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Occurrence of endospores within conidia and hyphal cells of morphologically atypical isolates of *Ophiostoma querci* (Georg.) Nannf.

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Ultrastructural observations of two *Ophiostoma querci* isolates presented in this paper showed endospores within hyphal cells and *Sporothrix* conidia. The endospores were apparently included in a matrix of an electron transparent material or were associated with unidentified granules scattered throught cytoplasm of enclosing cells. The endospores may be liberated by breakdown of the enclosing cell. When free they were observed giving rise to hyphae.

Key words: *Ophiostoma querci*, endospores.

INTRODUCTION

Ouellette and Gagnon (1960) reported, with use of a light microscope, minute bodies (0.5-1.0 µm) termed 'microendospores' within normal conidia and hyphae of *Ophiostoma ulmi* (Buism.) C. Moreau. They were observed predominantly in older or slowly growing cultures and were readily stained with Sudan black, but inconsistently with other stains. The presence of microendospores in *O. ulmi* and other *Ophiostoma* spp. was questioned by Brotzman and Campana (1968), Mariat and Diez (1971), Sansome and Brasier (1973), Chamberland and Ouellette (1977), Garrison et al. (1977) as well as by Kulkarni and Nickerson (1983).

Investigations performed by Mariat and Diez (1971) as well as by Sansome and Brasier (1973) revealed that spores could be produced within hyphal cells of *Sporothrix schenckii* Hektoen et Perkins and *O. ulmi*. In the latter case, they were morphologically similar to externally produced conidia.

In view of controversial opinions concerning the occurrence of microendospores and suggested implications of endogenously produced conidia in hyphae of

S. schenckii and *O. ulmi*, we have searched for such structures in isolates of *O. querci* (*Ophiostoma querci* and *O. quercus* are both correct names. We used *O. querci* according to G e o r g e v i ć, 1926). At first, two isolates 88 A and 88 were selected. *Ophiostoma querci* isolates are not strictly uniform in their morphology, mainly in the *Sporothrix* anamorph. Atypical isolates (e.g. 88, 88 A) differed from other *O. querci* isolates in colony morphology (felted for the atypical isolates compared to scant, floccose for typical isolates), synnemata formation (they were absent during 1-2 months of observations in atypical isolates), and length of *Sporothrix* primary conidia (6.6-39.6 µm for atypical isolates compared to 6.6-26.6 µm – P r z y b y ł, 1992; P r z y b y ł, M o r e l e t, 1993). Moreover, the minute bodies in hyphal cells and *Sporothrix* conidia in these cultures were more conspicuous than in other cultures of *O. querci*.

This paper reports our findings obtained using both light and transmission-electron microscopy. This report, however, is only a part of a much larger study on the ultrastructure of conidia and hyphae of *O. querci* isolates.

MATERIALS AND METHODS

The isolates 88 A and 88 of *Ophiostoma querci* were obtained in 1985 and 1986 from brown discoloured sapwood of the trunks of *Quercus robur* trees growing in the Krotoszyn Forest District (P r z y b y ł, 1995). Isolates were stored under paraffin oil, and subsequently transfered on fresh medium once a year.

O b s e r v a t i o n s b y m e a n s o f l i g h t m i c r o s c o p e. Monoconidial cultures of the studied isolates were obtained by planting single *Sporothrix* anamorph conidia from dilute suspensions on malt agar (Difco; pH 5.4) in Petri dishes. The cultures were kept at room temperature. Observations were performed on 10 and 30-day old cultures. The following stains were used in this study: neutral red, crystal violet, cotton blue in lactophenol, Sudan IV and Heidenhain's iron haematoxylin according to J o h a n s e n (1940) and O u e l l e t t e and G a g n o n (1960).

O b s e r v a t i o n s b y m e a n s o f t r a n s m i s s i o n - e l e c t r o n m i c r o s c o p e. For transmission-electron microscopy (TEM), cultures were grown on malt agar (Difco, pH 5.5) at room temperature for 10 and 30 days. Samples were fixed in 4 % glutaraldehyde with 0.1 M sodium cacodylete buffer for 6 hr. Postfixation (for 2 hr) was performed in osmium tetroxide in the same buffer.

Subsequently the material was stained with a water solution of uranyl acetate and then dehydrated in a graded series of ethyl alcohol (30, 50, 60 and 80 %), acetone (90 and 100 %), and propylene oxide. Samples were embedded in Epon 812 (L u f f, 1961). Ultrathin sections were cut with an LBK Ultra-microtome III and stained with uranyl acetate and lead citrate (R e y n o l d s, 1963). Observations were carried out using a JEOL TEM-1200 Ex transmission-electron microscope.

RESULTS

Light microscope observations. The *Sporothrix* conidia and hyphal cells taken from young (10-day old) cultures were generally characterized by having a uniformly dense cytoplasmic organisation. Both single conidia and hyphal cells contained single vacuole-like areas in which dark or glistening small-sized bodies (about 0.5 µm) occurred sporadically (Figs. 1, 2).

The dark minute bodies were more visible in 30-day old cultures. Conidia appeared less dense than in the young cultures. This is especially so for primary conidia in which large, visually transparent areas were seen, which were occasionally, hyaline (Fig. 3). An increase in the number of the minute particles (exceeding 5 in some conidia) was observed.

Small-sized and spore-like bodies (1.0-1.5 µm) near the distinct invagination of the conidial wall were observed (Fig. 4). Sporadically they formed chains (Fig. 5) and gave rise to short hyphae.

The intensity of staining of the minute bodies did not increase with the age of the culture, using cotton blue in lactophenol, neutral red, crystal violet and haematoxylin. The minute bodies were stained red, violet, and brown using neutral red, crystal violet, and haematoxylin, respectively. When using Sudan IV, the red particles occurring near unstained ones were revealed in 10-day old cultures.

Transmission-electron microscope observations. Young (10-day old) and old (30-day old) cultures of 88 and 88 A isolates were observed using TEM.

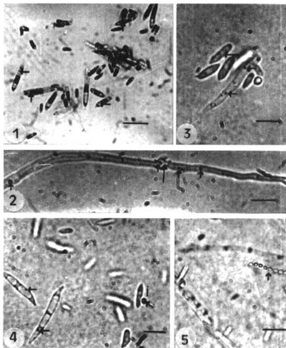
Typical conidia are characterized by an electron-light cell wall, outside of which pigment may appear as electron-dense granules, forming a thin layer. The conidia generally contained a nucleus, mitochondria (rather elongated), lipid drops, endoplasmic reticulum, vacuoles, osmiophilic inclusions, multivesicular bodies and the tubular complex (Figs. 6, 7, 8). These structures were also observed in the hyphal cells (Figs. 9, 10, 11). In addition, septa with central, simple pore and Voronin bodies were observed. Multivesicular bodies, characterized by various internal organization are closely associated with septa (Fig. 10).

Endospores formed in *Sporothrix* conidia and in hyphal cells were of various shapes (Figs. 11, 12, 13). They were more often observed in conidia than in hyphal cells.

Fig. 11 shows a micrograph of oval and fusiform endospores enclosed in a primary conidium. A distinct cell wall of endospores appeared to be present in one electron-light layer. The majority of endospores contained a nucleus, mitochondria, endoplasmic reticulum, multivesicular bodies, lipid drops, and the tubular complex (Figs. 11-15). These structures were easily observed, depending upon the plane of sectioning. Some endospores exhibited an electron-dense organization in which the structures were not recognizable. Endospores were apparently included in the matrix of the electron transparent material or were associated with unidentified

granules scattered throughout cytoplasm of the enclosing cell (Figs. 14, 15). Occasionally some structures seen around the entire endospore were electron-dense osmiophilic bodies and osmiophilic inclusions or clearly resembled the tubular complex and multivesicular body (Figs. 16, 17). Fractured cell walls of conidia containing endospores were found with TEM (Fig. 14). We suspect that endospores may be liberated by breakdown of the cell wall. When free they formed hyphae (Fig. 18).

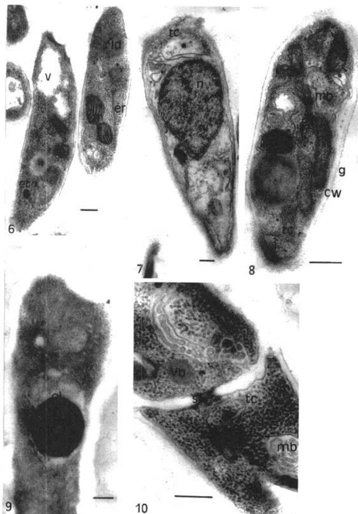
Examination by TEM confirmed the existence of chain-formed endospores with light microscopy (Figs. 19, 20).



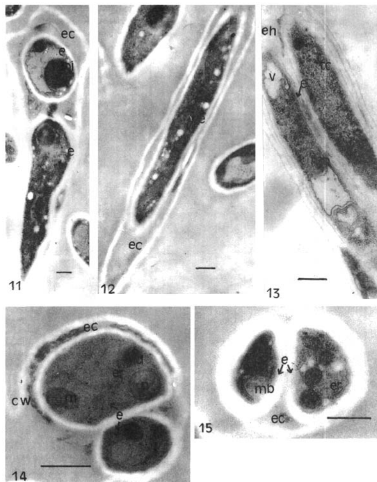
Figs. 1-2. Light micrographs of conidial and hyphal cells in 10-day old culture. Vacuole-like area (long arrow) and minute bodies (short arrow) within conidia and hyphal cells
1 – stained with neutral red, 2 – unstained, Bar – 10 μm

Figs. 3-4. Light micrographs of conidia in 30-day old culture. Vacuole-like area (long arrow) and minute bodies (short arrow) within conidia. A distinct invagination of conidial wall (i) is seen on Fig. 4
3 – unstained, 4 – stained with haematoxylin, Bar – 10 μm

Fig. 5. Light micrograph of chain of spore-like bodies (arrow) in 30-day old culture unstained
Bar – 10 μm

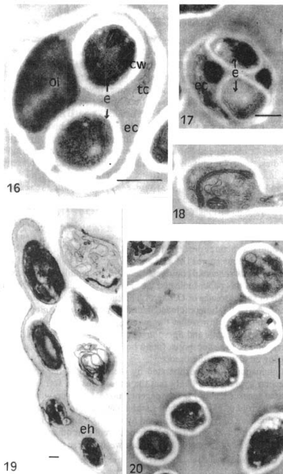


Figs. 6-10. TEM micrographs of longitudinal section through normal conidial and hyphal cells
cw – cell wall, g – electron-dense granules, n – nucleus, m – mitochondria, ld – lipid drops, er – endoplasmic reticulum, mb – multivesicular body, tc – tubular complex, oi – osmiophilic inclusions, v – vacuole, vb – Voronin body, s – septa; 6 – Bar = 1 μm, 7-10 – Bar = 0.5 μm



Figs. 11-15. TEM micrographs of longitudinal section 11-13 and cross section, 14-15 through conidial and hyphal cells

cw – cell wall, e – endospore (es), ec – enclosing conidium, eh – enclosing hyphae, n – nucleus, m – mitochondria, ld – lipid drops, er – endoplasmic reticulum, mb – multivesicular body, tc – tubular complex, oi – osmiophilic inclusions, v – vacuole; 11 – Bar = 0,5 μm, 12-15 – Bar = 1 μm



Figs. 16-17. TEM micrographs showing some structures resembling the tubular complex (tc) and lipid drops (ld) in matrix of enclosing conidium (ec) around the entire endospores (e)
Bar = 1 µm; cw – cell wall, oi – osmiophilic inclusions, ld – lipid drops

Fig. 18. TEM micrograph showing germinating endospore
Bar = 1 µm

Figs. 19-20. TEM micrographs showing endospore forming chain
eh – enclosing hyphae; Bar = 1 µm

DISCUSSION

The minute bodies were apparent within the cytoplasm of *Sporothrix* anamorph conidia and hyphal cells of atypical morphological isolates of *Ophiostoma querci* in examination by conventional light microscopy. They stained well both with cytoplasm and vital stains (especially with neutral red and crystal violet) as well as with nuclear stain – Heidenhain's iron haematoxyline. It was impossible to identify the minute bodies using these standard methods. However, these cytological studies confirmed the observations of Ouellette and Gagnon (1960). In the opinion of Sansome and Brasier (1973), the cytoplasmic bodies observed within hyphae and conidia of two isolates of *O. ulmi* in light microscopy were probably nuclei or ergastic substances. Some of the bodies reported here, occurring near other minute bodies, reacted positively with Sudan IV may indicate the presence of lipid structures in conidia and hyphae of 10-day old cultures of *O. querci* isolates. Kulkarni and Nickerson (1983) showed that in *O. ulmi* the refractile bodies present in glucose-salt media, containing L-proline grown yeast and blastospores, were lipid storage inclusions. Chamberland and Ouellette (1977) identified, using the electron microscope, two types of osmiophilic inclusions based on their opacity. Ultrastructural observations of Garrison et al. (1977) also revealed that "the intercellular structures described as microendospores (Ouellette, Gagnon, 1960) and endogenous spores (Mariat, Diez, 1971) in hyphae of *Ophiostoma ulmi* and *Sporothrix schenckii* could represent the osmiophilic inclusions". Observations presented in this paper indicated the endospore within *Sporothrix* conidia and hyphal cells of some isolates of *O. querci*. The endospores contained their own organelles. Thus our observations excluded their identification as osmiophilic inclusions.

It remains to be shown that the minute bodies observed by means of light microscope in hyphal cells and *Sporothrix* conidia of morphologically atypical isolates correspond to the endospores found both in conidia and hyphae of the same isolates using an electron microscope.

The endospores may be liberated by breakdown of the enclosing cell and germinate into hyphae. It is likely that the endospores are formed by a fungus as a response to senescence or possibly degeneration. Moreover, we suppose that they might play the role of chlamydospores which can survive unfavourable conditions for the development of typical conidia and hyphae. Of particular interest is the question of endospore development and their occurrence in other *Ophiostoma querci* isolates. We have begun research towards answering these questions.

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