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Research article

SCLEROTIA OF THE ACELLULAR (TRUE) SLIME MOULD *Fuligo septica* AS A MODEL TO STUDY MELANIZATION AND ANABIOSIS [#]

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Abstract: Acellular (true) slime moulds (*Myxomycetes*) are capable of a transition to the stage of sclerotium – a dormant form of plasmodium produced under unfavourable environmental conditions. In this study, sclerotia of *Fuligo septica* were analyzed by means of electron paramagnetic resonance (EPR) spectroscopy. The moulds were cultivated *in vitro* on filter paper, fed with oat flour, and kept until the plasmodia began to produce sclerotia. The obtained sclerotia differed in colour from yellow through orange to dark-brown. The EPR spectra revealed a free radical, melanin-like signal correlated with the depth of the colour; it was strongest in the dark sclerotia. Sclerotization only took place when the plasmodia were starved and very slowly dried. Only the yellow sclerotia were able to regenerate into viable plasmodia. This suggests that myxomycete cytoplasm dehydration is an active process regulated metabolically. Plasmodial sclerotization may therefore serve as a convenient model system to study the regulation of cytoplasmatic water balance, and sclerotia as a convenient

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Abbreviations used: DOPA – 3,4-dihydroksyphenylalanine; DPPH – 1,1-diphenyl-2-picrylhydrazyl; EPR – electron paramagnetic resonance

material for EPR measurements, combining the quality of plasmodia with the technical simplicity of the measurements characteristic of dry spores. Darkening of the sclerotia is most probably a pathological phenomenon connected with the impairment of water balance during sclerotization.

Key words: Aquaporins, Dehydration, EPR, Melanin, Myxomycetes, Pigmentation

INTRODUCTION

True slime moulds (*Myxomycetes*) are a separate taxon of protozoal organisms, related to rhizopoda and primitive fungi [1]. They are a specialised group of *Eucaryota*, as evidenced by their complex life cycle, but in their vegetative phase, they reveal simple organization, forming large multinuclear plasmodia devoid of cell walls. They have preserved various simple and primeval characteristics, and developed fewer narrow specializations than the fungi often used as model eucaryotes (e.g. the yeast *Saccharomyces cerevisiae* [2]). They live in a wide range of ecosystems, from deserts to forests, from water pools to the surface of the snow in mountain ranges [1]. Some can be efficiently and cheaply cultured *in vitro* as model organisms [1, 3].

Besides their other applications in biological and biomedical research, they provide material for the biophysical analysis of free radicals and transient metal ions, using electron paramagnetic resonance (EPR) spectroscopy [4, 5]. So far, two types of material have been used for EPR measurements: spores and plasmodia. The dark and bright spores of several species of slime moulds [4-7], and material isolated from spores by acid hydrolysis have been checked for the presence of melanin, and for the characteristic EPR free radical signal of this polymer. Even in bright spores, narrow free radical EPR signals could be detected [4, 5]; however, the strongest signals originated from dark spores with high melanin contents [4-7].

The myxomycetal plasmodium is in fact a single cell consisting of a huge amount of cytoplasm with numerous synchronously dividing nuclei. Due to its high water content, the plasmodium is more difficult to study with EPR than dry spores are [4, 5, 8, 9]. The usual protocol with normal plasmodia and microplasmodia involves investigation using EPR spectroscopy at 77 K (in liquid nitrogen) [4, 5]. Such studies have revealed that one species, *Physarum nudum*, produces melanin in response to irradiation of the plasmodium with white light [10]. The dark plasmodia of *Metatrichia vesparium*, surprisingly, did not reveal melanin signals, but high amounts of manganese II (measurements of small samples exceptionally at ambient temperature; [11]). The plasmodia of *Ph. nudum* and *Ph. polycephalum* revealed quite strong signals of complexes of non-heme iron II with nitric oxide [9]. The microplasmodia of *Ph. polycephalum* and *Ph. nudum* cultivated in liquid cultures additionally revealed signals of nitrosyl-haeme complexes, probably due to the presence of haemin in the medium [9].

Another vegetative form of slime moulds is the sclerotium [1]. This dormant stage is an irregular associate of small cell-like elements called macrocysts (spherules) consisting of dehydrated cytoplasm surrounded with a polysaccharide cell wall [1, 12]. Sclerotia are produced in response to harmful factors in the environment (drought, low levels of food, low or high temperatures). Under favourable conditions, such resting forms can reproduce vegetative plasmodia along with some preserved features of the original plasmodium (e.g. the ability to sporulate [12]). Studies on the control of sclerotization may thus be important to understand the process of cytoplasm dehydration with the preservation of life functions, i.e. anabiosis [1, 12]. Controlled water removal from cells with the engagement of special protein water pores, like aquaporins, is an important mechanism to control a surprisingly wide spectrum of biological processes, including angiogenesis in tumour tissue [13], cell migration during corneal re-epithelialization [14], and water transport in endothelium [15]. The plasmodial model of dehydration due to sclerotization is, therefore, very interesting for basic and biomedical investigations.

Being a dry and highly condensed form of plasmodium, the sclerotium should be EPR-measureable under normal conditions. However, this has not yet been tested systematically. The main aim of this study was to investigate this possibility using a common, but rarely cultured species, *Fuligo septica*. This organism usually produces yellow plasmodia, yellow sclerotia and dark spores. Its plasmodia may also produce dark-brown or even black sclerotia. Their dark colour may be associated with melanization. Here, using EPR spectroscopy, we confirm that this dark pigment is of the melanin type. We also suggest potential reasons why some of the sclerotia become dark.

MATERIALS AND METHODS

Preparation of DOPA- and cysteinylDOPA melanins

All the reagents were obtained from Sigma-Aldrich, (St. Louis, MO, USA). 3,4-dihydroksyphenylalanine (DOPA) melanin, the synthetic counterpart of eumelanin, was synthesized by DOPA autooxidation [16]. The aqueous solution of D,L-DOPA was bubbled with air for 4 days at ambient temperature, while the pH was kept constant at 8 (NH_4OH). The synthetic equivalent for natural phaeomelanin was prepared by enzymatic oxidation of L-DOPA and L-cysteine [17]. A solution of 994 mg of L-DOPA and a double excess of L-cysteine (448 mg) in 500 ml of Sőrensen buffer (Na_2HPO_4/KH_2PO_4, 0.01 M, pH 6.8) was supplemented with 200,000 ù of yeast tyrosinase in 25 ml of the same buffer, stirred for 1 hour and bubbled for 4 days with air. Afterwards, both pigments were precipitated from the solution by lowering the pH to 3-3.5 (HCl), and then purified with serial washes and dialyses towards re-distilled water for 4 days. The substances were then dried for 4 days in air at 37ºC, and sealed in glass capillaries. Every sample contained 8.2 ± 0.1 mg of dry powdered melanin.

In vitro* cultivation of *Fuligo septica

Cultures of *Fuligo septica* (L.) F.H. Wiggers were established *de novo* in our laboratory from spores. The plasmodia were then cultured on filter paper (medium density) with water and oat flour *ad libitum*. The cultures were maintained in Petri dishes (10, 12, 15 cm) under septic conditions in the dark, at room temperature (19ºC). The plasmodia were fed and watered 1-2 times a week. A half of each filter paper was exchanged every 2 months.

Induction of sclerotization

Sclerotia were produced spontaneously by the cultivated plasmodia. Once a plasmodium started to transform into a sclerotium, the feeding was stopped, and when the process of sclerotization had finished, the dish was left open to achieve an air-dry state. We also tested the possibility of obtaining sclerotia by limitation of feeding, watering or by keeping the dishes open. We applied these procedures to normal plasmodia, which initially did not reveal any characteristics of sclerotization.

Sample preparation and EPR measurements

The dried sclerotia were broken into smaller pieces, which were put directly into a quartz finger dewar, and introduced into the resonant cavity of the spectrometer. The masses of the sclerotia were measured after EPR measurements. The EPR measurements were carried out at room temperature using an X-band Varian E-3 spectrometer with a rectangular TE 102 resonant cavity at the following parameters: field 3265 ± 50 Gs; modulation amplitude and frequency, respectively, 5 Gs and 100 kHz; microwave power and frequency, respectively, 4 mW and 9.25 GHz; scan time 180 s (double digital acquisition, 1024 points per spectrum), gain 25000. Powder samples of dry melanins were similarly measured as a control. The intensities of the EPR signals were expressed as peak-to-peak amplitudes, normalized per constant mass of 100 mg and constant gain of 25000. A powder sample of 1,1-diphenyl-2-picrylhydrazyl (DPPH) served as a free radical standard, to indicate the position of the free radical signal ($g = 2.0037$). We paid particular attention to preserving the geometry of the measurements and to placing the samples each time in the same position relative to the modulation field [18].

Statistical analysis of the results

To compare the results, the sclerotia were divided into three groups, according to their colouration. This division was based on an arbitrary judgement of their colour: yellow, orange, or dark (brown and black). We checked both the mean values of the mass and EPR signal amplitudes ± SD of the groups, and we presented the individual results of particular samples. The significance of differences between the means was tested using the two-tailed, independent Student's *t*-test for populations of equal and nonequal variations. The statistical significance of the differences between the variations was tested using the F Snedecor test.

RESULTS AND DISCUSSION

Fuligo septica produces *in vitro* sclerotia of various colours and viabilities

Examples of *in vitro* cultures of *F. septica* are given in Fig. 1, which shows young (Fig. 1A) and old (Fig. 1B, C) cultures of this slime mould, all fed with oat flour. Sometimes very sophisticated, fractal (Fig. 1D) or even 3-dimensional (Fig. 1E) plasmodium structures were observed. The sclerotia produced by the cultures are shown in Fig. 2A-F. This type of material was dry and very hard (comparable to glass). The plasmodia were usually yellow (Fig. 1A), and very exceptionally, orange (Fig. 1C). The sclerotia were of various colours which allowed for their division into three groups by pigmentation: yellow (n = 16), orange (n = 17), or dark (brown or black, n = 10). A brown tint could also be found in the old mucosal traces of plasmodia on the surface of old cultures (Fig. 1B). 60% of the yellow sclerotia maintained the ability to reproduce viable plasmodia, whereas 100% of the orange and dark sclerotia were devoid of this ability.

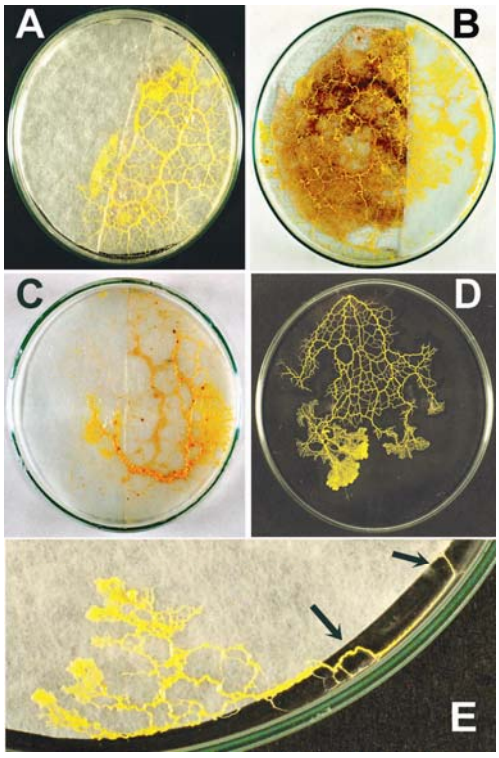

Fig. 1. *In vitro* cultures of *Fuligo septica*. A – 2 weeks old, B – 4 months old (note the dark coloration of the filter paper in the old part of the culture), C – 2 months old (note the unique orange colour of the plasmodium), D – fractal structures of a plasmodium in search of food on the cover deck of a Petri culture dish, E – three-dimensional structures (arrows) of cytoplasm tubes formed at the edge of the cover deck, side walls and bottom of a Petri culture dish.

Sclerotization is an active process

In order to test whether the dehydration of the cytoplasm connected with sclerotization is a passive water loss or a controlled physiological process, we left a few culture dishes open (Fig. 2G). This never lead to the production of sclerotia, only to the desiccation of the plasmodia. Sclerotization is controlled by the organism, and sclerotia can often be found beside living plasmodia, on the walls of culture dishes (Fig. 2C, E). In order to produce sclerotia, the plasmodium must undergo temporary starvation, but the water supply must be maintained. Only mature sclerotia could be additionally desiccated by keeping the culture dish open. There is another reason for which the process of sclerotization cannot be considered as a simple, passive water loss by the plasmodium. Spherules, i.e. the small cell-like constituents of the sclerotium, can be produced even in liquid cultures of microplasmodia. In such an environment, water availability does not limit the growth of the slime mould, thus the transition to a dormant stage is obviously for other reasons [12].

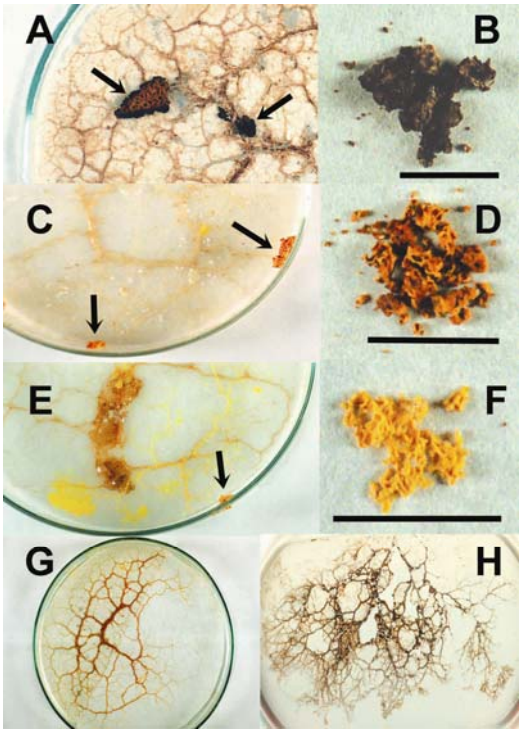

Fig. 2. Sclerotization and melanization of *Fuligo septica*. The formation of black (A), orange (C), and yellow (E) sclerotia (arrows) on filter paper (A) or on Petri dish walls (B, C). Black (B), orange (D), and yellow (F) slerotia enlarged (scale bar – 1 cm). G – a dried plasmodium which did not produce sclerotia in an open dish, H – old, dried mucosal traces of a plasmodium on the cover deck, which have darkened due to a spontaneous autooxidative melanization.

Sclerotia reveal melanin-like paramagnetism but do not contain phaeomelanin

Although EPR spectroscopy is one of the best methods for qualitative and quantitative melanin measurements in biological samples [19], difficulties may arise due to the high water content and non-resonant absorption of microwaves [8]. Therefore, we tested the feasibility of EPR measurements of dry sclerotia. Previously, we found this method suitable for measurements of living plasmodia and spores, so we hoped to identify any EPR signals which may be correlated with the degree of dark colouration of *Fuligo septica* sclerotia.

We found that the sclerotia reveal a strong singlet EPR line with the g constant around 2.00 (Fig. 3), which is typical of free radicals [19, 20]. The signal was stable and detectable even in old material. The position and shape of the signal corresponded well with the position and shape of the DOPA-melanin signal (Fig. 3), but the latter turned out to be narrower than the signals of sclerotia ($P < 0.001$, Tab. 1). However, the signals of particular groups of sclerotia did not differ from each other in their average linewidths ($P > 0.05$, Tab. 1) and shapes. The lack of hyperfine splitting strongly suggests that no phaeomelanin was produced during sclerotization (Fig. 3, [21]).

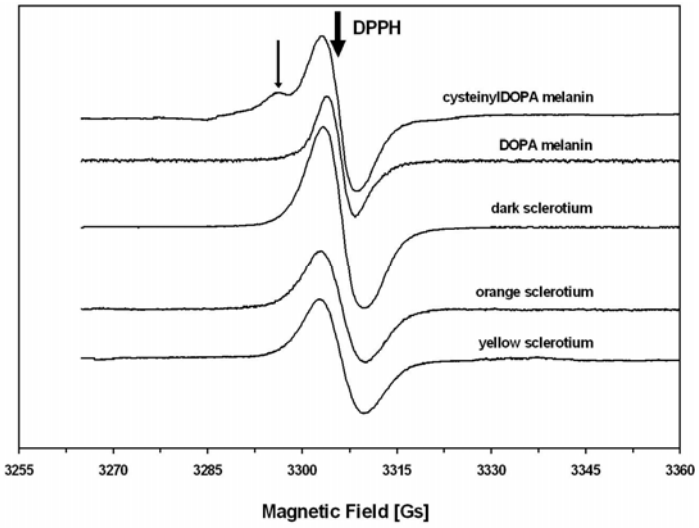

Fig. 3. The EPR signal of a yellow, orange and dark sclerotium of *Fuligo septica*, together with the signals of powder samples of DOPA- and cysteinylDOPA melanins. No hyperfine splitting (thin arrow) could be detected in any of the measured samples except the cysteinylDOPA melanin. Parameters of measurements – see Materials & Methods. Thick arrow – the position of the EPR signal of DPPH (a free radical standard).

It cannot be excluded that the signal at $g = 2.00$ might partly originate from free radicals other than melanin-like centres. A potential source of such signals may be the pool of "metabolic" semiquinones [20] or free radicals trapped in the

Tab. 1. Linewidth (ΔH, means ± SD) of the EPR signals of synthetic melanins and sclerotia of various colours.

Material	ΔH [Gs]	SD [Gs]
DOPA melanin	4.27	± 0.13
CysteinylDOPA melanin	5.68	± 0.19
Dark sclerotia	6.45	± 0.41
Orange sclerotia	6.88	± 0.50
Yellow sclerotia	6.73	± 0.50

solid-state structure of dry sclerotia [22]. In the first case, they would have revealed much lower intensity than, for example, those of black sclerotia, and they would not be detectable in the dormant, metabolically mute stages [20], like spores or sclerotia. In the second case, they should be narrower (2-3 Gs) and reveal a complicated structure [22]. We observed such signals in the bright spores of slime moulds [4, 5]; however, they were of a much lower intensity than the ones reported on here. The most convincing thing is the appearance of the strongest signals in the dark-brown and black sclerotia. Such sclerotia appeared to be dead, and thus metabolically inactive. Even if the observed signals did not originate from the produced dark pigment but from side-produced free radicals, they would indicate the intensity of melanogenesis. In previous studies, we observed dark *M. vesparium* plasmodia, which did not contain melanin, but manganese II ions [11]. Such material, even though measured alive at ambient temperature, did not reveal any detectable free-radical signals, but the very strong, 6-line signal of Mn II ions. When diluted, the plasmodial pigment had a pink colouration [11]. Finally, it was shown [23] that the bright spherules of other species (*P. polycephalum*) contain about 15% melanin in their polysaccharide cell wall; however, this was never confirmed with EPR measurements.

Pigmentation of the sclerotia correlates with their free radical signal amplitude and mass

The amplitude of the EPR free radical signal of the dry sclerotia partially correlated with the plasmodial colour in the sense that dark sclerotia revealed significantly stronger signals than the rest of the investigated material, and the orange ones had stronger signals than the yellow ones (Fig. 4A, B). This observation confirms the notion that the dark pigmentation is due to the presence of a melanin-like, paramagnetic pigment in the material, most probably a product of the polymerization of phenolic compounds during sclerotization. A similar process may take place in living plasmodia, which sometimes darken in old parts (Fig. 1C), or in the mucosal trace (Fig. 1B, 2H) produced by them when progressing in quest of food.

It is very unlikely that the increase in the comparatively wide free radical signal (over 6 Gs, which is similar to natural melanins) coupled with the deepening of

dark pigmentation is not connected with melanization, but with another process, but not with oxidative respiration, as such dark sclerotia are dead.
Moreover, the dark plasmodia tended to be heavier than the other ones (Fig. 4C, D). The signal correlated, therefore, not only with the pigmentation but also with the mass of the produced sclerotium (Fig. 4E).

Sclerotia are suitable for EPR measurements

Our study directly and unambigously classed the third type of slime mould material as appropriate for EPR measurements. Besides spores and wet plasmodia or pellets of microplasmodia, sclerotia can easily be measured using this biophysical technique. Their low water content, comparable to that in spores, makes it possible to carry out measurements at room temperature, whereupon the sclerotia can be used for further studies or cultivation. On the other hand, from the life-cycle point of view, sclerotia are a particular form of the vegetative plasmodium and they reveal at least some of its properties [1, 12]. Spores often differ from plasmodia in their physiological characteristics, e.g. they often contain high concentrations of melanin, while vegetative plasmodia are completely devoid of this pigment [4-7]. Large samples of plasmodia must be measured at 77 K, because of their high water content. Therefore, the sclerotia provide an interesting equivalent – they reveal some properties of spores, but they are a form of vegetative plasmodia, and they appear to possess their own intriguing features, as can be concluded from this study. Moreover, they are usually available in higher amounts than spores.
Dark sclerotia revealed highly variable intensities of EPR signals, which is not the case for bright sclerotia (Fig. 4A, B). This resulted in a much wider spread of data around the mean values (Fig. 4A). A few dark sclerotia also had a soft consistency, similar to fat, which was most probably due to their higroscopic properties, and which was not the case for orange or yellow material. The moistest sclerotia were difficult to couple in the resonant cavity [8], and their amplitudes were artificially lowered (Fig. 4A). Therefore, we did not include them in the calculation of the mean EPR signal amplitudes for Fig. 4.

Melanin is a side-product and/or evidence for deregulation of the plasmodial metabolism

Melanin has various biological properties, and the ability to produce this polymer possesses adaptative values [24]. One of the important functions of this pigment is general protection against harmful environmental conditions, in particular high doses of UV radiation or oxidative stress [25-26]. This is probably one of the reasons for melanin production in the dark spores of some species of fungi and myxomycetes, and even some seeds of higher plants [20]. Nevertheless, as the dark sclerotia never reproduced living plasmodia, their melanization does not seem to be protective. On the contrary, only the yellow sclerotia, with the lowest content of melanin, maintained the capability to regenerate.

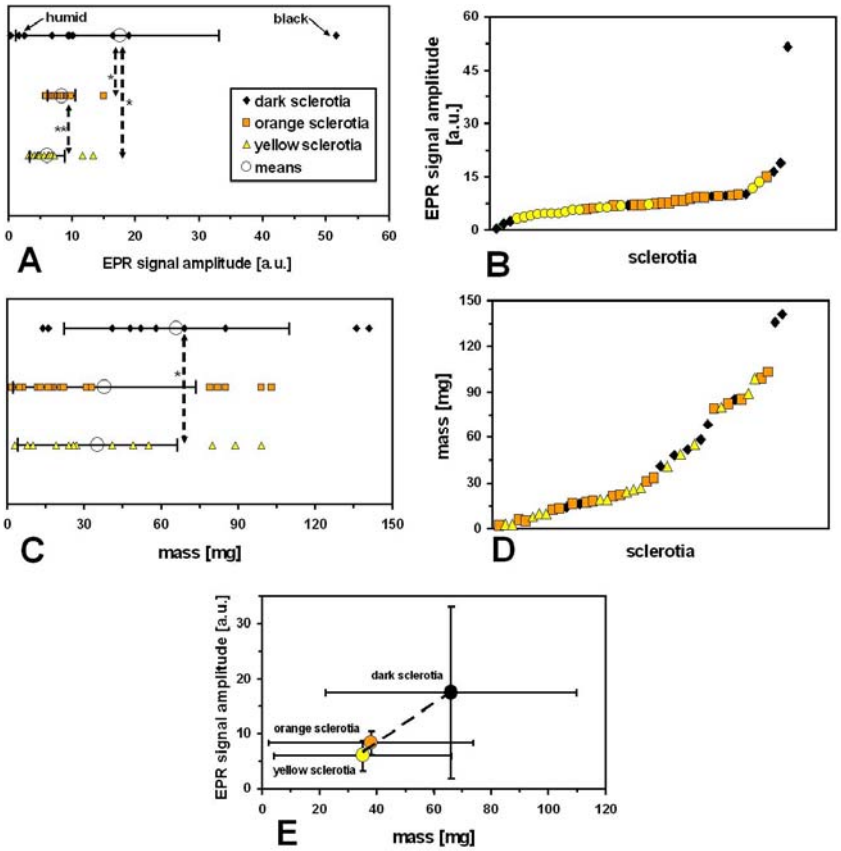

Fig. 4. The quantitative analysis of EPR signals (A, B) and mass (C, D) of the sclerotia of *Fuligo septica*. A, C – the values of particular samples of various colours, and the corresponding mean values (circles) of the dark (n = 10), orange (n = 17), and yellow (n = 16) sclerotia ± SD (error bars). * - 0.05 ≥ P ≥0.01; ** - 0.01 ≥ P ≥0.001. B, D – particular samples ranked by growing EPR signal amplitudes (B) or growing masses (D). Note the concentration of the yellow and orange points in the initial parts of the curves and the spread of the black points. E – mean masses and EPR amplitudes of the yellow, orange and dark sclerotia (means ± SD).

If melanization of the sclerotia appeared in the dead material (unable to reproduce viable plasmodia), its production must have not been associated with the activation of any special genes. It rather reveals an autooxidative nature [16]. It involves the spontaneous oxidative polymerization of phenolic compounds and free aromatic amino acids. A similar process takes place in response to wounding or infection of plant tissues or fungal mycelia. It is connected with an abrupt increase in the level of oxygen free radicals, hydrogen peroxide, nitric oxide, phenolic compounds and the activity of some enzymes, such as polyphenol oxidase, which may be responsible for the production of melanin

[24, 27, 28]. The spontaneous darkening of mucus accumulated in old slime mould cultures (Fig. 1B, 2H) is also symptomatic. A similar effect was observed during the acid hydrolysis of the plasmodia of *P. polycephalum* and *P. nudum,* with no regard to their plasmodial melanin content. Even if they revealed initially very weak EPR signals, the material gradually turned black and its EPR free radical signal increased [29]. A strong increase in the paramagnetic properties during acid hydrolysis of myxomycete spores in parallel to their gradual darkening was also reported [6, 7].

The yellow and orange colouration of the sclerotia is connected with low amounts of melanins, excluding, however, yellow or orange pheomelanins (Fig. 3), and with the presence of other pigments, a rich list of which can be found in the plasmodia of various slime moulds [1, 30]. A larger linewidth of the EPR signals of sclerotia, as compared to the synthetic DOPA- and cysteinylDOPA melanin can be explained by the ion-exchange properties and high concentration of various metal ions frequently adsorbed by natural melanins [16, 19, 31]. The metal ions can by themselves catalyze the oxidation of phenolic compounds to melanins [32, 33], and slime moulds (including *F. septica*) have been reported to accumulate metal ions in high concentrations in their plasmodia [11, 34].

From our observations, one can conclude that water removal during sclerotization is an active process, which, when reversed, restores viable plasmodia. Improperly controled, particularly in large plasmodia, it tends to generate large but dead, often melanized sclerotia. Interestingly, the yellow sclerotia seemed to be able to maintain the state of dehydration and glass-like texture when stored. Such intracellular glasses were discovered by EPR in dehydrated dormant stages of higher plants – pollen and seeds [35]. Meanwhile, in our study, at least a few dark sclerotia revealed higroscopic properties. In vegetative plasmodia, the ability to produce melanin has been reported so far only for *P. nudum* [10, 36] and only when the organism underwent irradiation with white light. This process was reversible and never testified to impairment of the vital functions of the plasmodium. Melanin was also found in the cell wall of *Ph. polycephalum* spherules from liquid cultures of microplasmodia [23]. Here, however, the pigment was isolated by acid hydrolysis, and was not characterized by EPR spectroscopy. It would be of great interest to study the sclerotization of other species of myxomycetes from this point of view, as the presence of melanin in spherules or sclerotia of acellular slime moulds seems to be quite a common feature of this dormant stage, resembling in this aspect spores of myxomycetes.

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REFERENCES

1. Stephenson, S.L. and Stempen,H. **Myxomycetes. A Handbook of Slime Molds**, 1st edition, Timber Press, Inc., Portland, Oregon, 1994 (paperback edition printed 2000).
2. Towpik, J. Regulation of mitochondrial translation in yeast. **Cell. Mol. Biol. Lett.** 10 **(**2005) 571-594.
3. Rakoczy, L. [The acellular slime moulds (*Myxomycetes*) – a model system for the modern biology. in: **Application of the In Vitro Cultures in Plant Physiology**] (Dubert, F., Ed.), 1st edition, The Franciszek Górski Department of Plant Physiology, Polish Academy of Science, Kraków, Poland, 1995, 301-308.
4. Płonka, P.M. and Rakoczy, L. Electron paramagnetic resonance spectroscopy (EPR) as a method for studying the biology of the acellular slime moulds (*Myxomycetes*). **Acta Physiol. Plant.** 19 suppl. (1997) 233.
5. Płonka, P.M. and Rakoczy, L. [Usefulness of the EPR method in the research on slime moulds. in: **Application of the In Vitro Cultures in Plant Physiology**] (Dubert, F. and Skoczowski, A., Eds.), 1st edition, The Franciszek Górski Department of Plant Physiology, Polish Academy of Science, Kraków, Poland, 1997, 175-180.
6. Rakoczy, L. and Panz, T. Melanin revealed in spores of true slime moulds using the electron paramagnetic resonance method. **Acta Protozool.** 33 (1994) 227-231.
7. Loganathan, L. and Kalyanasundram, I. The melanin of the myxomycete *Stemonitis herbatica*. **Acta Protozool.** 38 (1999) 97-103.
8. Sarna, T. and Lukiewicz, S. The double role of water in quantitative electron spin resonance (ESR) determinations on samples of biological materials. **Folia Histochem. Cytochem. (Krakow)** 9 (1971) 203-216.
9. Płonka, P.M. and Rakoczy, L. [Heme and non-heme iron complexes of nitric oxide in the plasmodia of acellular slime moulds cultured *in vitro*]. **Zesz. Probl. Post. N. Roln.** 473 (2000) 249-259.
10. Płonka, P.M. and Rakoczy, L. The electron paramagnetic resonance signals of the acellular slime mould *Physarum nudum* plasmodia irradiated with white light. **Curr. Top. Biophys.** 21 (1997) 83-86.
11. Rakoczy L. and Płonka, P.M. [Accumulation of manganese in plasmodia of the acellular slime mould (*Myxomycetes*) *Metatrichia vesparium*]. **Ochr. Środ. Zas. Nat.** 18 (1997) 299-308.
12. Rakoczy L. [Preservation of the ability to sporulate of the myxomycete *Physarum polycephalum* in its dormant stage – spherules. in: **Application of the In Vitro Cultures in Plant Physiology**] (Dubert, F. and Skoczowski, A., Eds.), 1st edition, The Franciszek Górski Department of Plant Physiology, Polish Academy of Science, Kraków, Poland, 1997, 467-473.
13. Verkman, A.S. More than just water channels: unexpected cellular roles of aquaporins. **J. Cell Sci**. 118 (2005) 3225-3232.

14. Levin, M.H. and Verkman, A.S. Aquaporin-3-dependent cell migration and proliferation during corneal re-epithelialization. **Invest. Ophthalmol. Vis. Sci.** 47 (2006) 4365-4372.
15. Verkman A. Role of aquaporins in endothelial water transport. **J. Anat.** 200 (2002) 528.
16. Felix, C.C., Hyde, J.S., Sarna, T. and Sealy, R.C. Interactions of melanin with metal ions. Electron spin resonance evidence for chelate complexes of metal ions with free radicals. **J. Amer. Chem. Soc.** 100 (1978) 3922-3926.
17. Deibel, R.M.B. and Chedekel, M.R. Biosynthetic and structural studies on pheomelanin. **J. Amer. Chem. Soc.** 104 (1982) 7306-7309.
18. Lukiewicz, S.J. and Sarna, T. Double internal standard for quantitative demonstration of free radicals. **Folia Histochem. Cytochem.** 9 (1971) 127-128.
19. Sarna, T. and Plonka, P.M. Biophysical studies of melanin: paramagnetic, ion-exchange and redox properties of melanin pigments and their photoreactivity. in: **Biomedical ESR. Biological Magnetic Resonance Series. vol. 23. (**Eaton, S.S., Eaton, G.R. and Berliner, L.J., Eds.), 1st edition, Kluwer Acad. Publ., The Netherlands-New York-Boston, 2005, 125-146.
20. Commoner, B., Townsend, J. and Pake, G.W. Free radicals in biological materials. **Nature** 174 (1954) 689-691.
21. Sealy, R.C., Hyde, J.S., Felix, C.C., Menon, I.A., Prota, G., Swartz, H.M., Persad, S. and Haberman, H.F. Novel free radicals in synthetic and natural pheomelanins: Distinction between dopa melanins and cysteinyldopa melanins by ESR spectroscopy. **Proc. Natl. Acad. Sci. U. S. A.** 79 (1982) 2885-2889.
22. Yordanov, N.D. and Pachova, Z. Gamma-irradiated dry fruits. An example of a wide variety of long-time dependent EPR spectra. **Spectrochim. Acta A Mol. Biomol. Spectrosc.** 63 (2006) 891-895.
23. McCormick, J.J., Blomquist, J.C. and Rusch H.P. Isolation and characterization of a galactosamine wall from spores and spherules of *Physarum polycephalum*. **J. Bacteriol.** 104 (1970) 1119-1125.
24. Plonka, P.M. and Grabacka, M. Melanin synthesis in microorganisms – biotechnological and medical aspects. **Acta Biochim. Pol.** 53 (2006) 429-443.
25. Slominski, A., Tobin, D.J., Shibahara, S. and Wortsman, J. Melanin pigmentation in mammalian skin and its hormonal regulation. **Physiol. Rev.** 84 (2004) 1155-1228.
26. Wood, J.M., Jimbow, K., Boissy, R.E., Slominski, A., Plonka, P.M., Slawinski, J., Wortsman, J. and Tosk, J. What is the use of generating melanin? **Exp. Dermatol.** 8 (1999) 153-164.
27. Talarczyk, A. and Hennig, J. Early defence responses in plants infected with pathogenic organisms. **Cell. Mol. Biol. Lett.** 6 (2001) 955-970.
28. Sommer, A., Ne'eman, E., Steffens, J.C., Mayer, A.M. and Harel, E. Import, targeting, and processing of a plant polyphenol oxidase. **Plant Physiol.** 105 (1994) 1301-1311.

29. Rakoczy, L. and Płonka, P.M. [Plasmodia of acellular slime moulds – materials for verification of the procedure used for natural melanin purification.] **Zesz. Probl. Post. N. Roln.** 473 (2000) 267-277.
30. Majcherczyk, A., Rakoczy, L. and Hüttermann A. A method for separation of pigments from plasmodia of the true slime molds, *Physarum polycephalum* and *Physarum nudum*. **Anal. Biochem.** 160 (1987) 178-183.
31. Okazaki, M., Kuwata, K., Miki, Y., Shiga, S. and Shiga, T. Electron spin relaxation of synthetic melanin and melanin-containing human tissues as studied by electron spin echo and electron spin resonance. **Arch. Biochem. Biophys.** 242 (1985) 197-205.
32. Jara, J.R., Solano, F., Garcia-Borron, J. Aroca, P. and Lozano, P. Regulation of mammalian melanogenesis II: the role of metal cations. **Biochim. Biophys. Acta** 1035 (1990) 276-285.
33. Napolitano, A., Di Donato, P. and Prota, G. Zinc-catalyzed oxidation of 5-S-cysteinyldopa to 2,2'-bi(2H-1,4-benzothiazine): Tracking the biosynthetic pathway of trichochromes, the characteristic pigments of red hair. **J. Org. Chem.** 66 (2001) 6958-6966.
34. Zhulidov, D.A., Robarts, R.D., Zhulidov, A.V., Zhulidova, O.V., Markelov, D.A., Rusanov, V.A. and Headley, J.V. Zinc accumulation by the slime mold *Fuligo septica* (L.) Wiggers in the former Soviet Union and North Korea. **J. Environ. Qual.** 31 (2002) 1038-1042.
35. Buitink, J., Dzuba, S.A., Hoekstra, F.A. and Tsvetkov, Y.D. Pulsed EPR spin-probe study of intracellular glasses in seed and pollen. **J. Magn. Reson.** 142 (2000) 364-368.
36. Rakoczy, L. and Płonka, P. [Pigment changes in irradiated plasmodia of the acellular slime moulds *Physarum polycephalum* and *Physarum nudum.* in: **Application of the In Vitro Cultures in Plant Physiology**] (Dubert, F., Ed.), 1st edition, The Franciszek Górski Department of Plant Physiology, Polish Academy of Science, Kraków, Poland, 1995, 309-315.