A NOVEL EXPERIMENTAL PLATFORM FOR THE MICROSCOPIC OBSERVATION OF GERMINATION AND POST-GERMINATION EVENTS IN PHYTOPATHOGENIC MICROFUNGI

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Abstract

This method describes the optimisation of compound microscopy for the observation of fungal spore germination, and post-germination events including appressorial formation, migration of nuclei, and development of the vacuolar-microtubular system. Central to this method is the cellulosic microscope slide, developed as an extension of the method for observing fungal mycelial growth devised by Harrower (1985). The cellulosic microscope slide is a transparent experimental platform developed as a dry substrate specifically to enable the observation of fungal spore germination under various desiccation and relative humidity regimes, but which also proved to be invaluable for the observation of post-germination events.

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Introduction

The microscopic observation of fungal spore germination and post-germination events is often done using an agar-based medium and high quality microscopes. Since Köhler illumination is the normal aim in most critical microscopic studies, it is often the case that such a system is diminished in value when an agar block preparation is used as the mountant for spores under observation. It is also recognised (Kuo 1999), that the spores of many plant pathogenic fungi, including the target organism used in our studies, *Colletotrichum gloeosporioides*, do not germinate on glass slides unless the spores have been triggered to germinate by being immersed in some sort of 'plant decoction'. Other workers have noted the involvement of epicuticular waxes in the germination of various members of the genus *Colletotrichum* (Podila *et al.* 1993, Staples and Hoch 1987).

A critical examination of spores for the assessment of germination and observation of the subsequent formation of appressoria and other post-germination events is often difficult. This paper describes a novel experimental platform which was developed during the course of a mango research project on the conidial biology of the phytopathogenic microfungus *C. gloeosporioides*, the aetiological agent of anthracnose disease.

The platform was developed as a research tool specifically to enable the observation of spore germination under various desiccation and relative humidity regimes, both of which required perfectly dry substrates at the commencement of the experiments, with subsequent rehydration under strictly controlled conditions. During our research, it

was found that spores of *C. gloeosporioides* do not germinate satisfactorily on untreated glass microscope slides, so these could not be used. This confirmed the finding of others (Kuo 1999) and led to the development of the cellulosic microscope slide for our research project. It was subsequently found that this experimental platform was also invaluable for the observation of post-germination events such as appressorial formation (Fig. 1) that requires a hard-surfaced substrate (Liu and Kolattukudy 1998).

It was also found that when spores and germlings on the platform were stained with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI), nuclei could be readily observed and their migration monitored (Fig. 2). Likewise, staining of spores and germlings with the fluorochrome 6-carboxyfluorescein diacetate (CFDA) enabled clear observation of the vacuolar-microtubular system in those structures.

The cellulosic microscope slide offers distinct advantages over conventional media for research involving observation of germination and post-germination events of the spores of phytopathogenic microfungi. It provides a thin, hard, dry, and transparent substrate comprised of cellulose acetate, a plant-derived substance, which is tightly adhered to a standard glass microscope slide. Because of these characteristics, and the fact that the substrate is nutrient-limited, germ tubes and hyphae grow in a single plane across the surface of the cellulose without excessive branching or three-dimensional growth, thus facilitating clear

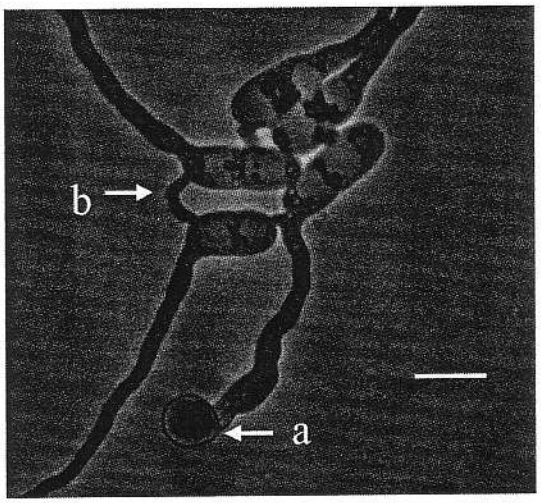

Figure 1. Germinated conidia of *Colletotrichum gloeosporioides* on cellulosic microscope slide. **(a)** melanised appressorium. **(b)** anastomosis tube. Bar = 10 µm.

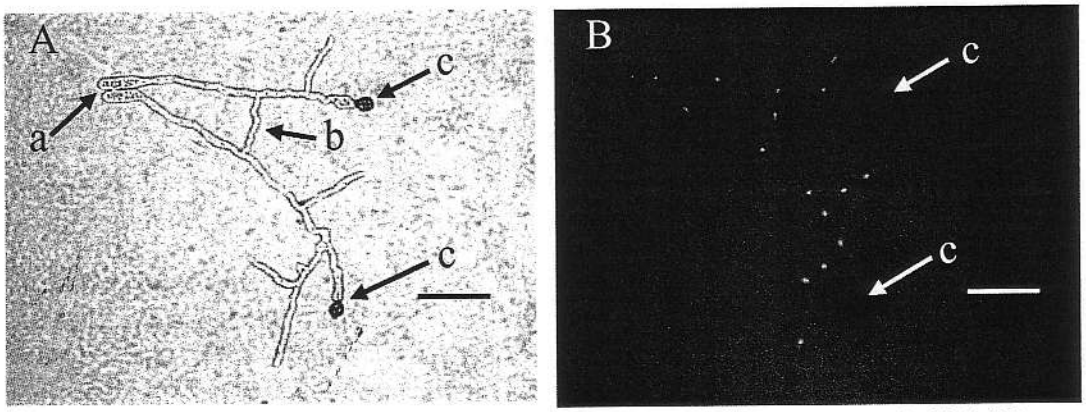

Figure 2. A. Bright Field micrograph of germinated *Colletotrichum gloeosporioides* conidia stained with DAPI on a cellulosic microscope slide **(a)** conidia **(b)** hyphal anastomosis tube **(c)** appressoria. **B.** Micrograph of the same specimen as in A taken a few moments later with fluorescence microscopy showing fluorescing nuclei. Bar = 30 µm.

observation of events as they occur. Dynamic observation of individual spores or germlings is enabled by using a New England Finder to locate particular spores or germlings for observations over time. Appressorial formation, which requires a hard substrate, occurs readily on cellulosic microscope slides. Furthermore, the cellulose strips used in making the slides can be prepared well in advance of requirements and can be stored in sterile distilled water. Cellulosic microscope slides can then be prepared within minutes and are ready for immediate use.

Agar-based media, comprising various nutrient mixes solidified by agar, are the conventional media used for most fungal cultures. These soft media are ideal for studies of fungal growth such as of mycelial growth rates, effect of substrate water potential and effect of different nutrients and their concentrations, but they are inferior to the cellulosic microscope slide for studies of spores and germlings. This is because the germ tubes and hyphae readily penetrate the soft agar-based media making them difficult to trace and observe. Furthermore, the nutritious environment encourages rapid mycelial growth into the media, across their surfaces, and aerially, and this three-dimensional growth, together with diminished Köhler illumination, makes observation of the development of germlings very difficult. A further drawback is the lack of appressorial formation on nutrient agar media. A soft agar medium is readily penetrated by hyphae thus obviating the need for the fungus to form appressoria, the specialized structures which evolved to penetrate the hard surfaces of plants. Study of these structures is therefore not possible if nutrient agar media are used.

Various slide culture methods involving nutrient agar have been developed in an attempt to overcome some of these drawbacks; for example, Harris (1986) developed a method, as a modification of other slide culture methods, whereby a thin block of inoculated nutrient agar was sandwiched between two sterile cover glasses and incubated in a Petri dish containing water agar. The slide was subsequently disassembled and mounted in a conventional manner after growth occurred. This technique was clumsy and frequently did not permit proper Köhler illumination.

Other workers have used whole leaf clearing techniques to enable observation of germination and post-germination events on and under the leaf surface (Liberato *et al.* 2005). These techniques are time-consuming, often involve the use of hazardous chemicals, and do not permit dynamic observation of events as they occur.

Whilst the cellulosic microscope slide was developed as a research tool for a specific need, it is also well suited as a teaching aid for the observation of germination of spores and post-germination events of microfungi.

Method

The novel platform is termed a 'cellulosic microscope slide'. This is a dry, transparent, experimental platform developed during the course of studies on mango anthracnose caused by *C. gloeosporioides*, as an extension of the method for observing fungal mycelial growth devised by Harrower (1985). Squares approximating 15 x 15 mm were cut from clear cellophane gift-wrapping paper (such as is used for cut flowers), and de-plasticised by boiling the squares twice in two changes of sterile reverse osmosis water (SROW) with a drop of surfactant (Tween 80™) in accordance with the method devised by Harrower (1985). The resulting cellulose squares (larger strips may be used depending on coverslip dimensions) were autoclaved at 121°C for 15 mins and stored in SROW in sterile tubes until needed. A 0.1 g/10 mL solution of gelatin (Acumedia™) and hot SROW was then prepared. A drop (about 20 µL) of this solution was placed in the centre of a glass microscope slide and spread thinly using a bent sterile glass rod. A strip of moist autoclaved cellulose was placed over the gelatin, the slide was held vertically, and a piece of tissue paper was held against the lower edge of the cellulose to draw off excess moisture from under the cellulose. Alternatively, a strip of absorbent paper (the paper interleaves used to protect new microscope slides are ideal) can be placed over the cellulose strip and gentle thumb pressure applied to express excess moisture and air bubbles. The reverse side of the slide was then

slowly and gently flamed to drive off all remaining moisture. Alternatively a warm hotplate may be used with constant attention to the drying process. This warming also had the effect of removing wrinkles in the cellulose and caused it to adhere very strongly to the slide.

Cellulosic microscope slides are best inoculated with a spore suspension of the fungus, at a designated spore density, by means of an atomiser which will produce a well-dispersed and more or less even distribution of spores over the cellulose. The authors used a cheap, plastic perfume dispenser for this purpose. Köhler illumination can be maintained and the thin film of spores and hyphae results in excellent photographic images.

Because the cellulosic microscope slide is a dry substrate, inoculated slides need to be incubated in high humidity conditions in order to hydrate and germinate the spores. For this purpose we adapted 50 mL plastic screwtop centrifuge tubes (TPP®) as small humidity chambers. A small wad of tissue paper was inserted into the bottom of each tube and thoroughly moistened to create a high-humidity atmosphere within the tube. A 76.2 x 25.4 mm cellulosic microscope slide fitted neatly into one of these tubes, thus keeping the inocula away from the sides of the container regardless of the tube's orientation. The tubes were small enough to facilitate their random placement in the incubator.

We postulate that either the gelatin or the cellulose acetate, which appears to simulate a leaf surface, provides a stimulant for spores of those fungi which are recalcitrant to germinate on glass slides, although they will often germinate on an agar medium where some component of the medium acts as a stimulant.

A further modification for some fungal species, which is often helpful, is to soak the sterilised cellulose squares in a plant leachate for 10 mins. This leachate is then absorbed into the cellulose acetate conferring stimulant activities. Moreover, an examination of nuclear behaviour in spores, germ-tubes and other post-germination structures is possible using a fluorochrome such as DAPI (4',6-diamidino-2-phenylindole). Other fluorochromes may also be used to examine other internal structures.

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