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Dual culture of *Crinipellis perniciosa* and potato callus

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Abstract. Inoculation of potato callus cultures with basidiospores of the cocoa pathogen *Crinipellis perniciosa* resulted in the development of a mycelium which was morphologically identical to that found in green cocoa brooms. These dual cultures could be maintained for periods of several months. The nuclear condition of this mycelium was found to be variable, in contrast to previous reports. Basidiospores of the L-biotype of *C. perniciosa* were also able to form dual cultures, although a biotrophic phase in its life cycle has yet to be demonstrated *in vivo*. Attempts to form stable heterokaryons between genetically distinct biotrophic mycelia were unsuccessful.

Introduction

The mycelial dimorphism of the agaric *Crinipellis perniciosa* (Stahel) Sing., causal agent of witches' broom disease of cocoa (*Theobroma cacao* L.), is well-documented [Calle, Cook and Fernando, 1982; Evans, 1980; Pegus, 1972; Wheeler, 1985]. A mycelium, composed of swollen, flexuose, unclamped and relatively wide (5–20 μm) hyphae develops after the infection of meristematic cocoa tissues by basidiospores. This mycelium grows intercellularly among the cortical tissues for 4–6 weeks until the brooms begin to necrose. As the host tissues die, a narrower (1–3 μm), dikaryotic mycelium bearing clamp connections emerges. This grows both inter- and intracellularly, degrading the host tissues prior to basidiocarp production on the surface of the dead brooms.

The mycelium found in living brooms was observed by Pegus [1972], Evans [1980] and Calle et al. [1982] to have uninucleate compartments. For this reason it is frequently referred to as primary and monokaryotic, inferring a functional similarity to unmated single spore cultures of outcrossing basidiomycete species [Boidin, 1986; Burnett, 1975]. The mycelium found in dead host tissues was found to have binucleate compartments and has therefore been described as secondary or dikaryotic for similar reasons. However, since both the cocoa biotype (C-biotype) and solanaceous biotype (S-biotype) of *C. perniciosa* have been shown to be

homomictic (primary homothallic) [Delgado and Cook, 1976; Griffith and Hedger, 1994a], while a third biotype found associated with lianas (L-biotype) has been shown to be outcrossing [Griffith and Hedger, 1994a]. The continued use of such terms related to the basidiomycete reproductive strategies to describe the disease cycle in *C. perniciosa* is thus potentially confusing and indeed inaccurate. In this paper, the two phases of mycelial development will be referred to as biotrophic (found in green brooms) and saprotrophic (found in dead brooms).

When basidiospores are germinated on agar media, the hyphae initially bear some resemblance to the biotrophic mycelium seen in green brooms [Brownlee, Hedger and Scott, 1990; Evans, 1980; Hedger, Pickering and Aragundi, 1987]. However, over a period of 7–14 days after germination there is a gradual transition to the saprotrophic mycelium [Griffith and Hedger, 1994a; Hedger et al., 1987]. Attempts to maintain the biotrophic mycelium on agar media have been unsuccessful, although Evans [1980] was able to maintain this mycelial form for several weeks in dual culture with cocoa callus.

We describe here an investigation of the possibility of maintaining the biotrophic mycelium of *C. perniciosa* in dual culture with callus of a number of plant taxa, thus providing a convenient model system for elucidating host-pathogen interactions and investigating the developmental biology of the mycelium.

Materials and methods

Plant tissue culture. Potato callus cultures from two varieties, Maris Piper and Desirée, were the kind gift of Dr. M. G. K. Jones of Rothampsted Research Station. Callus cultures of tobacco and tomato (cv Moneymaker) were obtained from sources within the Institute of Biological Sciences at Aberystwyth. All were maintained on Murashige and Skoog [1962] basal medium (supplied as a powder by Flow Laboratories Ltd.), supplemented with 30 g/l sucrose, 2 g/l casein hydrolysate (Sigma, C-7290), 8 g/l Agar No. 2 (Lab M Ltd.), 0.25 mg/l 6-Benzyl Amino Purine (Sigma B-5898) and 2 mg/l 2, 4-D (Sigma, D-4517). Having adjusted the pH to 5.8 with NaOH, the medium was autoclaved (15 min, 15 psi) and dispensed into 90 mm Petri dishes (30 ml/plate). Three 0.5–1 cm^3 pieces of callus were transferred onto each plate of the fresh medium and after sealing with Parafilm (Alcan Ltd.), these were incubated in the dark at 25 °C.

Basidiocarp production. Basidiocarps of *C. perniciosa* were produced on a bran-vermiculite medium which was covered by a peat-based casing layer and subjected to fluctuating moisture conditions, as described by Griffith and Hedger [1993].

Inoculation of callus dual cultures. Basidiospores were obtained by placing a fresh basidiocarp over a plate of 1.5% water agar at 25 °C until a faint spore print was visible (*c.* 3 h). The callus was then inoculated by placing a 5mm^2 piece of water agar containing spores face down on the top of the callus. Any dual cultures established were incubated as described above and subcultured onto fresh medium at fortnightly intervals.

Microscopic examination of dual cultures and nuclear staining. The method of Stephenson and Gooday [1984] was used. A piece of callus (2–3 mm^3) was teased into small pieces on a microscope slide, placed at 4 °C for 1 h, fixed for 1 h in 70% ethanol and squashed with a coverslip in a drop of DAPI stain solution (4 µg/ml DAPI [Sigma-D1388] in 100 mM Na_2HPO_4 buffer [pH 7.5], stored at 4 °C in a dark bottle). Hyphae were examined under bright field illumination and stained nuclei were observed by epifluorescence microscopy (Carl-Zeiss, filter no. 46-63-01-9901, excitation at 360 nm and fluorescence at 450 nm) of the same preparations.

Assessment of somatic incompatibility. Potato callus was inoculated with basidiospores derived from basidiocarps of pairs of isolates found to belong to different somatic compatibility groupings (SCGs). Small pieces of callus tissue from dual cultures were isolated on MYEA (3% malt extract, 0.5% yeast extract, 1.5% agar). After incubation at 25 °C for 7 days, 5 mm plugs of agar containing the mycelia which grew from the callus tissue were plated on 20% clarified V-8 juice agar [Aragundi, 1982] along with inoculum plugs from each of the parental cultures. This medium was prepared by adding 15 g $CaCO_3$ to 1l V-8 juice (Campbell's Soups Ltd., Norwich, UK), followed by vigorous stirring for 5 min and centrifugation at 10,000 g for 10 min. 200 mls of clarified V-8 juice and 20 g agar was made up to 1l and autoclaved. These plates were sealed with insulating tape, incubated inverted for 4 weeks at 25 °C and examined for incompatibility reactions, including the production of mycelial barrages and zones of crimson pigmentation between the mycelia [Hedger et al., 1987; Griffith and Hedger, 1994b].

Results

Establishment of dual cultures. Inoculation of cocoa callus with basidiospores of *C. perniciosa* (all biotypes) resulted in the appearance of a fluffy clamped mycelium near the point of inoculation, followed by the death of the callus within a few weeks and outgrowth of the saprotrophic mycelium. Others have had more success in establishing the biotrophic phase in cocoa callus [Evans, 1980; Muse, 1989] but the slow growth rate of cocoa callus appears to lead to overgrowth by the fungus and eventual death of the host tissue, possibly due to toxin production [Muse, 1989].

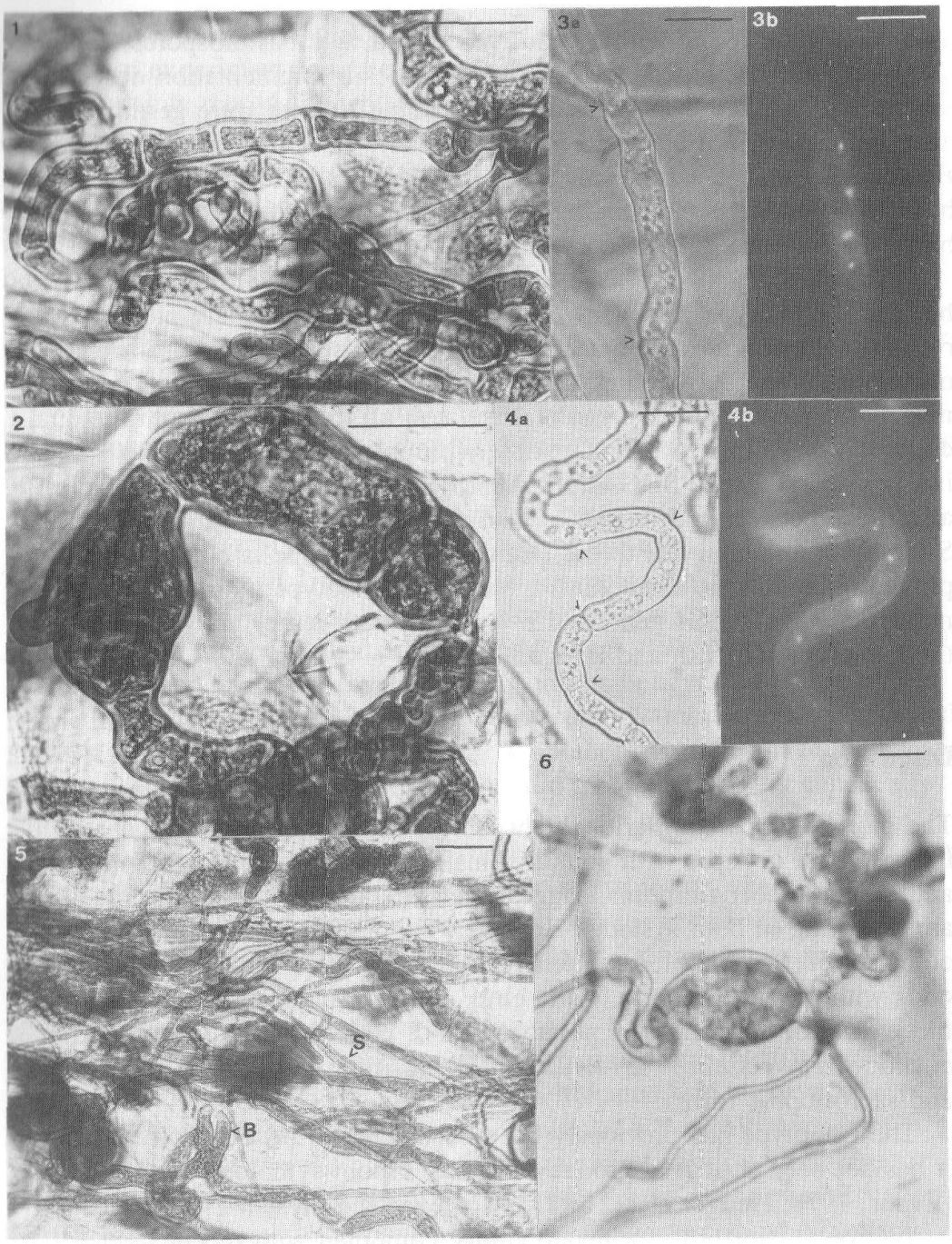
However, a similar result was obtained when more rapidly growing callus cultures of tomato and tobacco were inoculated, and again these cultures were overgrown and killed by the saprotrophic mycelium within two weeks. Microscopic examination of the callus after 5–7 days revealed the presence of the thin saprotrophic hyphae within the callus, also forming aerial hyphae around the point of inoculation. As was found to be the case with basidiospores germinating on agar media [Griffith and Hedger, 1994a], the formation of clamp connections on the saprotrophic mycelium was delayed for two weeks or more on these hyphae.

In contrast, all the potato callus remained healthy in appearance for 4–8 weeks and up to three months if the callus was subcultured fortnightly. Microscopic examination of pieces of inoculated callus tissue near points of inoculation revealed the presence of hyphae which were swollen (5–15 μm), thick-walled and unclamped (Fig. 1). Some cells were distinctly swollen and up to 20 μm diameter (Fig. 2). The hyphae were always intercellular and in older dual cultures were found throughout the callus. The nuclear condition of this mycelium was found to be variable, usually with one to four nuclei per hyphal compartment (Figs. 3–4), although five to ten nuclei per cell were observed in a few cases.

The presence of the fungal mycelium had no apparent effect on the health of the callus tissue, which retained its pale green colour and usual growth rate (doubling time *c.* 14 days). In older dual cultures, however, the callus became darker green in appearance and aerial hyphae characteristic of the saprotrophic mycelium could be seen on the surface of the callus, usually near the callus/agar interface (Fig. 5). In these senescent dual cultures, narrower hyphae (1–4 μm diameter) were also visible. Clamps connections were absent on the narrower mycelium found in the callus but they were observed where the mycelium had grown onto the surrounding agar. Griffith and Hedger [1994a] found that the formation of clamp connections on the hyphae of basidiospore germlings grown on agar did not occur until 7–10 days after germination. In places, the transition between the two mycelial forms could be seen and on two occasions, this transition appeared to be associated with the presence of highly swollen cells (Fig. 6).

When plugs of clamped saprotrophic mycelium taken from agar media were used to inoculate potato callus, no biotrophic infection occurred and the callus was rapidly overgrown and killed. However, basidiospore germlings left to grow on water agar for up to 4 days after deposition did give rise to the biotrophic mycelium when inoculated onto callus tissue. No differences in growth pattern and morphology were observed between dual cultures involving the three biotypes of *C. perniciosa*.

Dual cultures with inocula from different SCGs. When callus cultures were inoculated with basidiospores from pairs of somatically incompatible isolates, the hyphae observed within the callus tissues were indistinguish-



Figs. 1–6. Mycelia of *Crinipellis perniciosa* maintained in dual culture with potato callus. Scale bar in all cases = 20 μm. (1) Biotrophic mycelium of an Ecuadorian C-biotype isolate (226); (2) Grossly swollen mycelium in the same dual culture; (3–4) Hyphae of isolate 226 stained with DAPI, viewed in light field (a) and by epifluorescence (b). Arrows indicate the position of septa (3 a,b) A hyphal compartment containing four nuclei S = septum. (4 a,b) Hyphal compartments containing two nuclei each; (5) A mixture of biotrophic and saprotrophic mycelium of an Ecuadorian L-biotype isolate (SCFT) in senescent callus tissue. S and B indicate saprotrophic (thin) hyphae and biotrophic (swollen) hyphae respectively. Note the absence of clamp connections on the saprotrophic mycelium; (6) A grossly swollen cell at the transition point between the biotrophic and saprotrophic mycelial forms in a senescent callus culture.

able from those found within callus inoculated with basidiospores from a single isolate. The mycelial barrages and zones of pigmentation observed when basidiospores from pairs of incompatible isolates were germinated on agar media were not observed in either healthy or senescent callus tissues. However, when the saprotrophic mycelium from senescent callus cultures had grown onto the surrounding medium, sectors delineated by mycelial barrages and lines of crimson pigmentation were observed.

Four pairings between basidiospores from C-biotype isolates and two S-biotype × C-biotype pairings were made. Mycelia from these pairings were reisolated from senescent callus tissue and from sectors of saprotrophic mycelium on the surrounding agar. However, when these were paired with mycelia of their respective parental isolates, all the mycelia from the dual cultures were found to be compatible with one or other of the two parental isolates and no novel SCGs were identified. A number of pairings were also made between basidiospores of the L-biotype and those of the C- and S-biotypes, although due to the fact that the L-biotype is outcrossing, it proved impossible to distinguish putative inter-biotype hybrids from the numerous novel SCGs generated by matings between primary mycelia of the L-biotype [Griffith and Hedger, 1994b].

Discussion

The morphological similarities between the biotrophic mycelium observed in these dual cultures with potato callus and that observed by Pegus [1972] and others in cocoa brooms, suggest that similar developmental processes are occurring in both systems. However, the observation that the hyphal compartments of the biotrophic mycelium were often multinucleate is at odds with previous reports [Calle et al., 1982]. This may be an artefact of the growth of the fungus in potato callus or perhaps an indication that a coenocytic mycelium is involved in the transition between the biotrophic and saprotrophic forms.

The L-biotype has previously been considered to be a saprotroph, since it has not been shown to produce witches' broom symptoms in any host [Evans, 1978; Hedger et al., 1987]. However, the present evidence of its biotrophic capability in potato callus, combined with recent field observations of its host specificity in nature [Griffith and Hedger, 1994b], suggest that the mycelial establishment of the L-biotype from basidiospores may involve biotrophic infection of its host, as has been demonstrated for the C- and S-biotypes.

There are several reports that the S-biotype but not the C-biotype of the fungus is able to produce witches' broom disease symptoms in a number of solanaceous hosts, including tomato and sweet pepper (*Capsicum annuum* L.) [Bastos, Andebrhan and de Almeida, 1988; Bastos and Evans, 1985; Ellen Dickstein, University of Florida, pers. comm., 1989]. The ability of

C. perniciosa to infect intact potato plants has not been demonstrated. Inoculation experiments at Aberystwyth were unsuccessful (data not shown), mainly due to the tendency of potato plants, subjected to the conditions of high humidity required for basidiospore infection [Frias, 1987], to succumb to other infections. However, the fact that all biotypes of the fungus produced a biotrophic infection of potato callus does not necessarily imply that intact potato plants are particularly susceptible hosts. Dual culture studies with other host-pathogen combinations have demonstrated that race-specific incompatibility reactions are not always expressed in callus culture [Gray and Sackston, 1987; Ingram, 1976; Miller and Maxwell, 1983].

Evans [1980] has proposed that the biotrophic mycelium of *C. perniciosa* is maintained by an unstable 'modifier' produced by actively growing host cells. In the absence of this (these) substance(s), an irreversible transition to the saprotrophic form occurs. The results of this study are consistent with this hypothesis. The failure of our tomato dual cultures despite the high growth rate of the callus suggests that the putative 'modifier(s)' may be absent in callus cultures of this species, although apparently not in whole plants [Bastos and Evans, 1985].

Somatic incompatibility in the higher fungi is believed to play a role in restricting genetic exchange between genetically distinct mycelia [Croft and Dales, 1984; Kay and Vilgalys, 1992]. A number of studies have indicated that the control of the expression of somatic incompatibility is genetically complex and that heterozygosity at any one of several loci can prevent heterokaryosis. In the outcrossing basidiomycetes, however, fusion between genetically distinct primary mycelia is a prerequisite for heterokaryon (i.e. secondary mycelium) formation and it has been suggested that somatic incompatibility mechanisms may be overridden during such mating events [Rayner et al., 1984].

Investigations of the genetic variability of C-biotype isolates, as assessed by somatic compatibility reactions and other parameters [Griffith, 1989; McGeary and Wheeler, 1988; Wheeler and Mepsted, 1988], have shown that populations of this biotype generally consist of a small number of geographically widespread clones (= somatic compatibility groupings). However, during a detailed study of the C-biotype in Amazonian Ecuador [Griffith, 1989], some local populations were found to consist of several sympatric clones. These populations were found to consist of two widespread SCGs along with a number of less common SCGs, whose incompatibility reactions with the two dominant SCGs were usually indistinct and sometimes difficult to classify. These observations raised the possibility that the smaller SCGs in these areas had arisen by genetic recombination between the dominant SCGs, and that this could have occurred through multiple infection of cocoa meristems and subsequent heterokaryosis during the biotrophic phase of growth.

The absence of any zones of antagonism within callus cultures inocu-

lated with pairs of incompatible C-biotype isolates suggests that the expression of somatic incompatibility in biotrophic mycelia is suppressed or absent. However, attempts to reisolate recombinant mycelia predicted to display non-parental somatic incompatibility phenotypes from these cultures were unsuccessful. It may be the case that heterokaryotic mycelia formed as described above become unstable and segregate during the transition to saprotrophic growth. Alternatively hyphal anastomosis and subsequent heterokaryosis may occur at a frequency too low to be detected by the approach used in the present study. Nevertheless, the use of dual cultures between potato callus and *C. perniciosa* does provide a useful system for the study of both the developmental and population biology of this fungus.

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