

In Vitro Production of Biotrophic-Like Cultures of *Crinipellis pernicioso*, the Causal Agent of Witches' Broom Disease of *Theobroma cacao*

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Received: 24 June 2005 / Accepted: 9 November 2005

Abstract. Witches' broom disease (WBD) of cacao, caused by the hemibiotrophic fungus, *Crinipellis pernicioso*, exhibits a succession of symptoms that are caused by the biotrophic phase of the fungus. However, the study of this biotrophic phase is limited by its exclusive growth inside the plant or in the presence of callus. Here we report for the first time a method for the growth and maintenance of the biotrophic-like phase of *C. pernicioso* on a defined medium with metabolites found in the diseased tissues. Our results suggest that glycerol is a key carbon source for this interaction. This is a crucial achievement toward understanding the biology of this fungus during the infectious phase of WBD.

Theobroma cacao is the source of cocoa beans, the raw material for the production of chocolate. Its worldwide production is hindered by several major fungal pathogens. *Crinipellis pernicioso* (Stahel) Singer (*Basidiomycota*, *Agaricales*, *Tricholomataceae*) is a hemibiotrophic fungal pathogen that causes witches broom disease (WBD) of cacao, which has drastically decreased cacao production in most of the western hemisphere [11]. This fungal pathogen appears to have originated in the Amazon basin, where it infects and causes disease on plant species from the families *Heteropteris*, *Sterculiaceae*, *Solanaceae*, and *Bignoniaceae* [7, 13, 14, 23, 24]. Because of the importance of this disease, a genome project is currently sequencing *C. pernicioso*.

WBD begins when wind-borne monokaryotic spores infect young meristematic tissues through stomatal openings [8]. The uninucleate biotrophic mycelia lack clamp connections and grow intercellularly within the living host tissues without causing the death of the

tissue [2]. An important aspect of this disease is that the infectious process, disease development, and ultimate death of the tissue all happen at or distal to the original infection point, thus limiting the spread and scope of this disease to new growth. Furthermore, all of the elicited plant responses of the living tissue result from a limited amount of fungal biomass [22]. The characteristic symptoms of WBD, caused only by the biotrophic phase of the fungus, are hyperplastic and hypertrophic growth of infected meristematic tissues, which are associated with the loss of apical dominance, resulting in brooming of the affected branches and the occurrence of parthenocarpic fruits, which occur when flower cushions are infected [11]. After 2 to 3 months, the fungus undergoes dikaryotization, produces clamp connections, and transforms to the saprotrophic phase [2]. The mechanisms involved in the change of the fungal phases (from biotrophic to saprotrophic) are not presently known, but it coincides with the cessation of green broom development and the subsequent senescence of the broom, which is rapidly colonized by the saprotrophic fungal mycelia. After multiple cycles of wetting and drying, the

fungal matrix inside the dry broom produces small pinkish basidiocarps on the surface of the broom and copious amounts of basidiospores. The process of basidiocarp formation from a single broom can be repeated for several years.

A number of different plant metabolites have been suggested in the past to have a role in maintaining or shifting the biotrophic phase of *C. pernicioso*. In the work of Brownlee et al. [3–5], cacao metabolites such as tannins and procyanidins were shown to have the ability to alter the growth of *C. pernicioso*. Furthermore, Evans [7] postulated that reducing sugars prevented the dikaryotization of the biotrophic phase. Unfortunately, none of these compounds have led to a method by which the biotrophic phase can be maintained for extended periods in culture. Therefore, to date, very little is known about the biotrophic phase of *C. pernicioso* because it has only been observed in vivo or maintained in vitro in the presence of cacao or potato callus [7, 14]. Germinating spores in typical culture media rapidly undergo dikaryotization, resulting in the saprotrophic phase [7, 14, 16]. Consequently, almost all current knowledge of this fungus is based on the saprotrophic phase despite the obvious biochemical, metabolic, and physiologic differences between the two phases. These differences are consistent with findings from our own recent work [26]. Therefore, one of the main objectives of our research is to develop methodologies to examine *C. pernicioso* at all stages of the disease process, with particular emphasis on the biotrophic phase, which could ultimately lead to a control for WBD.

In this article, we report for the first time the conditions that prevent the rapid in vitro shift of *C. pernicioso* cultures from the monokaryotic mycelia, which germinate from spores, to the saprotrophic/necrotrophic hyphae. A defined medium containing host metabolites was developed to support the stable growth of hyphae that were morphologically identical to the biotrophic hyphae found in infected cacao tissues. This achievement opens the possibility of studying *in vitro* the most important phase of *C. pernicioso* biology and could potentially be applied to other hemibiotrophic or biotrophic fungi.

Materials and Methods

Basidiospores. Basidiospores were prepared by the bran-vermiculite medium as described by Griffith and Hedger [12] from single-spore isolates and were collected and stored at -70°C in a buffered 16% (v/v) glycerol solution [9]. Spore preparations were provided by Dr. Alan Pomella (Almirante Cacao Agrícola, Itajuípe, Bahia, Brazil) and were maintained at -70°C before use. Before plating, spores were thawed at room temperature, and a volume representing approximately 250,000 spores was diluted in 2 volumes of sterile water containing kanamycin

($50\ \mu\text{g}\ \text{ml}^{-1}$) and streptomycin ($50\ \mu\text{g}\ \text{ml}^{-1}$), and the mixture was spread over a 15-cm diameter plate containing the spore germination medium.

Spore germination medium. Spore germination was carried out on a water-agar ($30\ \text{g}\ \text{l}^{-1}$) based medium containing glycerol ($20\ \text{ml}\ \text{l}^{-1}$) as sole carbon source with no nitrogen source. A final concentration of $5\ \text{mg}\ \text{l}^{-1}$ caffeine was added before autoclaving, and $10\ \text{mg}\ \text{l}^{-1}$ auxin (indole-3-acetic acid) was added as a sterile solution ($25\ \text{mg}\ \text{ml}^{-1}$ stock solution made up in 0.1 N KOH and filter sterilized) after autoclaving. This solid medium was designated LMCpS+. After adding and spreading the spore–water dilution (mentioned previously with antibiotics) over the surface, the plates were immediately wrapped with plastic film so that the surface of the plate was covered with a layer of water and spores. This was done to ensure a layer of free water, which is required for spore germination [8]. This method was used with spores that were relatively free of contaminating bacteria, yeast, or other fungal species. With contaminated spore preparations, the method was modified by using an additional step, which was to apply a $15\ \text{g}\ \text{l}^{-1}$ top agar after the addition of the spore solution. The top agar contained the same concentrations of antibiotics, auxin, caffeine, and glycerol—as mentioned previously—in the support layer. This additional step helped by confining the contaminating bacteria and yeast within the top agar. Plates were incubated at 28°C in the dark.

Microscopic evaluations. A stereomicroscope (Zeiss Stemi 1000; Zeiss International) was used to monitor the germination and growth of the *C. pernicioso* spores on a daily basis. Minicolonies derived from single spores were observed after 7 to 10 days. Colonies that showed rapid germination or growth, especially within the first 5 days, were not *Crinipellis* sp. (most were either *Penicillium* or *Trichoderma* spp.) and were removed aseptically. Agar blocks containing *C. pernicioso* mycelia were removed, placed onto a slide, squashed under a cover slip, and observed with a light microscope (using oil-emersion) to verify the presence of dolipore septa and the lack of clamp connections. Mycelia were stained with SYBR Green I (Invitrogen—Molecular Probes, Eugene, OR) as described by Meinhardt et al. [19] to verify nuclei number per cell with epifluorescence microscopy (Olympus BX40 microscope with a BX-FLA fluorescence attachment; Olympus, Melville, NY) and excitation filters (Olympus filter cube WB excitation 450 to 480 and emission 515).

Liquid growth medium. Biotrophic-like mycelia of *C. pernicioso* were aseptically cut from the LMCpS+ medium. The agar blocks (approximately 1 to 2 cm^3 agar and mycelia) were cut into numerous smaller blocks and added to 50-mL flasks containing a semidefined liquid growth medium comprising $5\ \text{g}\ \text{l}^{-1}$ yeast extract (Difco), $50\ \text{ml}\ \text{l}^{-1}$ glycerol, $2.5\ \text{g}\ \text{l}^{-1}$ K_2HPO_4 and $1\ \text{ml}\ \text{l}^{-1}$ trace elements [18] and designated LMCpL+. The inoculated medium was placed at 27°C at 120 rpm in the dark. The mycelia were microscopically monitored with a Nikon Elipse E800 microscope (Nikon, Melville, NY) equipped with the Media Cybernetics Digital Imaging System [Image Pro Plus version 4.0] every 48 hours to ensure that no alteration had occurred in the growth phase and to check for contamination.

DNA extraction. To verify the identity of the fungal mycelia, DNA analysis of the biotrophic-like phase of *C. pernicioso* was conducted. DNA was extracted from mycelia grown in LMCpL+ medium that had been verified by microscopy to have the characteristics of the biotrophic phase. DNA extraction was done according to Meinhardt et al. [20] (<http://www.scielo.br>). Microsatellite polymerase chain reaction (PCR) primers [20, 21] were tested with DNA extracted from the biotrophic-like and saprotrophic phases of the same isolates. The primers used were capable of distinguishing genetic variability among

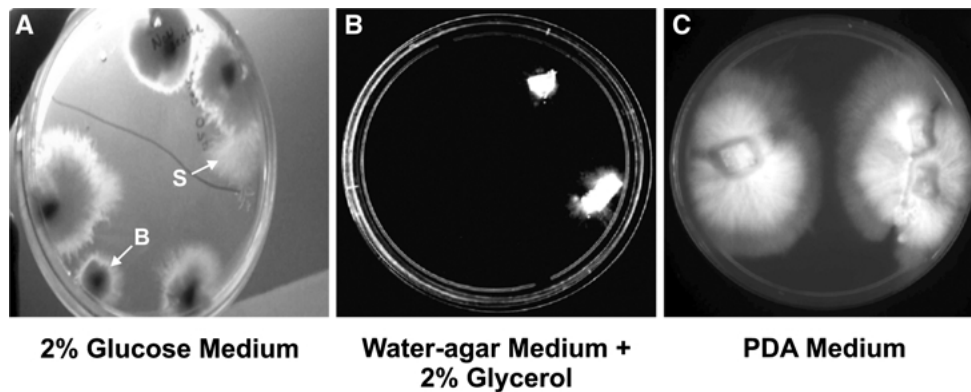


Fig. 1. Representative growth patterns of the different phases of *C. pernicioso* on agar media. (A) Typical slow-growing biotrophic phase. B = biotrophic-like colony; S = fast-growing saprotrophic sector. Small slow-growing colonies were moved from LMCpS+ medium to the same medium supplemented with 2% glucose without glycerol. Sectoring began between 10 and 14 days after transferring the colonies. (B) Agar blocks containing the biotrophic-like mycelia were removed from the primary plate, LMCpS+ medium, and transferred to a plate with water-agar plus 2% glycerol. The plate shows 14 days of growth of the biotrophic-like phase. (C) Agar blocks containing the biotrophic-like mycelia were transferred to a PDA plate where the mycelia transformed to the saprotrophic phase. The plate shows 14 days of growth after transference. The biotrophic-like mycelia rapidly transformed to saprotrophic (note the fast-growing sectors) on PDA medium. PDA = potato-dextrose agar.

the different clonal populations of *C. pernicioso* [25]. The ITS region containing the ITS1, ITS2, and 5.8S sequences were amplified with the universal ITS1 and ITS4 primers [28]. PCR-restriction length fragment polymorphism analysis was done with the restriction enzyme *HinfI* [1].

Results and Discussion

Initially, we reasoned that substances present in living infected tissues could be determinants for the maintenance of the biotrophic phase of *C. pernicioso*. Therefore, we tried to mimic the conditions found in the infected tissue by adding specific compounds to artificial culture medium. Spores were germinated at low densities on water-agar media to identify *C. pernicioso* monokaryotic mycelia and isolate them from contaminants that are frequently associated with spore preparations. The germinating spores were then transferred after 7 days to other media containing different classes of compounds at different concentrations. In most cases, the cultures rapidly shifted to binucleate mycelia with clamp connections, which are characteristics of the saprotrophic phase of this fungus. The first promising results were achieved by inoculating spores onto a nutrient-poor solid medium containing 5 mg l^{-1} caffeine, 10 mg l^{-1} auxin, and 0.05% glucose. In this case, we could clearly detect the slow-growing colonies with mononucleated hyphae, dolipore septae and no clamp connections, which are distinct well-accepted features of the biotrophic mycelia [7, 14, 23, 8, 2]. However, these colonies were unstable, and they transformed into the saprotrophic/necrotrophic hyphae within 15 days, which would typically begin as a faster-growing sector of mycelia at the border of the colonies. (Fig. 1A). Sub-

sequently, our group found that green brooms contain a significantly higher amount of glycerol than normal tissues [26], and thus we tried this carbon source to replace glucose. Spores plated onto the LMCpS+ medium showed small slow-growing minicolonies approximately 7 days after inoculation. These slow-growing colonies had white fluffy mycelia that normally stopped growing after 4 to 6 weeks on the solid medium (Fig. 1B), although the cells remained viable for up to 3 months on this plate. The viability was tested by transferring the colonies to the LMCpL+ liquid medium and evaluating the growth of the biotrophic-like colonies. The cessation of growth on the solid medium could have been caused by the lack of a nitrogen source in the solid medium. Biotrophic-like colonies transferred to typically fungal media such as malt extract or potato dextrose agar converted to the saprotrophic phase after 7 to 10 days of growth (Fig. 1C). Biotrophic-like colony growth in the LMCpL+ liquid medium, which contained a nitrogen source (amino acids from the yeast extract), was more vigorous and showed multiple round colonies of varying sizes (not shown). The size variation of the colonies showed no effect on the biotrophic characteristics and appeared to be derived from mycelia that had broken off the original colonies imbedded in the agar. It is interesting to note that the biotrophic-like mycelia appear to be produced only under conditions in which nutrients are limited. This seems to mimic the *in vivo* situation because the biotrophic hyphae do not have haustoria and grow exclusively outside the plant cell within the intercellular space (apoplast), subsisting on the limited nutrients found there. This may also explain

the low density found for the biotrophic hyphae in the green broom.

To test other carbon sources, the biotrophic-like phase of *C. pernicioso* was grown on LMCpL+ for 10 days and transferred to LMCpL medium containing different compounds (supplemented with a 2% [w/v or v/v] concentration of each of the different compounds) such as, ethanol, fructose, glucose, glycerol, sorbitol, or sucrose. The transferred mycelia transformed into the saprotrophic phase of the fungus (Fig. 1C) in all carbon sources except glycerol.

The next step was the removal of caffeine and auxin. The working hypothesis was that the maintenance of the biotrophic phase needed a plant hormone, such as auxin, which was very tempting because this could connect the physiology of both the fungus and the plant. However, no differences in the growth pattern of the mycelia were observed after removal of the hormone, clearly showing that glycerol was the fundamental compound. Nevertheless, there was a clear benefit in maintaining auxin and caffeine in the medium because the spores are normally collected along with various contaminants, such as yeasts, that are more sensitive to the caffeine and auxin concentrations used than the monokaryotic biotrophic-like hyphae. Therefore, these compounds are useful in obtaining clean cultures, but only glycerol is required to maintain the biotrophic mycelia.

Lipids have been found to be one of the signals that promote and maintain the filamentous phenotype of *Ustilago maydis*, which resembles the infectious mycelia found in planta [17]. Furthermore, Wei et al. [27] provided evidence that glycerol played a significant role in nutrient transfer from infected plants to the fungal pathogen *Colletotrichum gloeosporioides* f. sp. *malvae*. In addition, the sucrose nonfermenting-related protein kinase from *Colletotrichum gloeosporioides* f. sp. *malvae*, which is overexpressed during penetration in planta, was found to have higher expression when the fungus was grown in glycerol [10]. Taken together, all of the evidence suggests that glycerol, and possibly lipids, could play a significant role in altering the fungal response during in planta growth. The levels of glycerol used to germinate *C. pernicioso* and maintain the biotrophic phase in vitro are comparable with those observed by Scarpari et al. [26], who found significantly higher levels of glycerol in infected green broom tissue of *T. cacao* compared with uninfected tissues. These higher levels of glycerol found in the infected tissues were completely gone in dry broom tissues, which have been shown to contain only the saprotrophic phase of *C. pernicioso* [2]. These high levels of glycerol in planta may maintain the biotrophic phase during the development of WBD.

As mentioned previously, *C. pernicioso* spores are collected with various contaminating microorganisms. Therefore, it was important to prove that the colonies with different morphology were indeed *C. pernicioso*. To do that, DNA was extracted from the saprotrophic mycelia of four different *C. pernicioso* isolates (CP02, CP09, Bp10, and FA42) and from four biotrophic-like colonies developed from spores from the isolate Bp10. In all cases, PCR produced ITS1-5.8S-ITS2 bands of similar size for all of the samples, and the DNA fragments that were generated from these bands produced exactly the same restriction pattern after being digested with the restriction enzyme *Hinf*I [1] (data not shown). Additionally, no differences in PCR banding patterns were observed between the samples for several microsatellites primers used [20, 21, 25] (data not shown). Furthermore, cDNAs derived from mRNA extracted from the biotrophic-like mycelia of *C. pernicioso* were verified by sequence analysis and matched exactly the genomic DNA sequences (unpublished data). Together, these results clearly show that the morphologically different biotrophic-like and saprotrophic colonies are indeed the same organism: *C. pernicioso*.

Epifluorescence microscopy confirmed the cytotopic nature of the colonies (Fig. 2). Biotrophic-like cultures presented mycelia lacking clamp connections (Fig. 2A) that were uninucleated (Fig. 2B), whereas mycelia with clamp connection (Figs. 2D and 2E) were binucleated (Fig. 2E) [6, 25]. The transformation of biotrophic to saprotrophic mycelia has been previously characterized by a slight narrowing of the mycelia accompanied by the formation of clamp connections. In addition, during the period of transition, we detected highly swollen cells (Fig. 2C), which are structures previously reported by Griffith and Hedger [14].

To date, this procedure has been repeated at least 12 times during a 2-year period using four different *C. pernicioso* isolates representing the two main genotypic groups found in the State of Bahia, Brazil [25]. As mentioned previously, this is the first report of an *in vitro* method to prevent the direct shift of the biotrophic-like mycelia of *C. pernicioso* to the saprotrophic mycelia, thus creating conditions to maintain the biotrophic-like phase of *C. pernicioso*. Mycelia obtained with this procedure are now being used in multiple physiologic, biochemical, and genetic analyses of this developmental stage. In particular, this methodology has allowed the production of cDNA libraries that are now being sequenced as part of the *C. pernicioso* functional genome project (<http://www.lge.ibi.unicamp.br/vassoura>). These analyses will allow a detailed examination of the complete host-pathogen interaction involved in WBD at the molec-

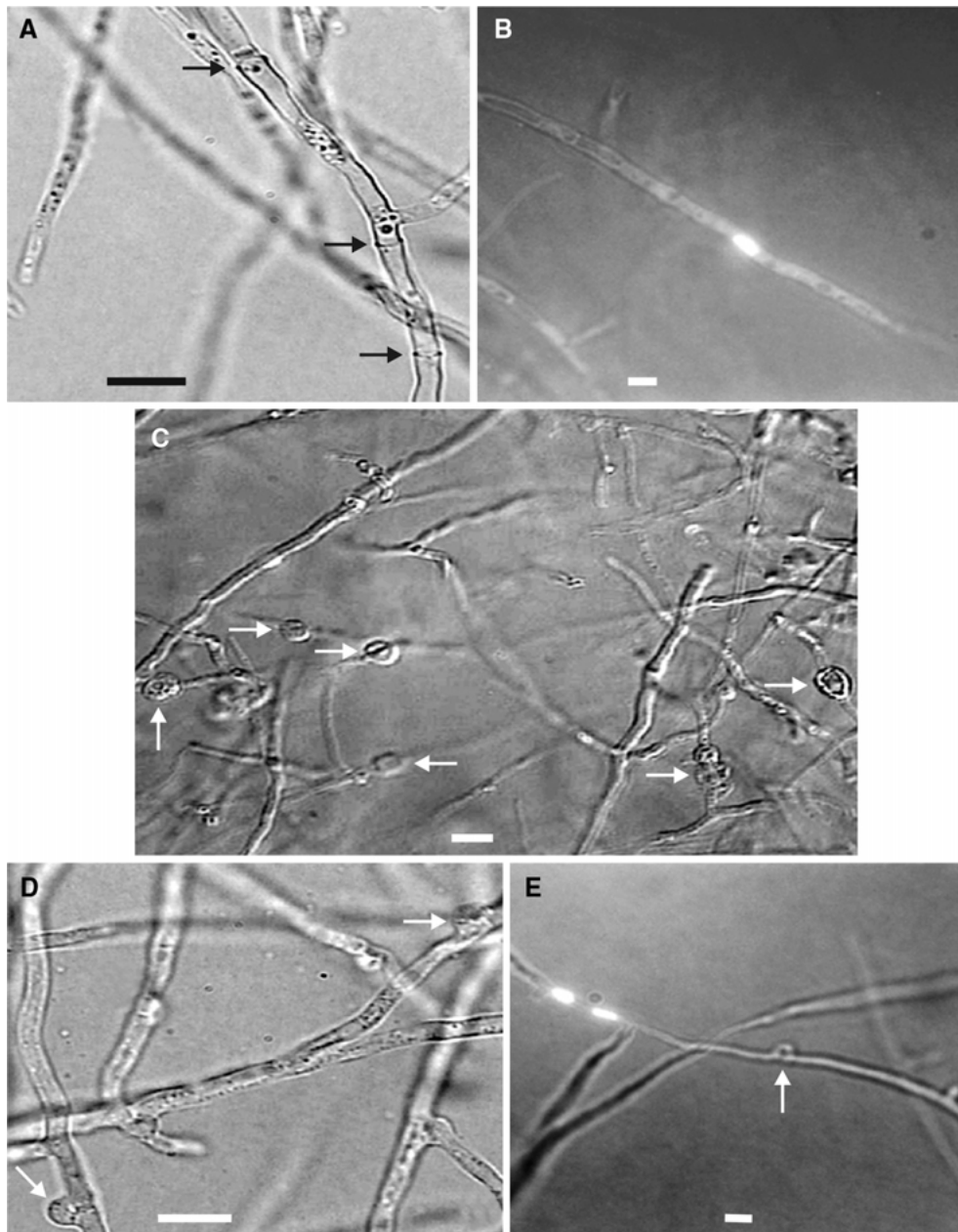


Fig. 2. (A and D) Micrographs of the biotrophic-like and saprotrophic phases of *C. pernicioso*. (A) Biotrophic-like characteristics (larger mycelia, dolipore septa, and no clamp connections). Black arrows point to septa. (D) Saprotrrophic-phase mycelia. White arrows point to clamp connections. (B and E) Nuclear condition of the different phases of *C. pernicioso*. (B) Epifluorescence micrograph of the uninucleated biotrophic-like mycelia. (E) Binucleated mycelia of the saprotrophic phase, which also has clamp connections (white arrow). (C) Highly swollen cells (white arrows) that occur during the period between biotrophic and saprotrophic phases of *C. pernicioso* growth *in vitro*. The micrograph was taken with Nomarski phase filters. Solid bars = 10 microns. All photos are in gray scale.

ular level and could help determine the signaling mechanisms involved in phase changes.

ACKNOWLEDGMENTS

The investigators thank the ongoing corporate support given by Almirante Cacau Agrícola (Masterfoods) and Cargill. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo

(Nos. 00/10545-4 and 02/09280-1); Conselho Nacional de Pesquisa e Desenvolvimento (No. 68.0032/01-0; 47.1609/03-0); and Secretaria de Agricultura do Estado da Bahia.

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