ASSESSING FUNGAL GROWTH IN THE UNDERGRADUATE LABORATORY

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Abstract

This paper describes a model experiment used in introductory microbiology and in mycology classes. The model introduces students to several important concepts and essential techniques in basic fungal physiology. It also introduces new students to several important concepts in microbiology/mycology.

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Introduction

In many introductory microbiology courses and, indeed, in mycology courses, relatively little attention is given to physiological characteristics. In microbiology courses many standard physiological tests are used in the characterisation and identification of prokaryotes, but the fungi are rarely discussed apart from, amongst other basic facts, the most basic concepts of exozyme production and the difference between pathogens and saprobes. It is recognised that more comprehensive experiments are needed to illustrate the more complex functions of fungi, thus enabling a clearer understanding of their overall role in various habitats.

The exercise described here illustrates several important physiological aspects in fungal biology, allows the student to practise data processing and presentation skills and clearly demonstrates to students the often complex pre-experimental planning and efficient pre-preparation of media required for efficient data acquisition in mycology.

Materials and Methods

The lecturer may provide each group of students with a named culture chosen because of its known physiological attributes with respect to substrate water potential (ψ). Alternatively, students who performed the 'Gravity Plate Experiment' (Harrower 2004) may choose one or more of the isolates made earlier in the course by the same group of students—thus providing a continuum of information. Cultures are normally grown on half strength Potato Dextrose Agar (HPDA) or some other suitable general purpose medium at 25°C.

The experiment is based on comparing the radial growth rate of a fungal isolate on media adjusted in various ways. This is most easily done as follows.

(1) A standardised plug of inoculum is aseptically cut from a culture of a fungus grown on (HPDA) as a general purpose mycological growth medium with depth approximately 5 mm.

(2) Two diameters (thus 4 radii—r1, r2, r3 and r4) are marked on the base of each plate with a thin-point waterproof marking pen. These are drawn at right angles through the point of inoculation. Using a sterile mounted needle, students transfer an inoculum plug to fresh media and invert the plug to each of 5 plates of the test medium. Inversion of the plug helps minimise the chance of spores initiating secondary colonies.

(3) All test plates are incubated in a randomised manner for 24 hours or 48 hours at or about 25°C.

(4) Usually, at the end of this period, a small amount of mycelial growth can be seen growing from the inoculum block. The periphery of such growth is marked on the radii and the date and time recorded. If no mycelium has emerged then the edges of

those inoculum blocks are marked on the base lines as 'point zero'.

(5) In most cases, the rate of mycelial extension over/through a medium is more or less constant since the medium is homogeneous. A final reading of each radius (r) must be taken before the mycelium reaches the edge of the medium. This is critical since it would not be known when the mycelium reached the edge of the plate. Intermediate markings of the mycelial periphery might be made since each round of marking events takes so little time. The final measurement is that which is taken last before the mycelium reaches the edge of the plate.

(6) For each plate, there are four measurements of radial extension. There should be at least five replicates of each treatment and these replicate plates are randomised within the incubator. Either the mean linear extension per plate (p) is calculated (i.e. p = [r1 + r2 + r3 + r4]/4) (r = radius) and then the mean radial extension per treatment is calculated as (p1 + p2 + p3 + p4 + p5)/5, since five replicate plates were used. Alternatively, each of the 20 radial extensions can be summed and averaged.

(7) The mycelial growth rate can then be calculated by dividing the mean radial extension by the incubation period between the first mark and the last mark closest to the edge given to the nearest 0.1 day. This is why the time of measurement is essential. This takes so little time that students can perform this stage within a lunch break to avoid timetabling constraints. Thus a mycelial growth rate in mm/d can be found and a statistical analysis, such as a 1-way ANOVA can be used to indicate a significant growth in mean growth rate and, perhaps, differences in response between fungal species.

Various physiological parameters may be tested. A range of temperature or a range of carbon sources may be tested, but these are often thought by students to be too mundane. The preferred experiment described here is to examine the effect of substrate water potential (ψ) on growth. Various methods can be used to measure the water potential of a medium, including vapour pressure osmometry and freezing point depression osmometry. Here it is preferred to work from first principles and to use such techniques to verify calculated values for water potentials.

The water potential of a medium may be closely related to solute concentration by the equation (Griffin 1972):

$$\psi = -RT\upsilon m\varphi.\ 10^{-9}\ \text{MPa}$$

$$\text{thus,}\quad \psi = -24.7\ \upsilon m\varphi\ (\text{at } 25^{\circ}\text{C}).$$

where R = the gas constant (8.31×10^{7}), T = temperature in °K, υ = ions/molecule, m = molality and φ = osmotic coefficient at molality m and at 25°C. Data relating to the osmotic coefficients of various compounds are available in Robinson and Stokes (1955).

Firstly, the students should prepare sufficient basal media using distilled water. Here, 10 different water potentials are used with five replicates, thus requiring 50 × 20 mL (or 1.0 L) of Basal Medium (BM). The BM has the following composition (g/L): Na_2HPO_4 0.725; KH_2PO_4 0.725; $MgSO_4.7H_20$ 0.120; NaCl 0.100; NH_4NO_3 0.400; Glucose 1.800; Yeast Extract 1.100; Malt Extract 1.000; Agar 15.00.

Table 1. Relative quantities of KCl in each WPM.

WPM	KCl (M)	ψ (-MPa)
1	0.0	0.12
2	0.1	0.46
3	0.2	0.90
4	0.3	1.35
5	0.4	1.79
6	0.5	2.23
7	0.6	2.67
8	0.7	3.11
9	0.8	3.55
10	0.9	4.00

The water potential (ψ) of the basal medium is -0.12 megapascals (MPa) and this can either be calculated or measured. Next, the BM is divided into 10 equal parts and KCl is added to reduce the water potential. Other solutes often used in the adjustment of water potential are glycerol (Chandler *et al.* 1994) or mannitol (Smith *et al.* 1990). However, the use of an organic source has the potential to alter the nutritional parameters of the test media simultaneously with the water potential, thus increasing the number of variables in the experiment. For that reason alone, KCl is preferred. The test media are labeled WP1, WP2 ... WP10 and the quantities of KCl added can be calculated from the molarities shown above in (Table 1). Test plates can be labelled according to the corresponding Water Potential Medium (WPM). The media are then placed in closed Shott bottles

Results

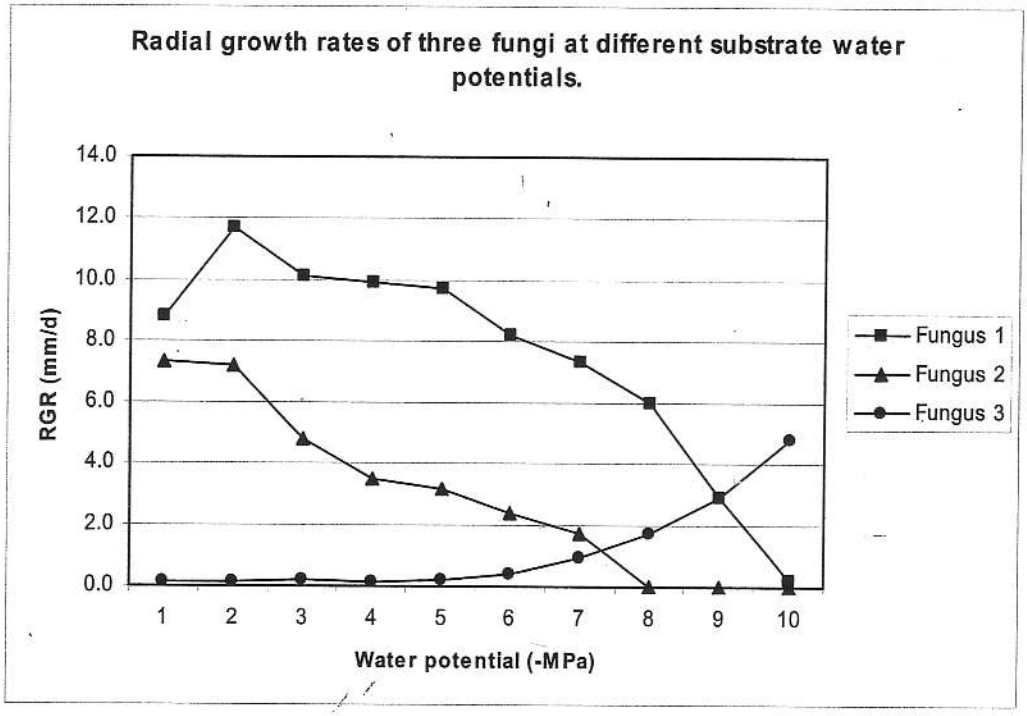

Figure 1.

and autoclaved at 121°C for 15 minutes. After autoclaving, it is essential to thoroughly mix the ingredients of each bottle by swirling gently and then allow the media to cool such that, when pouring plates, no steam is lost which will affect the final water potential of the plate. This follows the protocol detailed by Brown and Harrower (1997).

Discussion

These data can lead to discussions regarding how a hypha actually extends. An excellent summary of this has been provided by Gooday (1995). The extension rate is, of course, dependent on nutrient availability and type, i.e. C-source, N-source and various environmental parameters. By keeping these constant by using the same BM, we ensure that water potential (ψ) is the only variable acting on hyphal extension. It also introduces the concept that a fungal hypha is a dynamic entity reliant on water influx generating a hydrostatic pressure at the hyphal tip. With regards to medium (substrate) water potential (ψ), the discussion should get to the point where students realise that water influx generating a hydrostatic pressure at the tip is due to the difference between the medium ψ (ψ_{ext}) and the internal water potential (ψ_{int}) and the energy used by the fungus to maintain this differential. That is, the difference between ψ_{int} and ψ_{ext} is the cause of the net inward flux if $\psi_{int} < \psi_{ext}$.

Data such as that in Figure 1 shows that there are at least three different physiologies. One (Fungus 1) shows a typical fungus such as *Alternaria* or *Pythium* where there is some compensation, probably by the production of compatible solutes, which allows the fungus to explore a range of media of relatively high water potential. At about ψ = -2.0 MPa the radial growth slows due to the inability of the fungus to intake sufficient water. This may be due to the inability of these fungi to produce sufficient compatible solutes rapidly enough, or there may be a balance reached through resource partitioning where the energy required to produce sufficient compatible solutes does not permit sufficient resources to be left to produce new hyphal tip materials. Eventually growth ceases since there is no net influx of water to create hydrostatic pressure at the tip. Here a discussion of the role of tubular microvacuoles in delivery of materials to the hyphal tip may be introduced and of the overall role of the Spitzenkörper in regulating delivery and demand. Students may be introduced to an old, but excellent, paper by Griffin (1981) which covers much of the applicable theory of hyphal-water relations.

The plot of radial growth of the intermediate type fungus (Fungus 2) shown in Figure 1 demonstrates that fungi can differ in their response to substrate water potential. Some genera, such as *Aspergillus* and *Cladosporium*, may have some species which exhibit the growth pattern similar to 'Fungus 1' and others which exhibit that of 'Fungus 2'. This may assist in the ecological distribution of different species within a genus. This is important, since it allows a discussion to take place on physiological ecology and the introduction of the concept of substrate (water potential) specificity. The third plot (Fungus 3) on Figure 1 shows the type of result that would be expected for an almost xerophylic fungus such as several members of the genera *Aspergillus* and *Penicillium* (Pitt and Hocking 1997). This could lead to a discussion of food preservation technologies and the concept of extremophiles. Some fungi demonstrate an increased rate of radial growth at lower water potentials compared to a higher water potential. This could allow for an introduction of the concept of resource partitioning in mycelia, since those fungi are probably putting resources into the removal of water to arrest tip burst rather than into linear growth *sensu stricto*.

Many students have difficulty with the concept of 'higher' and 'lower' when dealing with only negative values. To get around this problem a discussion of temperature is useful. Over the temperature range of -1°C to -10°C it is obvious that -1°C is higher than a temperature of -5°C but -10°C is lower than -5°C. After this students find it easier to understand the relative ranking of water potentials, which are always negative—apart from absolutely pure water.

This is a useful student exercise which opens up many avenues for discussion including:

1. the effect of substrate water potential;
2. the dynamics of hyphal growth;
3. the ecophysiology of fungi;
4. a discussion of the difference between growth on a homogeneous medium (an agar) compared to growth in a heterogeneous substrate (e.g. in soil or in a processed food);
5. the importance of accurately making media for special purposes;
6. statistical analyses of data and data presentation;
7. the accuracy of measurement and its limitations; and,
8. there is often a need for further experiments to be planned to clarify interpretations of data.

This is a simple but effective exercise which can be used to enable relatively novice microbiologists, especially mycologists, better understand some important strategies for fungal survival and growth.

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