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Development of Natural Culture Media for Rapid Induction of *Fonsecaea pedrosoi* Sclerotic Cells In Vitro▽

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***Fonsecaea pedrosoi* is the main agent of chromoblastomycosis, a skin disease presenting verrucous lesions, in which round, thick-walled sclerotic cells are found. In vitro induction of sclerotic cells is time-consuming (20 to 45 days) and temperature dependent. We present two new natural media that reduce the sclerotic-cell induction time to only 2 days.**

Chromoblastomycosis is a subcutaneous mycosis with verrucous-nodular lesions, usually localized on the lower limbs of rural workers (12), appearing after accidental inoculation with thorns bearing dematiaceous fungi, such as *Fonsecaea pedrosoi* or *Cladophialophora carrionii* (11, 15). Laboratory diagnosis is performed by direct microscopic examination of skin scrapings after treatment with 10% KOH. Round, brownish fungal cells (called sclerotic or muriform cells) characterized by multiseptate division are observed in small aggregates or isolated in the lesion (9).

The vast majority of in vitro work done with *F. pedrosoi*, including tests with drugs, has used only hyphae (2) or the conidial stage of the life cycle of the fungus. In contrast to sclerotic cells, hyphae and conidia are never found in the lesions. Initial attempts to produce sclerotic cells in vitro were made in 1957 using a "synthetic lymph medium" made with alcoholic extracts of hair and nails in which spherical bodies, articulated in hyphal fragments similar to chlamydospores, were obtained (13). In 1985, *F. pedrosoi* strains cultured under constant shaking in Sabouraud medium (pH 2.5) after 30 days produced round, yeast-like cells with no septation (7). In 1993, a chemically defined medium (pH 2.5, 25°C) with 0.1 mmol liter^{-1} Ca^{2+} or the calcium chelant EGTA at 2 mmol liter^{-1} induced sclerotic cells after 21 days of culture under constant shaking (8).

The two media developed in this study were made from tree fruits. *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum. (cupuassu) is a native Amazonian tree that has a slightly fibrous, yellowish mesocarp, containing potassium (34.3 mg/100 g of mesocarp [fresh weight]), phosphorus (15.7 mg/100 g), magnesium (13.0 mg/100 g), and amino acids (10). *Bactris gasipaes* Kunth (peach palm) is an American palm tree, containing potassium (289.3 mg/100 g of mesocarp), calcium (24.7 mg/100 g), and magnesium (17.6 mg/100 g). It is rich in fatty acids, with oleic (46.3%), palmitic (38.2%), and palmitoleic (7.4%) acids (14).

Three different *F. pedrosoi* strains, obtained from skin scrapings, were used in this study. All samples presented the classic sclerotic cells (Fig. 1A and B) and were isolated in Mycosel (Becton Dickinson) and perpetuated in Sabouraud agar (Merck, Germany). These media promoted the formation of greenish-black colonies (Fig. 1C), which were analyzed by microculture (Fig. 1D); dematiaceous hyphae with cylindrical, intercalary or terminal, loosely branched conidiophores with small conidia compatible with *F. pedrosoi* (5) were observed.

T. grandiflorum and *B. gasipaes* fruits were washed in the laboratory. The mesocarp was separated from the seeds, diluted 1:3 with distilled and deionized water, homogenized using a shaker, and centrifuged at 4,000 rpm for 5 min. The supernatant was collected and filtered through a 0.22-μm-pore-size filter (Advantec); the pH was adjusted to 2.7 with 1 M HCl; and the medium was autoclaved. The media and the processes for their production were registered with the Brazilian National Institute for Intellectual Property (11a).

Five fungal colonies, each with a diameter of around 2 to 3 cm (Fig. 1C), cultured for 15 to 20 days in potato agar (Merck, Germany), were harvested, suspended in 10 ml of distilled and deionized water, homogenized in a vortex mixer for 30 s, and filtered through a nylon membrane to separate hyphae from conidia. The conidia were collected and centrifuged at 4,000 rpm for 5 min, and the pellet was resuspended in deionized water to a final volume of 1 ml for counting using a Neubauer chamber. Sclerotic cells were induced from previously isolated conidia in 24-well cell culture plates (TPP, Switzerland) filled with one of the two new media at a concentration of 10^3 conidia/ml, with 20 μg/ml of gentamicin. For optical microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM), sclerotic cells were prepared as described previously (4).

More than 90% of conidia cultured for 48 h differentiated into sclerotic cells very similar to those found in lesioned tis-

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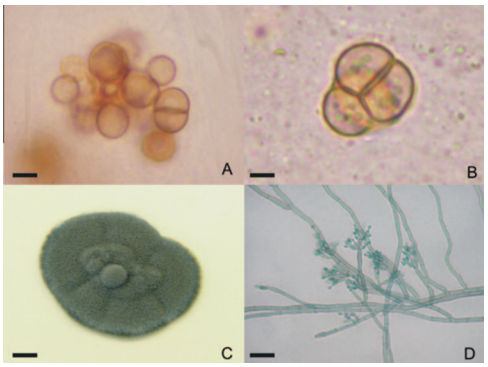

FIG. 1. *Fonsecaea pedrosoi* sclerotic cells were obtained after scraping of chromoblastomycosis lesions. The cells gave rise to hyphae and conidia after in vitro culture. (A and B) Skin scrapings were collected and analyzed after clarification with 20% KOH, revealing well-defined, septated, sclerotic cells. (C) Greenish-black colonies grew from culture of this material on Mycosel. (D) Characteristic dematiaceous hyphae originating terminal cylindrical conidiophores with small subhyaline conidia were observed upon microculture. Bars, 4 μm (A), 3 μm (B), 0.5 cm (C), and 10 μm (D).

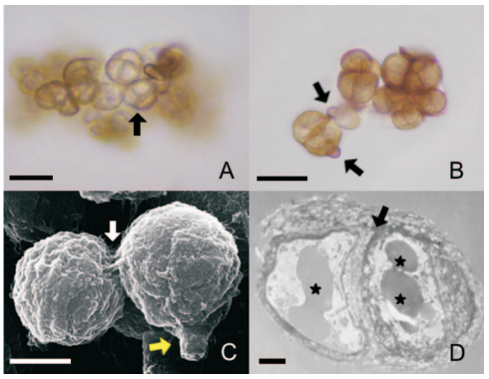

FIG. 2. The morphological characteristics of sclerotic cells induced in either of the two natural media are similar. (A and B) Sclerotic cells obtained after culture of conidia in *Theobroma grandiflorum* or *Bactris gasipaes* natural media were analyzed by optical microscopy with no special staining. (A) Brownish, multiseptated cells (arrow) were evident. (B) Remnants of conidia adhering to the newly formed sclerotic cells (arrows) were observed. (C) SEM of aggregates of sclerotic cells demonstrates septated division (white arrow) and remnants of conidia (yellow arrow). (D) TEM of a sclerotic cell shows typical thick-walled septation (arrow) and vesicles containing electron-dense material (asterisks). Data are representative of one of three independent experiments, which were performed in triplicate. Bars, 10 μm (A and B), 5 μm (C), and 1 μm (D).

sue, with multiseptate division, a very thick wall, and a brownish color. After 15 days with no changes in the medium, there was differentiation from sclerotic cells toward hyphae. When the medium had been changed before 15 days, there was no hyphal formation, and it was possible to keep sclerotic cells viable for 15 days more, and so on. These cells became enlarged and septated, forming small aggregates, with no individualization of sclerotic cells. The longest culture time was 1 year, indicating the long-lasting viability of these cells.

The sclerotic cells induced in vitro in the two media displayed the same characteristics of size, color, and type of cellular division and multiseptation (Fig. 2A). An interesting characteristic of *F. pedrosoi* differentiation was observable for the first time: the conidial cellular wall breaks at one point, and a new sclerotic cell is formed by the expansion of the cytoplasm that was previously contained by the conidial wall (Fig. 2B and C). Multiseptation can be observed well by both SEM and TEM (Fig. 2C and D). Besides the well-defined septation, TEM also made it possible to observe vesicles and electron-dense granules near the thick cellular wall.

The techniques presently available for inducing sclerotic cells from *F. pedrosoi* hyphae or conidia are based on the chemically defined Butterfield medium, which, following addition of 800 μM DL-propranolol, can induce sclerotic cells after 45 days of culture at pH 2.5 with constant shaking (1).

Phytopathogenic fungi are common in the species used to develop the new media. Examples include *Crinipellis perniciosa*, the etiologic agent of witches' broom disease in *T. grandiflorum*, and a *Mycospharella* sp. that is responsible for brown leaf spot in *B. gasipaes*. Also, the pathogenic forms of chromoblastomycosis are observed in plant tissues, for example, in histological specimens of the cactus species *Ritterocereus griseus* and *Ritterocereus deficiens* (15), indicating that different plant species can be natural substrates for the growth of pathogenic fungi. Here we report that plant species can be employed in in vitro media to grow *F. pedrosoi*.

By using these natural media, it was possible to reduce the time required for the induction of sclerotic cells from about 45 days to only 48 h without the addition of other chemical components and without the use of specific temperature conditions or shaking. SEM and TEM enabled us to evaluate the morphological similarities between our in vitro-generated sclerotic cells and those of others (3, 6) and between in vitro-generated sclerotic cells and those from lesions. The main similarities are size, multiseptation, and the formation of a thick, pigmented cellular wall. Additionally, the vesicles observed inside the sclerotic cell cytoplasm by TEM are consistent with previous findings (3).

The new culture media presented here significantly reduce the time necessary for the induction of sclerotic cells, making labor-intensive conditions such as constant shaking or controlled temperature unnecessary, and open up new possibilities for studying *F. pedrosoi* pathogenic forms in vitro using a simple and economical technique.

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