivalent to 1 g soaked in 6400 ml was 0.600. The absorbance value of 0.600 was equivalent to that obtained from 2.4 µg/ml freeze-dried mycelium (Figure 4). Thus the total amount of mycelium present on 1 g of inoculated grain is approximately equivalent to 2.4 × 6400 µg, or 15 360 µg. Since 1 g of rice grains contains, on an average, 35 grains, then each

TABLE 2. Absorbance values from ELISAs of wells coated overnight with extracts from mycelial homogenates from the equivalent of 1 cm² plugs from live solid cultures. Results are expressed as averages of the four immunoassay replicates for each of the 10 different sets of mycelial plugs homogenized

Sample	Average absorbance of four replicates	SD
A	0.396	±0.032
B	0.375	±0.066
C	0.342	±0.060
D	0.282	±0.024
E	0.315	±0.014
F	0.298	±0.023
G	0.318	±0.014
H	0.323	±0.032
I	0.261	±0.045
J	0.261	±0.023

Cumulative average (A to J) of absorbance values=0.317.

inoculated grain has the equivalent of 15 360/35 µg of freeze-dried mycelium per grain, i.e. 439 µg per grain or $439 \times 1.31 \text{ m} = 575 \text{ m}$.

Ergosterol

In assays on extracts from freeze-dried and liquid-grown cultures ergosterol levels were found to increase with the age of the culture and the production of aleuriospores (Table 3); young non-sporulating cultures contained 5.98 µg ergosterol/g mycelium whereas extracts from heavily sporulating six-day-old cultures contained 15.04 µg ergosterol/g mycelium. During growth studies of *H. lanuginosa* on rice grains (Figure 5) ergosterol was not detected in freshly inoculated rice grains or in grains 24 h after inoculation but it was detected after 48 h and the ergosterol level continued to rise throughout the seven days that ergosterol was monitored.

TABLE 3. Ergosterol content of *H. lanuginosa* mycelium grown in liquid culture.

Days	µg ergosterol/g mycelium	Spores
3	5.98	Absent
4	12.54	Some spores
6	15.04	Many spores

In an earlier, separate experiment the ergosterol content was determined in (a) 1 mg of freeze-dried mycelium alone; (b) 1 g uninoculated rice grains; and (c) 1 g rice grains inoculated with *H. lanuginosa* and sampled after a four-day incubation. The inoculated grains were from the same batch as those above for the diffusate/soak ELISA estimates. The following values were obtained:

- (a) mycelium alone=3.3 µg ergosterol/mg freeze-dried mycelium;
- (b) uninoculated rice grains=6.5 µg ergosterol/g grain (equivalent to 1.97 mg freeze dried mycelium/g grain);

- (c) inoculated rice grains = $18.8 \mu\text{g}$ ergosterol/g grain (equivalent to 2.97 mg freeze dried mycelium/g grain).

Thus each inoculated g of grain contained an additional $12.3 \mu\text{g}$ of ergosterol equivalent to 3.73 mg freeze-dried mycelium or $107 \mu\text{g}$ per grain.

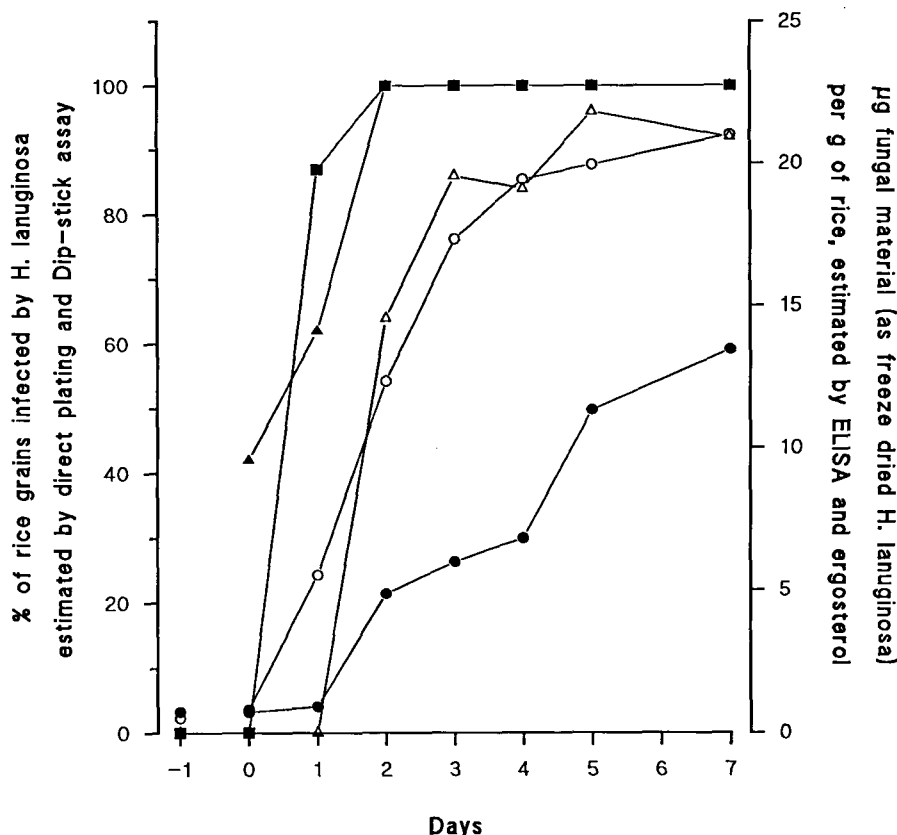


FIG. 5. Comparison of conventional methods with immunoassay procedures and quantification of the ELISA assay to estimate fungal biomass in inoculated rice paddy grains. Rice was inoculated at 0 days, while -1 days refers to uninoculated rice. Biomass by (●) ergosterol determination, (○) ELISA; % infection by (■) dip-stick assay, (▲) direct plating of non-surface-sterilized grains and (Δ) direct plating of surface-sterilized grains.

Theoretical Calculations

Hypothetical Length of Hyphae per 1 μg Fresh Weight and 1 μg Dry Weight. Specific gravity (density) of fungal hyphae was assumed to be 1.3, based on previous studies (Warnock, 1971) and the mean diameter of the hyphae, determined by direct measurement of 20 hyphae using a graduated eyepiece calibrated against a graduated stage piece, was taken as $3 \mu\text{m}$.

Since volume $V = \text{mass}/\text{density}$,

$$V = 1 \text{ g fresh weight hyphae}/1.3 = 0.769 \text{ cm}^3.$$

Assuming the hyphae to be cylindrical, then the length, L , of the hyphae is calculated:

$$L = V/\pi r^2,$$

where the radius of the hyphae $r = 1.5 \times 10^{-4}$ cm. Thus:

$$L = 0.769/\pi (1.5 \times 10^{-4})^2 \text{ cm};$$

$$L = 10\,879\,125 \text{ cm/g fresh weight of mycelium};$$

$$L = 10.9 \text{ cm}/\mu\text{g fresh weight of mycelium}.$$

Assuming the ratio of dry weight to fresh weight of mycelium is 12:1 (Kjoller & Struwe, 1982), then,

$$L = 10.9 \times 12 = 131 \text{ cm}/\mu\text{g dry weight of hyphae} = 1.31 \text{ m}/\mu\text{g dry weight of hyphae}.$$

Hypothetical Length of Hyphae per 1 cm Disc from Solid Culture. The hyphal length of mycelium in a 1 cm disc from a plate culture was estimated using the above calculations as follows:

$$\text{the surface of the 1 cm disc is } = \pi r^2 = 3.142 \times 5^2 \text{ mm} = 79 \text{ mm}^2$$

A square of 79 mm² would have sides 8.9 mm in width.

Total number of lengths of hyphae/side = width of one side/hyphal diameter

$$= 8.9 \text{ mm}/0.003 \text{ mm} = 2967$$

Thus the total lengths of fungal hyphae per one layer of a 1 cm disc

$$= 2967 \times 8.9 \text{ mm} = 26.4 \text{ mm}$$

Hypothetical Length of Hyphae per Rice Grain. Earlier measurements established that the average length and width of a grain of rough rice used in this work was 8 mm and 3 mm. Assuming that the surface area of a rice grain is approximately that of an open cylinder, then:

$$\begin{aligned} \text{the length of hyphae wound around cylinder once} &= \text{circumference} \\ &= \pi d \text{ (where } d = 3 \text{ mm)} = 9 \text{ mm.} \end{aligned}$$

The total number of possible coils of hyphae around the cylinder (grain) =

$$\text{height of cylinder/diameter of hyphae} = 8000 \mu\text{m}/3 \mu\text{m} = 2667 \text{ coils.}$$

The total length of hyphae wound around the cylinder (grain) =

$$\begin{aligned} &\text{number of coils} \times \text{length of one coil} \\ &= 2667 \times 9 \text{ mm} = 24\,000 \text{ mm} = 24 \text{ m.} \end{aligned}$$

The rice used in this study was in the form of paddy. This can be considered as having three surfaces on which mould growth could occur:

- (i) outer surface of rice husk;
- (ii) inner facing surface of rice husk;
- (iii) outer surface of rice grain.

The total length of hyphae wound around three cylinders (representing the three surfaces of the grain) would be 72 m.

Method Comparison and Assay Quantification

Results for the growth of *H. lanuginosa* in inoculated rice paddy grains are shown in Figure 5. Dip-stick assay and direct plating results represent the percentage of mould-infected grains. Ergosterol and ELISA results have been calculated in μg fungal material per g of paddy grain using the conversion factors of $3.3 \mu\text{g}$ ergosterol being equivalent to 1 mg freeze dried mycelium and 0.600 absorbance units being equivalent to $2.4 \mu\text{g}$ freeze-dried mycelium per ml.

Growth of *H. lanuginosa* was detected after one day by ELISA, dip-stick and direct plating of untreated grains (NSS). There was a significant rise in ergosterol content, ELISA response and direct plating of surface-sterilized (SS) grains by the second day indicating penetration of the grain by fungal mycelium. ELISA and dip-stick assay results for pre- and post-inoculation samples showed no response while direct plating indicated that 40% of grains were infected on inoculation.

DISCUSSION

In this study we have demonstrated that, using a fungal-specific monoclonal antibody that does not recognize spores, the ELISA method can be used as a reliable quantitative method for estimating the mycelial mass of live or freeze-dried mycelium from pure cultures or on rice grains, provided that a series of reference standards are carried out with each ELISA test to eliminate day-to-day variations. We found that freeze-dried mycelium from young, non-sporulating, liquid-grown cultures of *H. lanuginosa* provided a convenient, consistent source of reference material. We have shown that estimates of the mycelial mass or length on/in 1 cm^2 plugs by ELISA are of the same order of magnitude as those obtained by direct linear measurement, 2096 m and 346 m, respectively, but that estimates from the dilution plate method are much lower, in the region of 0.18 m. The latter figure is based on the assumption that the average length of a hyphal fragment that can form a colony-forming unit (c.f.u.) is $100 \mu\text{m}$. This is almost certainly a gross underestimate because clumping of hyphal fragments is known to occur and it is difficult to eliminate this problem without destroying the viability of the fragments. Many workers, particularly those measuring fungal biomass in soils, have noted that the correlation between estimates of mycelial mass by the dilution plate method (c.f.u.) and estimates by direct linear measurements or enzyme activity is poor (Frankland *et al.*, 1981; Kjoller & Struwe, 1982). In general, however, the differences found by other workers have been due to overestimates caused by the presence of spores rather than underestimates. In this study the problem with spores has been avoided but we have found that the viability of hyphal fragments of *H. lanuginosa* is generally poor.

Our estimated figure for mycelial mass of the 1 cm^2 disc by direct linear measurements (346 m) is almost certainly an underestimate because the filters used to catch the mycelial fragments did not fit exactly and a small amount of the mycelial suspension was known to slip past the filters at their edges. The estimated length of mycelium by the ELISA is dependent upon the theoretical conversion presented earlier that $1 \mu\text{g}$ dry weight of mycelium is equivalent to a hyphal length of 1.31 m. It is possible that the antigen/epitope recognized by the *Humicola*-specific MAb, EC6, may become diminished or altered in the freeze-drying process so that estimates of live mycelial mass by ELISAs using freeze-dried material as standards will give inflated values. If one assumes that a mat of hyphae on/in a 1 cm^2 disc from a solid plate culture is at least 10 layers deep (0.03 mm) and the total length of mycelium one layer deep is 26.4 m (from calculation in results section) then the total length of mycelium present in a 1 cm^2 plug would be $26.4 \times 10 \text{ m} = 264 \text{ m}$ which is close to the figure obtained by direct linear measurement. It is reassuring that the differences between the measurement of mycelial mass of live mycelia by direct visual measure-

ment, ELISA and theoretical calculations are relatively small; 364 m, 2096 m and 264 m, respectively.

Other workers, notably Gams *et al.* (1987) and Warnock (1971), have calculated that the length of hyphae of 1 μg of fresh mycelia is in the region of 0.25 m and 5.2 m, respectively. Therefore, using the fresh weight to dry weight conversion of 12:1, 1 μg freeze-dried mycelium would be equivalent to 4 m and 62.4 m; both these figures are higher than the one used in this study (1 μg dry weight mycelium = 1.31 m). Baath & Soderstrom (1979, 1980) have pointed out that the conversion of mycelial mass to length is greatly influenced by changes in the radius of the hyphae because the radius is squared in the calculation. Furthermore when they used the gross mean diameter of fungal hyphae they found that it gave smaller linear values than calculations based on the distribution of hyphal diameters. We found that the hyphal widths of *H. lanuginosa* varied considerably from 2 to 5 μm . The figure of 3 μm for the hyphal diameter that we used in our calculations is based on the average of 20 hyphae measured microscopically using a graduated eyepiece calibrated against a graduated stage and does not take full account of the distribution.

Quantitative estimates of mycelial mass by ergosterol were made only with freeze-dried mycelium and inoculated grains. The observation that spores of *H. lanuginosa* contain relatively large amounts of ergosterol has not apparently been reported before. The presence of ergosterol in spores complicates the quantitative assessment of mycelial mass and has serious implications for the use of this method with other spore bearing fungi. When ELISA and ergosterol methods were used to determine the mycelial mass of *Humicola* on inoculated rice grains the values obtained were similar (439 μg and 107 μg freeze-dried equivalents per grain, respectively). Using the theoretical conversion that 1 μg of freeze-dried mycelial mass is equivalent to 1.32 m the above values are equivalent to 575 m and 140 m, respectively. The distribution of fungal hyphae on these inoculated grains seen in scanning electron micrographs (Dewey *et al.*, 1989) indicates that in some areas there is a weft of mycelium several hyphal layers deep but that it is not uniform over the whole grain. The ELISA estimate of hyphal length per grain of 575 m, is much nearer to the theoretical estimate. The three surfaces of a rice grain covered completely with 1 layer deep of mycelium would have a hyphal length of 72 m, therefore the value derived from the ELISA method of 575 m is equivalent to a grain covered eight layers deep in hyphae and the ergosterol estimate of 140 m is equivalent to a grain covered two layers deep. Both means of estimation give feasible results.

Results of the method comparison and quantification study showed that the ELISA and dip-stick assays can be successfully used to detect growth of *H. lanuginosa* at an earlier stage than would be possible by monitoring ergosterol content or direct plating of surface-sterilized grains. The immunoassays have additional advantages in rapidity and specificity with results obtained within 24 h compared with five days by conventional mycological methods.

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