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Abstract

Nuclei of vegetative hyphae of the puffball species *Lycoperdon pyriforme* are small and difficult to stain. A simple mitotic staining technique utilizing a modified version of Ziehl-Neelsen's carbol-fuchsin stain, originally developed for examining bacteria and small chromosomes of plants, has proven useful for observing fungal nuclei in this gasteromycete species. Cells of vegetative hyphae were pre-treated for 2 hours with an aqueous solution saturated with naphthalene, fixed in a 3:1 mixture of 100% ethanol and glacial acetic acid, and hydrolyzed for 10 minutes in a 1:1 mixture of 100% ethanol and 12 M hydrochloric acid. Cells were rinsed with distilled water before exposure to the carbol-fuchsin stain. Microscopic examination of hyphae revealed that the nuclei were darkly stained, whereas the rest of the tissue remained relatively clear. Examination of 800 apical and adjacent subapical cells from six isolates revealed that the hyphae are predominantly binucleate. This nuclear staining technique may prove useful in studies of the life cycle and genetics of this and other fungal species.

Introduction

The puffballs are members of the gasteromycetes or "stomach fungi" in which the developing hymenium or gleba of the basidiocarp is enclosed by one or more outer layers of tissue (i.e., the peridium). Unlike other members of the phylum Basidiomycota, gasteromycetes are not capable of actively dispersing basidiospores without the assistance of some mediating force or agent (e.g., the force of a falling rain drop, wind, or insects) (Miller and Miller, 1988). Gasteromycetes have been traditionally united by the above characters, but the growing body of evidence indicates that this diverse group (e.g., puffballs, stinkhorns, bird's nest fungi, earthstars) does not constitute a monophyletic assemblage (Alexopoulos et al., 1996). The life cycles of gasteromycetes vary, as do the form and structure of different species, the methods for dispersing spores, and the habitats that these organisms occupy (Brodie, 1975; Miller and Miller, 1988). In delineating the life cycle of puffball species, in particular *Lycoperdon pyriforme* Schaeff.:Pers., the maintenance of the dikaryon appears to be one feature that separates this group from the others.

Dikaryotic hyphae are composed of binucleate cells and often possess clamp connections which are used as a diagnostic feature to distinguish them from uninucleate or monokaryotic cells (Buller, 1933). Clamp connections are a result of mitotic events. Each nucleus in the cell divides synchronously with one pair of daughter nuclei migrating directly into one of the two newly forming cells before becoming separated by an intervening crosswall. The other pair of daughter nuclei form within a hyphal peg that extends from the anterior hyphal cell back toward the adjacent subapical cell. Anastomosis of the hypal peg to the adjacent subapical cell allows one of these daughter nuclei to enter. Another crosswall in the hyphal peg develops forming a barrier between the cytoplasm of the apical and subapical cells, reestablishing the dikaryotic condition in the two daughter cells.

Clamp connections have been observed in a number of gasteromycetes including the bird's nest fungi (Brodie, 1975; Olchowecki and Brodie, 1968), the stinkhorns (i.e., *Phallus hadriani* Vent. per Pers. and *Simblum sphaerocephalum* Schl.) (pers. observ.), the sphere thrower (i.e., *Sphaerobolus stellatus* Tode: Pers.) (Buller, 1933) and a uniloculate gasteromycete (i.e., *Limnoperodon incarnatum* Escobar) (Esocbar et al., 1976). Cytological studies of puffball species found that clamp connections are generally absent from dikaryotic hypae (Dowding and Bulmer, 1964; Duncan and Keay, 1990; Huss, 1992).

The absence of clamp connections makes it virtually impossible to distinguish between monokaryotic (uninucleate) and dikaryotic (binucleate) hyphae unless nuclei are examined directly. Such information would be useful, especially when conducting sexual crosses in studies of mating systems in these fungi, and for determining the general ploidy condition of these fungi, a necessary prerequisite for the interpretation of isozyme data (Huss 1993, 1996).

Several nuclear staining techniques have been utilized

for cytological studies of the basidia, germinating spores, and vegetative hyphae of several puffball species, including *Lycoperdon pyriforme*. The nuclei of the puffballs are reported to be quite small, measuring about 1µm in diameter (Dowding and Bulmer, 1964; Duncan and Keay, 1990; Swartz, 1929). Many standard nuclear staining techniques are not efficient or effective due to the problems of over staining cytoplasm which often obscures the presence of small nuclei. Giemsa staining techniques have proven useful for observing nuclei among members of the Lycoperdaceae and other fungi (Duncan and Galbraith, 1973; Duncan and Keay, 1990; Robinow, 1957; Wilson, 1992). An equally effective technique (Dowding and Bulmer, 1964) utilies the Feulgen reaction (Feulgen and Rossenbeck, 1924) to stain nuclei using Schiff's reagent which contains basic fuschin. A different staining technique, which also utilizes basic fuschin, was originally developed by Ziehl (1882) and Neelsen (1883) for staining acid-fast bacilli (Drury and Wallington, 1967; Johansen, 1940). Dr. Zhongren Wang and Dr. Ralph E. Brooks (pers. comm.) modified this technique for examining the small plant chromosomes common to members of the Juncaceae. This paper presents the methodology and findings regarding the use of Ziehl and Neelsen's carbol-fuschin stain and modifications of Wang and Brooks technique for staining puffball nuclei.

Materials and Methods

Preparation of staining solution.--Carbol fuschin staining solution was originally utilized by Ziehl (1882) and Neelsen (1883) for staining acid-fast bacilli (Drury and Wallington , 1967; Johansen, 1940). The following recipe and methodology are based, in part, on unpublished modifications of these techniques by Dr. Li Maoxue in 1981 of Beijing University, People's Republic of China, and Dr. Zhongren Wang and Dr. Ralph E. Brooks at the University of Kansas in 1991.

Basic fuschin (3 g) was dissolved in 100 ml of 70% ethanol. Ten ml of this solution was transferred to 90 ml of 5% phenol in distilled water. Fifty-five ml of this second solution was mixed with 6 ml glacial acetic acid and 6 ml of 37% formaldehyde. The final staining solution was prepared by diluting this solution with 45% acetic acid at a ratio of 1:4; then adding 1.8 g of sorbitol for every 100 ml of staining solution prepared. This final staining solution was allowed to "mature" for several weeks before using.

Staining of fungal nuclei.--Cultures obtained by culturing glebal tissue of immature basidiocarps were used in this study. Cultures were obtained from personal collections (Huss, 1992) and from the American Type Culture Collection. Fungi were grown for several weeks over a sterile overlay of cellophane (dialysis membrane) on the surface of potato dextrose agar (Difco) in a petri dish. Sectors of mycelium left attached to the cellophane membrane were cut and transferred to deionized water saturated with naphthalene. This material was allowed to soak for two hours. Cellophane sectors with mycelium were soaked in Farmer's fixative (3:1 mixture of 100% ethanol to glacial acetic acid) for one hour. Fixation for more than one hour tended to result in increased cytoplasmic staining, but this was inhibited by refrigeration. Cellophane sectors with mycelium were hydrolyzed in a 1:1 mixture of 100% ethanol to 12 M hydrochloric acid for about 10 minutes. Sectors were rinsed in distilled water for 5 minutes and allowed to remain in water until ready for use. Single sectors were transferred to clean microscope slides, and a drop of staining solution added. Tissue was allowed to soak in stain several minutes before adding a cover glass. This resulted in shaper staining of nuclei. Photomicrographs were made using Kodak Technical Pan 2415 film under bright field conditions with a green filter.

Results and Discussion

The staining technique worked well with nuclei staining dark, while the rest of the cell with the cellophane background remained relatively clear. Condensation of the nuclei appears to be favored by pre-treating the tissue in a solution saturated with naphthalene. Wang and Brooks (pers. commun.) suggest substituting α-bromonaphthalene, which along with *p*-dichlorobenzene has been utilized as a substitute for colchicine in pre-treating cells (Dyer, 1979). The degree to which nuclei stained also seemed to be dependent on which isolate was examined.

The nuclei were quite small measuring about 1 µm in diameter (Fig. 1). Consequently, individual chromosomes were not observed, although occasionally nuclei with a miniature dumbbell appearance were seen, suggesting that active cell division was taking place. Most cells were binucleate or dikaryotic (Fig. 1A-C; Table 1). Some nuclei appear to be pairing with one another, although that did not always appear to be the case (Fig. 1B).

Adjacent cells lacked clamp connections between different cells, supporting previous reports that clamp connections are generally few in number or absent (Duncan and Keay, 1990). The dikaryotic condition is probably maintained by both dividing nuclei migrating into the newly developing cell before the crosswall or septum forms. Departure from this theme could occur if one nucleus failed to migrate to the newly formed cell before partitioning occurred ($n < 2$), or if the crosswall fails to form at all ($n > 2$). Aberrant dikaryons, where the num-

Table 1. Nuclear complement of apical (A) and adjoining subapical (S) cells in cultures of *Lycoperdon pyriforme* isolated from different localities.

Isolate	Source	Type of cell & total no. examined	Nuclear complement (%) 0	1	2	3	4
ARK-10C	Arkansas	A (n=50)	0	0	100	0	0
		S (n=50)	0	0	98	2	0
ATCC-52736[a]	Japan	A (n=50)	0	0	82	16	2
		S (n=50)	2	4	84	8	2
DON-A18c	Kansas	A (n=50)	0	2	86	6	6
		S (n=50)	0	2	90	6	2
LEA-17Ac	Kansas	A (n=50)	0	2	90	4	4
		S (n=50)	0	4	92	2	2
SQW-11B	Missouri	A (n=100)	0	1	96	3	0
		S (n=100)	0	1	96	3	0
SQW-64F	Missouri	A (n=100)	2	4	90	4	0
		S (n=100)	0	1	93	4	2

[a]American Type Culture Collection. All other cultures from personal collection (Huss, 1992).

ber of nuclei per cell is not equal to two, have been observed in the Lycoperdaceae (Duncan and Keay, 1990; Fig. 1D, Table 1). Generally these cells represent exceptions to the rule, since cells with excessive nuclei often degenerate reverting to the dikaryotic condition (Duncan and Keay, 1990). An examination of apical and subapical cells of actively growing hyphal tips of several isolates obtained from different geographical locations supports these observations (Fig. 1; Table 1). Examination of 800 apical and adjacent subapical cells from six isolates revealed that the hyphae are predominantly dikaryotic (Table 1). Cells departing from the normal expectation of two nuclei on average constituted less than 9% of the cells observed.

This staining technique adds to the current techniques available for staining fungal nuclei in puffballs and other fungi. Further application may prove useful in studies designed to determine the type of mating system that exists among members of this group. Light shed on sexual reproduction in puffballs would dispel some of the current confusion in the literature regarding the nuclear condition of colonies derived from single basidiospores (uninucleate or binucleate), and the presence or absence of homothallism and self-compatibility, or heterothallism and outcrossing (Dowding and Bulmer, 1964; Swartz, 1929; Huss 1993, 1996).

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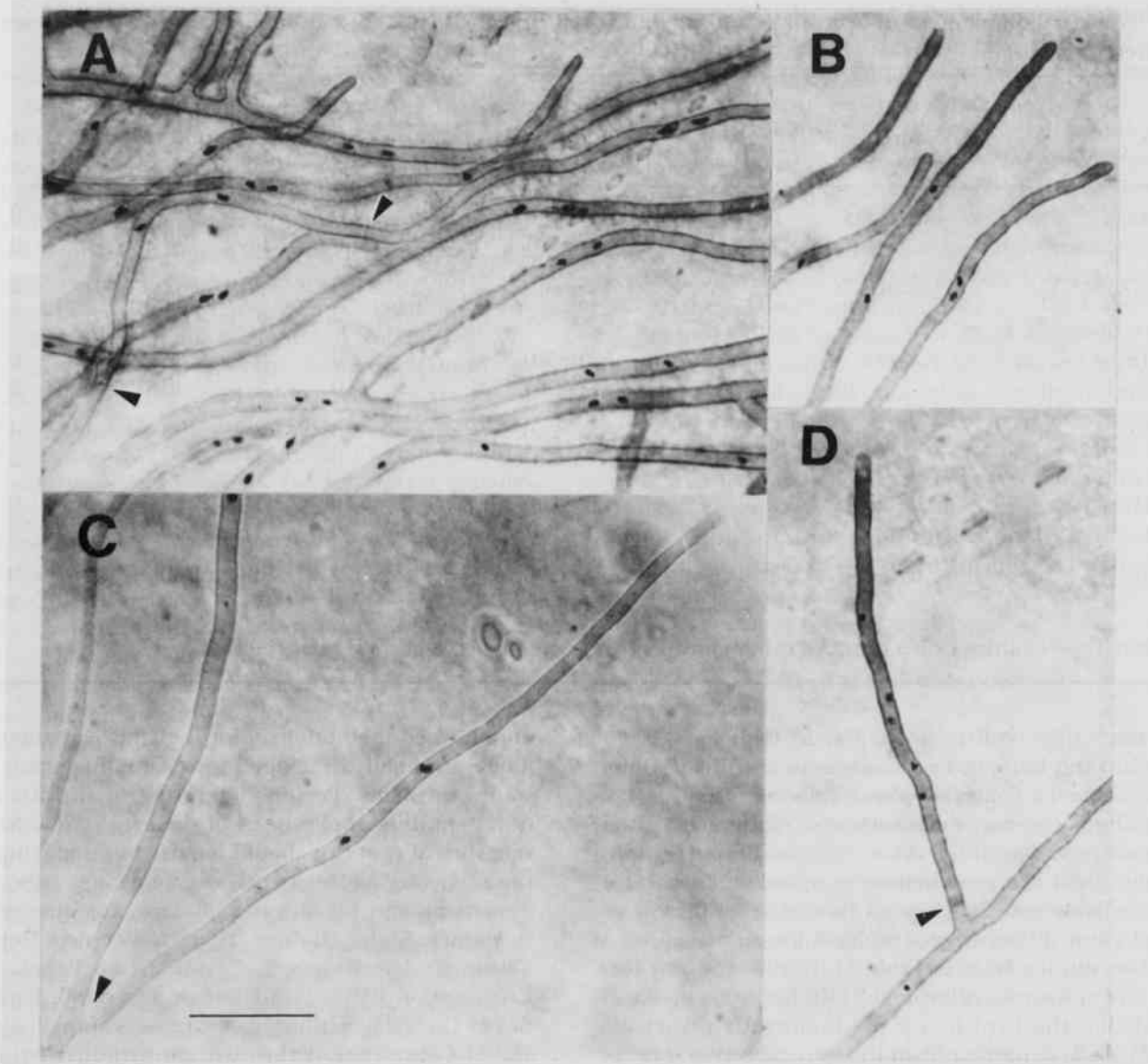

Fig. 1. Nuclei of *Lycoperdon pyriforme*. Septa indicated by arrows are difficult to see in these preparations. A-C. Hyphae showing binucleate cells. D. Aberrant cell containing 5 nuclei. Bar = 20 µm for all photographs.

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