Assessment of inhibition/growth-promoting properties of new agents on moulds: description of a simple bio-imaging technique

J. E. MOORE*†, G. McCOLLUM‡, A. MURPHY*, B. C. MILLAR*, D. NELSON‡, C. E. GOLDSMITH* J. S. ELBORN§#, A. LOUGHREY*, P. J. ROONEY* and J. R. RAO†‡

Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Lisburn Road, Belfast; †School of Biomedical Sciences, University of Ulster, Coleraine; ‡Agri-Food & Biosciences (AFBI), Newforge Lane, Belfast; §Northern Ireland Regional Adult Cystic Fibrosis Unit, Belfast City Hospital; and #Respiratory Medicine, Queen's University of Belfast, Department of Respiratory Medicine, Belfast City Hospital, Belfast, Northern Ireland, UK

Invasive fungal infections, particularly with *Aspergillus* spp. as well as the zygomycetes, continue to be associated with a high degree of mortality, especially in patients who are immunocompromised or immunosuppressed, particularly those with haematological malignancy. In addition, the laboratory detection of such fungal aetiological agents of serious systemic infection is compromised by poor laboratory isolation techniques, particularly from blood culture material or, in the case of patients with cystic fibrosis (CF), from bacterial competition and overgrowth by multi- and pan-resistant bacterial pathogens, including *Pseudomonas aeruginosa*.

Recently, several new ethnopharmaceutical agents have been described for their growth-inhibiting properties[1,2] as well as for their growth-promoting properties with the filamentous fungi.[3] In both scenarios, it is important to have a robust methodology that permits the reliable and accurate quantification of fungal growth with such agents. Unlike the bacteria and yeasts, assessment of any new antifungal compound is problematic when working with the filamentous fungi due to two main reasons: i) they do not grow well in broth culture, which is the preferred test medium, as they tend to grow on the surface of the liquid-air interface and not in the broth medium, and ii) when assessed, colonial growth tends to be in a highly irregular pattern and hence hard to quantify.

This study details a new method for the identification of fungal promoting/inhibiting properties using a seaweed extract derived from native seaweed grown on the west coast (Atlantic) of Ireland, through the employment of a simple bio-imaging technique, designed specifically to examine new compounds with filamentous fungi.

Three fungal isolates were employed in this study: *Scedosporium apiospermum*, isolated from the sputum of an adult patient with CF, *Aspergillus flavus* isolated from the hospital environment, and *A. fumigatus* isolated from the sputum of a patient with CF. The source and isolation methodology for these fungi are as described previously.[4,5] All isolates were passaged at least once on Nagano basal fungal medium containing glucose (16.7 g/L), agar (20 g/L), yeast extract (30 g/L) and peptone (6.8 g/L), where the pH was adjusted to 6.3. All plates were incubated aerobically at 22°C for seven days prior to further use. The identification of all fungi was confirmed through molecular techniques, including sequencing of the 5.8S, 18S and 28S ribosomal RNA (rRNA) genes and their associated internal transcribed spacer (ITS) regions, as described previously.[5]

Seaweed extract was obtained and initially irradiated to remove all contaminating microflora and microbiota. Seaweed extract was subdivided into 2 x 2.5 L samples and γ-irradiated to give an irradiation dose of 25 kGy. Irradiation was carried out using cobalt 60 (in a Gammabeam-650 irradiation unit, MDS Nordion, Kanata, Canada) at a dose rate of 1.5 kGy/h and at an environmental temperature of 4°C. Two red perspex dosimeters (AEA Technology, Harwell) were attached to the outside of each sample receiving irradiation, in order to measure the actual dose received. Following irradiation, the change in absorbance of the dosimeters was measured spectrophotometrically (Spectronic Unicam UV-500) at 603 nm and 640 nm and their thickness was measured using a digital electronic micrometer (RS Components, Corby). The corresponding dose received was then obtained from a calibration graph provided by the National Physical Laboratory (Teddington. Middlesex, UK). Following irradiation, all extracts were checked for sterility by enumeration on Mueller-Hinton agar, as well as in tryptone soya broth at 30°C for six days.

Irradiated seaweed was added as a supplement to double-strength (x2) Nagano basal agar, in an agar incorporation technique, to give a final concentration (v/v) of seaweed extract in the final agar medium of 5%, 10%, 20%, 30% and 50%. Prewarmed (45°C) sterile seaweed extract should be added to sterile basal agar immediately prior to plate pouring and not added initially prior to autoclaving, as experience showed that initial incorporation prior to autoclaving resulting in agar-setting interference and the formation of a sloppy agar which failed to set and could not be inoculated with any test mould. Unsupplemented Nagano (without seaweed extract) was employed as a control.

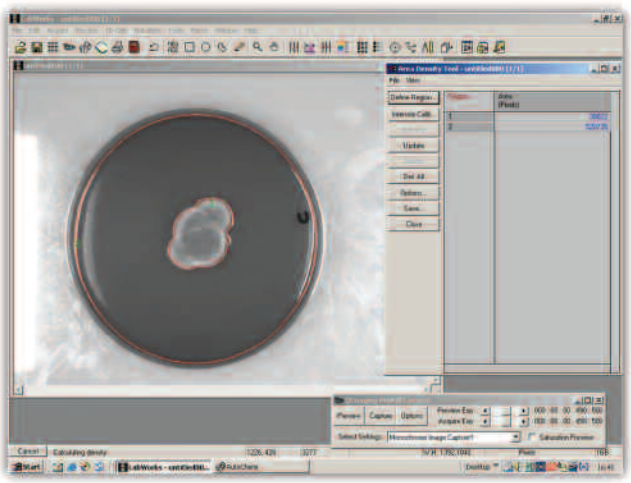

Fig. 1. Visual examination and measurement of fungal growth of an irregular-shaped pattern, as captured by the Autochemisystem UVP bioimaging system (UVP Products, Cambridge, UK), supported by LabWorks software.

Correspondence to: Professor John E. Moore
Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland,UK
Email: jemoore@niphl.dnet.co.uk

Table 1. Effect on growth of three filamentous fungi with supplementation of Nagano basal fungal medium with varying concentrations (0–50%[v/v]) of irradiated seaweed extract.

	Fungal growth units*						
	Supplementation with irradiated seaweed extract (v/v)						
	0% (Control)	50%	30%	20%	10%	5%	P value
Scedosporium apiospermum	0.0703	0.0096	0.0397	0.0711	0.0710	0.0651	0.1863 (NS)
Aspergillus flavus	0.7485	0.7699	0.7166	0.6343	0.7234	ND	0.2766 (NS)
Aspergillus fumigatus	0.2271	0.1792	0.1216	0.1891	0.2527	0.1743	0.1049 (NS)

*The arbitrary ratio 'fungal growth' was calculated for each concentration and is the ratio of total pixels of surface area occupied by fungal growth/total pixels of surface area of the plate.

NS: not significant; ND: not determined.

All three fungal taxa, as detailed above, were tested individually against all dilutions of supplemented Nagano medium, as well as with the unsupplemented control. Sterile dry cotton wool swabs were dipped into individual confluent fungal plates and were then stab inoculated in the geometric centre on all treatment concentrations and control plates, in duplicate. Inoculated plates were subsequently incubated aerobically at 22°C for seven days before examination.

Plates were examined on an Autochemisystem UVP bioimaging system (UVP Products, Cambridge, UK), supported by the LabWorks software package. Initially, the instrument was set on white light and to an exposure ratio of 490:500, with a constant focus of 47% (Fig. 1). Plates were read individually using the 'Area Density' tool, where the entire area of the plate was measured in pixel units and recorded, followed by the area (in pixels) of the irregular surface of the plate occupied by fungal growth. The irregular shape of fungal growth was accommodated through employment of the freehand drawing tool to map the shape of the growth.

The arbitrary ratio, fungal growth, was calculated for each concentration and is the ratio of total pixels of surface area occupied by fungal growth to the total pixels of surface area of the plate. Statistical analysis was performed using Student's *t*-test to compare fungal growth at each concentration against its control. Probability (P) ≤0.05 (5%) was considered significant.

The effect of supplementation of Nagano basal fungal medium with varying concentrations (0–50% [v/v]) of irradiated seaweed extract is shown in Table 1. Overall, statistically, there was no significant difference in the fungal growth value between the control (unsupplemented) medium and the supplemented medium for any of the fungal taxa examined (Table 1).

Examination of the activity of any ethnopharmaceutical agent with filamentous fungi is problematic, due to difficulties in the reliable measurement of activity *in vitro*. Previously, the authors described a method for the identification of the optimal basal medium for *Candida* spp., employing broth culture and measurement by spectrophotometry analysis.[6] However, in this case, measurement was uncomplicated, as the yeasts behaved more like bacteria than filamentous fungi, and hence were easier to measure spectrophotometrically. The complication in working with filamentous fungi is that broth culture cannot be used to measure *in vitro* growth routinely, as these moulds will only grow on the surface, although measurement of both dry and wet weight of fungal growth in broth culture may be attempted. Equally, it is not possible to use quantitative enumerative measurements, due to the release of spores from the mould's conidia, resulting in over-estimation of the biomass of filamentous organisms present on the agar surface.

Although the data indicate that this seaweed extract did not have growth-promoting nor antifungal inhibitory properties, the description of this simple bio-imaging and computer-assisted method is of value to aid characterisation of such properties with filamentous fungi. The instrumentation is commercially available, along with the supporting software; however, its application to the measurement of fungal activity has yet to be described. The irregular shape of fungal growth may be captured accurately using the freehand tool. The method is simple and, once the instrument is set up and calibrated, it lends itself to large-throughput screening.

In conclusion, this bio-imaging technique should be considered for the quantitative examination of new pharmaceutical compounds against filamentous fungi as a means of assessing their growth-promoting or inhibitory properties.

References

1 Meng F, Zuo G, Hao X *et al*. Antifungal activity of the benzo[c]phenanthridine alkaloids from *Chelidonium majus* Linn against resistant clinical yeast isolates. *J Ethnopharmacol* 2009; **125**: 494–6.

2 Duraipandiyan V, Ignacimuthu S. Antibacterial and antifungal activity of Flindersine isolated from the traditional medicinal plant *Toddalia asiatica* (L.) Lam. *J Ethnopharmacol* 2009; **123**: 494–8.

3 Barreto M, Critchley AT, Straker CJ. Extracts from seaweeds can promote fungal growth. *J Basic Microbiol* 2002; **42**: 302–10.

4 Nagano Y, Millar BC, Goldsmith CE *et al.* Development of selective media for the isolation of yeasts and filamentous fungi from the sputum of adult patients with cystic fibrosis (CF). *J Cyst Fibros* 2008; **7**: 566–72.

5 Nagano Y, Elborn JS, Millar BC *et al*. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Med Mycol* 2009 Aug 11;: 1-12.

6 Moore JE, McMullan R. Comparison of media for optimal recovery of *Candida albicans* and *Candida glabrata* from blood culture. *Ir J Med Sci* 2003; **172**: 60–2.