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EXPERIMENTAL ARTICLES

Zygote Formation in *Blakeslea trispora*: Morphological Peculiarities and Relationship with Carotenoid Synthesis

V. M. Tereshina, A. S. Memorskaya, and E. P. Feofilova

Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

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Abstract—Changes associated with zygospore formation in the mucorous fungus *Blakeslea trispora* were studied. Zygospores are dormant cells with thickened cell walls and large central lipid vacuoles containing large amounts of lycopene. We established for the first time that *B. trispora* gametangia of different sexes differ in their carotenoid content and revealed that zygote formation involves a novel structure that consists of densely intertwined hyphae. Using inhibitory analysis (blocking β-carotene synthesis with diphenylamine and 2-amino-6-methylpyridine), we showed that suppression of carotene producion results in the inhibition of zygote formation. Hence, we established a manifest dependence of zygote formation on β-carotene synthesis.

Key words: zygospores, zygote formation, *Blakeslea trispora*, β-carotene, lycopene.

The order *Mucorales* (phylum *Archemycota*, subclass *Mucoromycetidae*) includes about 400 species and occupies the central position in the class *Zygomycetes* [1]. Mucorous fungi are distinguished from the rest of representatives of this class by their peculiar sexual reproduction that is subject to regulation by isoprenoid hormone-like compounds [2]. Not all *Mucorales* representatives have been shown to use sexual reproduction, but it is this process that has recently received special attention in connection with the formation of peculiar dormant cells referred to as zygospores. They are characterized by a special chemical composition that enables the cells to remain viable for 40–50 years, with all vital activities drastically decelerated. Presumably, of paramount importance for this process are cell wall polysaccharides and sporopollenin (a product of oxidative polymerization of β-carotene that is characterized by a unique resistance to chemical and enzymatic degradation) [3]. However, β-carotene does not only serve as the precursor of sporopollenin and the sexual hormones of *Mucorales*. This carotenoid is a natural antioxidant [4], and it protects membranes from reactive oxygen species.

Nevertheless, no experimental evidence has yet been presented for the functional involvement of β-carotene in zygote formation.

The goal of this work was to clarify the question of whether zygospores can form in *Mucorales* without the involvement of β-carotene. For this purpose, we used the β-carotene-oversynthesizing fungus *Blakeslea trispora* as the research object. We investigated morphological changes occurring in its cells during zygote formation and elucidated the role of β-carotene in zygote formation in *B. trispora* using β-carotene synthesis inhibitors.

MATERIALS AND METHODS

We used the heterothallic mucorous fungus *Blakeslea trispora* Thaxter (strains VKM F-987 (–) and VKM F-989 (+)) as the main research object. The fungus was grown on wort (7°B) agar slants for 6–7 days at 26–28°C and stored at room temperature.

To obtain sporangiospores from sporangioles and stylosporangia, we grew *B. trispora* (+) on potato–carrot agar for 10–11 days at 25–26°C and 29–30°C, respectively [5], washed the mycelium with cold water, collected the spores with a nylon filter, separated them by centrifugation at 6000 g and 4°C, washed the spores with water, and lyophilized them. The process of spore isolation was monitored using a Jenaval light microscope.

To obtain zygospores, we placed fragments (about 1 cm^2 in size) of 6- to 7-day-old surface mycelia of opposite-sex *B. trispora* strains ((+) and (–)) on the opposite sides of a wort agar-filled petri dish. The cultures were grown for 14 days at 26–28°C in the dark. The resulting dark zone of zygospore-containing mycelium was separated with a scalpel and removed with tweezers. The material obtained was thoroughly disintegrated in a mortar with distilled water, yielding a suspension containing zygospores, fragments of substrate mycelium, and suspensors. The suspension was passed through a nylon filter to obtain a filtrate containing zygospores, small hypha fragments, and suspensors. Zygospores were separated as follows. We added a

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large volume of water, allowed the mixture to stand for 10 min (zygospores precipitated at the bottom of the glass), and removed the water by decantation. The procedure was repeated several times, until the water became transparent. We succeeded in obtaining a fraction that contained 95–97% of zygospores (based on the results of a microscopic assay). All the steps of this procedure were done at room temperature. No germination occurs under such conditions. The zygospores were dried by lyophilization and stored at –10°C.

Zygote formation and the separation of the zygospore and the sporangiospore fractions were monitored under a Jenaval light microscope (Germany); the magnification was 250–900×.

Scanning microscopy was used to investigate the structure of the zygospore surface. Zygospore-containing mycelium zone was dried, stained with gold, and examined under a Jeol JSM-T300 scanning microscope. The ultrastructure of zygospores was explored with a Jeol JEM 100 CXII electron microscope, using the method described in [6] to prepare the specimens.

To determine the carotenoid composition and content of zygo- and sporangiospores of *B. trispora*, we destroyed them by freezing and thawing (using liquid nitrogen) and subsequently grinding the spores with quartz sand. Carotenoids were extracted with acetone; this procedure was repeated 3–4 times. The carotenoids were transferred from acetone to hexane, and the solution was dried by passing it through anhydrous Na_2SO_4. Absorption spectra were recorded in the 350–530 nm range with a SF-56 spectrophotometer (LOMO, Russia). The total carotenoid content was calculated according to Vakulova *et al.* [7]. The carotenoid composition was determined by thin-layer chromatography on aluminum dioxide. For this purpose, evaporated carotenoid extract was applied, as a 20-mm-wide band, on an Al_2O_3 plate (the humidity was of the 3rd grade according to the Brockman scale) and chromatographed in an acetone–hexane (1 : 49) system. To prevent carotenoid oxidation, the system was supplemented with the antioxidant butylhydroxytoluene. Carotenoids were eluted with hexane containing 1% ethanol. The carotenoids were identified (1) using crystalline β-carotene and lycopene (obtained from the Uralbiofarm joint-stock company) as markers and (2) by comparing their absorption maxima in three solvents (hexane, acetone, and chloroform) with those available in the literature [8]. Carotenoid contents were determined using extinction coefficients of 3450, 2505, and 3100 for lycopene, β-carotene, and γ-carotene, respectively.

β-Carotene synthesis was inhibited by supplementing molten wort agar with (1) an aqueous solution of 2-amino-6-methylpyridine (AMPy), bringing its final concentration to 0.01 g/l, and (2) an ethanol solution of diphenylamine (DPA), bringing its final concentration to 10^{-5} M.

RESULTS AND DISCUSSION

While growing on wort agar, (+) and (–) mycelia come into contact after 24–26 h of cultivation. Thickened vacuolated hyphae that occasionally display coiling or intense branching and bundles of interwoven hyphae with numerous carotenoid inclusions form within the contact zone. The first young zygospores appear after 40–48 h. They are large cells with a regular rounded shape and a vacuolated content. At this stage, they are characterized by a rapid, spherical growth pattern and a lack of thickened cell walls (Fig. 1a). At a later stage (4–5 days), the forming zygospores display large vacuoles and rare large lipid droplets (Fig. 1b). Zygospore maturation begins on the 6th or 7th day: the cell walls become thickened, black pigment forms inside them, and significant changes occur in the internal structure of zygospores. They manifest themselves in an intense compartmentalization of reserve compounds, primarily lipids, resulting in the formation of 3–5 large vacuoles (Fig. 1c), including one huge central vacuole. Subsequently, the number of reserve vacuoles can decrease to one (Fig. 1d), which is characteristic of more mature (14-day-old) zygospores. Electron microscopic studies revealed that mature zygospores contain rounded mitochondria (with modified cristae), glycogen granules, vacuoles, and large lipid inclusions (Figs. 2a–2c).

Two new facts were established in our studies on zygote formation in *B. trispora.* It is well known that a zygospore in *Mucorales* is attached to two suspensors that, in many mucorous fungi, are of different size. In the case of *B. trispora*, they also differed in color intensity. One of the suspensors is bright yellow, apparently due to an elevated carotenoid content. This seems to be the suspensor of the (–) strain, which synthesizes a significantly larger amount of β-carotene than the (+) strain.

Another curious point concerning zygote formation was that it involved the development of a structure that was not described earlier. The structure had an indeterminate shape and consisted of densely interwoven hyphae that formed puffs. Zygospore-bearing brightly colored suspensors were in direct contact with this structure, whose functional role is still unknown.

Using scanning microscopy, we established that zygote formation in *B. trispora* occurs on the surface of agar-containing medium, where no asexual sporulation takes place and no aerial mycelium forms. We only detected zygospores with curved, claw-shaped suspensors that penetrated the substrate (Fig. 1e). This gives grounds for the suggestion that zygote formation involved substrate, not aerial, mycelium. The surface of a mature zygospore was smooth with rare wrinkles (Fig. 1f).

In order to clarify the question of whether *B. trispora* zygospores can form when β-carotene synthesis is inhibited, we conducted an inhibitor study using DPA

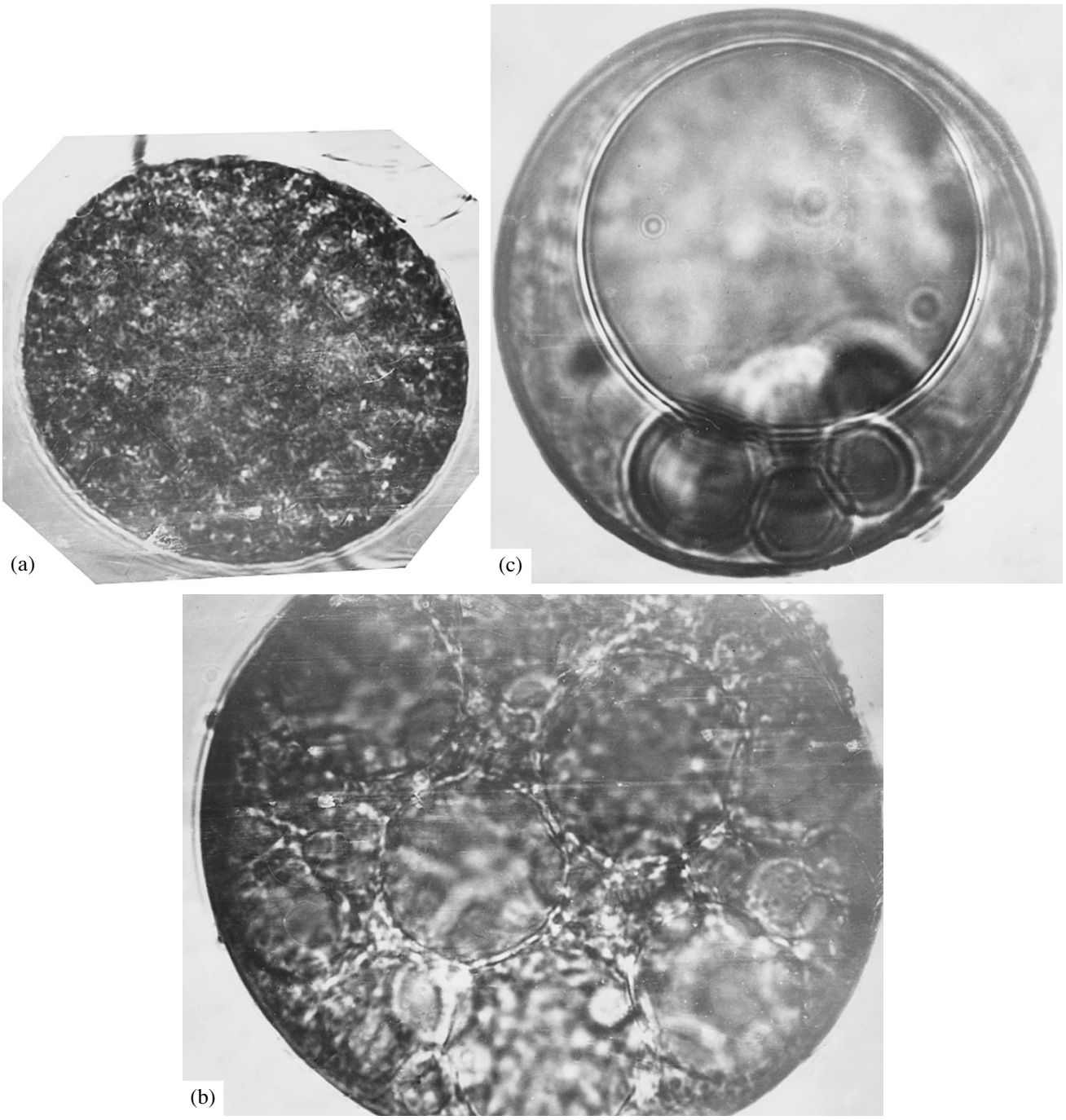
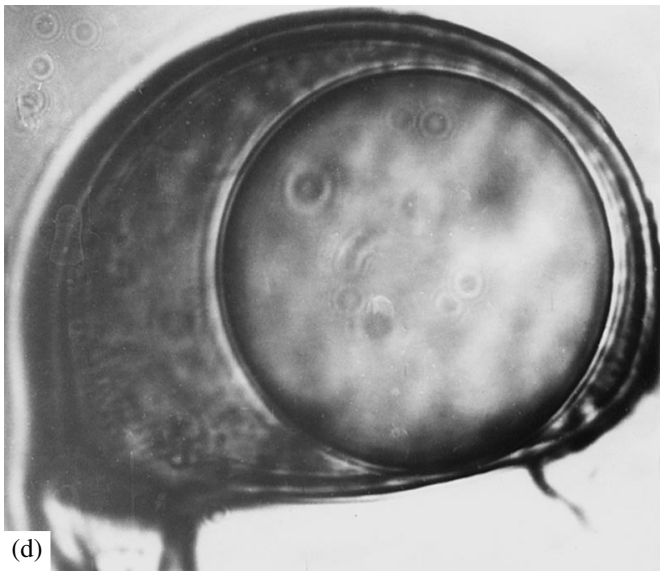
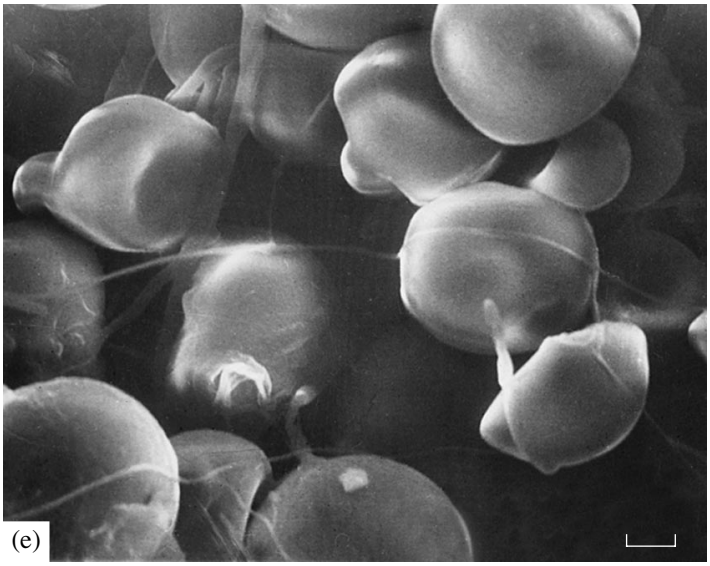


Fig. 1. Zygote formation in *B. trispora*: (a) a 3-day-old zygospore (3000×); (b) a 5-day-old zygospore (6000×); (c) a 10-day-old zygospore (2700×); (d) a 14-day-old zygospore (2400×); (e) mature zygospores in the zygote formation zone (640×); (f) a mature (14-day-old) zygospore (1300×).

[9] and AMPy [10]. DPA is known to cause a virtually complete blockage of β-carotene synthesis, and this results in the accumulation of the colorless polyene precursors phytoine and phytofluine in the mycelium.

Mycelium growth occurred in the presence of 10^{-5} M DPA, but carotenogenesis was partially inhibited and the process of zygote formation was retarded. Only weak interactions between hyphae occurred in a 48–50 h-old culture of the fungus. The contact zone (5–6 mm wide) was yellow in a 7-day-old culture and also displayed a very narrow (1- to 2-mm-wide) zygote formation zone. From these data, it can be concluded that partial inhibition of carotenogenesis by DPA resulted in a marked decrease in the zygospore number.





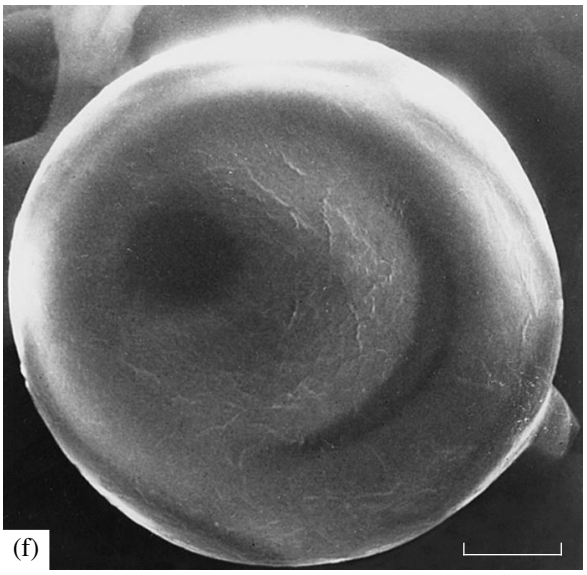


Fig. 1. (Contd.)

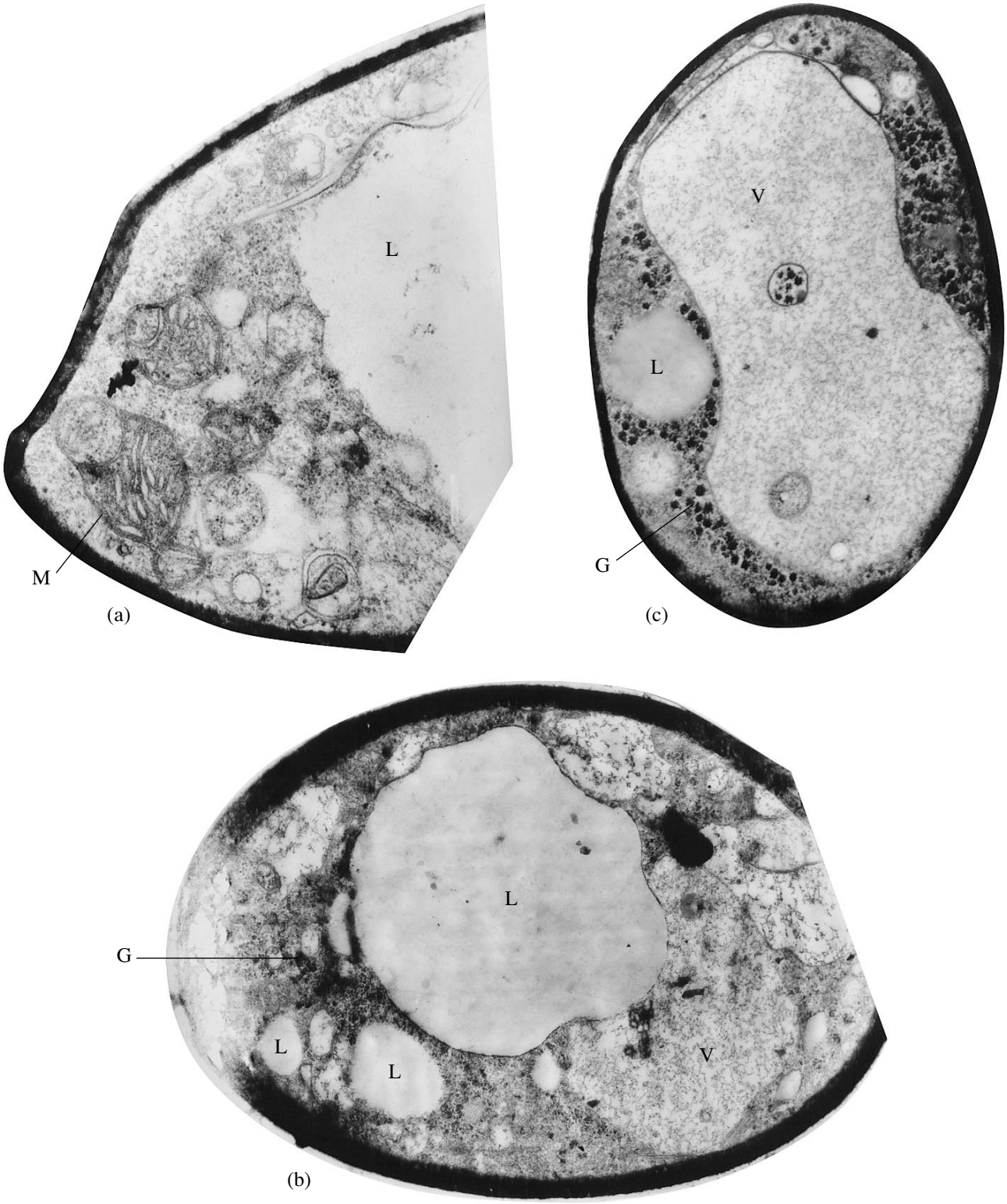


Fig. 2. Ultrastructure of mature zygospores of *B. trispora.* M, mitochondria; G, glycogen granules; V, vacuoles; L, lipid vacuoles. Magnification: (a) 28000×; (b) 20000×; (c) 14400×.

Composition of carotenoids in *B. trispora* mycelium and resting cells

Spore type	Carotenoids, % of dry biomass	Carotenoids, % of total		
		γ-carotene	lycopene	β-carotene
Submerged mycelium (5-day-old)	1.34	16.7	4.7	76.6
(+) Sporangiospores from sporangioles	0.04	62.5	25.0	12.5
(+) Sporangiospores from stylosporangia	0.05	62.0	17.2	20.8
Zygospores	0.45	50	33.3	16.7

Another inhibitor of β-carotene synthesis, AMPy, suppresses cyclization of lycopene and its conversion to the yellow pigment β-carotene by about 90%, which is accompanied by the accumulation of a red pigment lycopene in the mycelium. The result of the AMPy effect was also easy to detect visually. The addition of AMPy to wort agar resulted in retardation of zygote formation, and a 48-h-old culture resembled a 24-h-old culture in the control experiment. A broad bright red zone (the color is due to a high lycopene content) formed in a 7-day-old culture. Microscopic examination revealed that an insignificant (as compared to the control data) number of zygospores did form. The AMPy inhibition was incomplete, and about 10% of β-lycopene was still synthesized, which possibly accounts for this finding.

Hence, the inhibitor studies on β-carotene synthesis indicate that it is β-carotene, not lycopene and the colorless polyenes phytoine and phytofluine, that is the prerequisite for zygote formation in *B. trispora*. In addition, comparative research on the carotenoid composition of *B. trispora* cells at different stages of their ontogeny revealed new developmental patterns. From the data given in the table, it is evident that the hyphae of a submerged *B. trispora* culture predominantly contain β-carotene, which accounts for about 75–80% of the total carotenoid content [11], whereas the percentage of γ-carotene and lycopene is about 20–25%. Nevertheless, β-carotene does not dominate the pigments of spores resulting from asexual and sexual reproductive cycles, where it makes up only 15–20% of the total. Of paramount importance are the data that γ-carotene is the predominant pigment in sporangio- and zygospores. However, zygospores are also characterized by the maximum lycopene amount.

Thus, *B. trispora* cells exhibit different carotenoid composition patterns, depending on their developmental stage. Presumably, this is related to the degree of cell resistance to environmental factors. For instance, vegetative mycelium is particularly susceptible to deleterious environmental influences, while dormant cells (sporangio- and zygospores) are the most viable forms.

Reactive oxygen species (ROS) are currently considered to be the most destructive factors, and only cells possessing an antioxidant defense system can withstand their influence. Based on the recent data, lycopene produces the strongest antioxidant effect, the influence of γ-carotene is less significant, and β-carotene is still less efficient [4]. The presence of lycopene (up to 33% of the total carotenoids) in zygospores, which can retain their viability for a long time, can be regarded as one of the reasons why zygospores are particularly resistant to environmental factors.

However, sporopollenin, the biopolymer of dormant cells that provides for their protection from poisons, acids, and enzymes, can only derive from a carotenoid with two ionone rings, i.e., from β-carotene. This carotenoid forms in *B. trispora* mycelium at a particular developmental stage associated with the synthesis of secondary metabolites. Upon the transition to the anabiosis ontogenetic stage, β-carotene serves as the precursor of sporopollenin. Sporopollenin formation proceeds simultaneously with the accumulation of lycopene. Sporopollenin and lycopene are the compounds that render zygotes particularly resistant to environmental factors including ROS.

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REFERENCES

1. Cavalier-Smith, T., A Revised Six-Kingdom System of Life, *Biol. Rev.*, 1998, vol. 73, pp. 203–266.
2. Feofilova, E.P., Tereshina, V.M., and Vakulova, L.A., On the Biological Function of Trisporic Acids in Mucorous Fungi, *Mikrobiologiya*, 1994, vol. 63, no. 1, pp. 17–22.
3. Brooks, J. and Shaw, G., Sporopollenin: A Review of Its Chemistry, Paleochemistry and Geochemistry, *Grana*, 1976, vol. 17, pp. 91–97.
4. Martin, H.D., Ruck, C., Schmidt, M., Seil, S., Reutuer, S., *et al.*, Chemistry of Carotenoid Oxidation and Free Radical Reaction, *Pure Appl. Chem.*, 1990, vol. 71, no. 12, pp. 2253–2262.
5. Tereshina, V.M. and Feofilova, E.P., Two Sporulation Types in the Mucorous Fungus *Blakeslea trispora*: Morphological Characteristics, *Mikrobiologiya*, 1995, vol. 64, no. 5, pp. 681–685.
6. Edelman, R.E., Zygosporogenesis in *Zygorhynchus heterogamis* with a Proposal for Standardization of Struc-

tural Nomenclature, *Mycologia*, 1995, vol. 87, no. 3, pp. 304–318.

7. Vakulova, L.A., Kuznetsova, V.P., Kolot, F.B., Bab'eva, I.P., and Samokhvalov, G.I., A Rapid Method for Quantitative Determination of β-Carotene in Microorganisms, *Mikrobiologiya*, 1964, vol. 33, no. 6, p. 1061.
8. Foppen, F.H., Tables for the Identification of Carotenoid Pigments, *Chromatograph. Rev.*, 1971, vol. 14, pp. 133–298.
9. Feofilova, E.P. and Pakhlavuni, I.K., The Effect of Some Inhibitors of β-Carotene Synthesis on the Formation of Trisporic Acids by the Fungus *Blakeslea trispora*, *Mikrobiologiya*, 1972, vol. 29, no. 2, pp. 260–268.
10. Feofilova, E.P., Tereshina, V.M., and Memorskaya, A.S., Regulation of Lycopene Biosynthesis in the Mucorous Fungus *Blakeslea trispora* by Pyridine Derivatives, *Mikrobiologiya*, 1995, vol. 64, no. 6, pp. 734–740.
11. Tarasova, N.V., Boltyanskaya, E.V., Kalmykova, G.Ya., Feofilova, E.P., and Bekhtereva, M.N., Dynamics of the Development of *Blakeslea trispora* and β-Carotene Production, *Prikl. Biokhim. Mikrobiol.*, 1967, vol. 3, no. 1, pp. 173–176.