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EXPERIMENTAL ARTICLES

Effect of Oxylipins on *Neurospora crassa* Growth and Differentiation

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Abstract—The effect of the natural oxylipins 3(*R*)-hydroxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (3-HETE) and 18-hydroxy-(9*Z*,12*Z*)-octadecadienoic acids (18-HODE) on the growth and hypha aggregation, as well as on some light-depending processes, such as carotenoid biosynthesis, protoperithecia formation (sexual cycle), and conidiation (asexual cycle), of the ascomycete *Neurospora crassa* was studied. Hypha aggregation and growth slowdown were induced by 3-HETE, 18-HODE, and linoleic acid. At concentrations from 5 to 50 μM, these compounds had no significant effect on the light-induced carotenogenesis. At the same time, these 3-HETE and 18-HODE concentrations, unlike linoleic acid, induced the formation of protoperithecia in the dark. At the concentration of 5 μM, an additive effect of oxylipins and light was revealed. The studied oxylipins had different effects on the asexual reproduction of *N. crassa*: 3-HETE induced conidiation in the dark, whereas 18-HODE induced conidiation in the light. The possible involvement of oxylipins in the regulation of the processes of sexual and asexual reproduction of *N. crassa* is discussed.

Keywords: oxylipins, *Neurospora crassa*, hyphal growth, differentiation, carotenoids
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Oxylipins are a group of lipid compounds which contain oxygenated fatty acids. It is well known that, in fungi, these compounds affect the morphogenetic properties and growth, as well as the synthesis of secondary metabolites; they are involved in the processes of cell–cell interactions and cell transformation (in dimorphic fungi) [1–5]. The regulatory functions of oxylipins have been demonstrated as of yet only for some pathogenic fungi, although the presence of oxidized fatty acids and the pathways of their intracellular oxidation were reported for many fungi [1–5].

Oxygenation of fatty acids in fungal cells occurs both in the presence of reactive oxygen species and under the influence of lipid-oxidizing enzymes (β- and ω-oxidation of fatty acids, oxidation controlled by lipoxygenases, dioxygenases, etc.) [1, 2, 4–8]. This may result in production of a wide range of oxylipins with different biological properties.

In fungi, 3-hydroxy derivatives of saturated and unsaturated fatty acids resulting from incomplete β-oxidation, are the most abundant [9–12]. For instance, it was demonstrated that the yeast *Dipodascopsis uninucleata* var. *uninucleata* produced 3*R*-HETE upon addition of arachidonic acid to the medium [13] and synthesized 3-hydroxy-C20:3, 3-hydroxy-C20:5, 3-hydroxy-C14:2, and 3-hydroxy-C14:3 acids from their precursors [14]. Recently, 3-hydroxy derivatives of oxylipins were obtained by total chemical synthesis [15, 16], which made it possible to study these compounds by immunochemical and biological methods.

As a result of impaired β-oxidation of fatty acids, the pathway of their oxidation at the terminal carbon atom, which leads to the emergence of ω-hydroxylated oxylipins, becomes prominent. Cytochrome P450-dependent monooxygenases and NADPH: cytochrome P450 oxidoreductase are involved in this process [17].

Fatty acids, precursors of oxylipins, may also affect fungal development. In the case of *Aspergillus flavus*, exogenous linolenic acid accelerated the formation of sclerotia, whereas oleic and linoleic acids inhibited this process [6]. Unlike the wild type strain, a decrease in the rate of conidiation of dioxygenase-deficient *A. niger* mutants in the presence of linoleic acid was detected [18]. The formation of *Neurospora crassa* protoperithecia intensified in the presence of unsaturated fatty acids (linoleic, linolenic, and palmitoleic acids). Saturated fatty acids had no effect on this process [19].

Thus, despite intense scientific interest in the structures of natural oxylipins and their regulatory functions, the pathways of their biosynthesis, mechanisms responsible for signal transduction, and the effects of these compounds on fungal differentiation processes remain poorly studied. Today, the role of

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oxylipins in the development of *N. crassa* is uncertain, although this ascomycete is known to possess its own pathways of oxylipins biosynthesis. It was demonstrated that *N. crassa* glyoxysomes are responsible for β-oxidation of fatty acids [20]. The genes encoding lipoxygenases and dioxygenases were detected [1, 21], although the role of this enzymes in the *N. crassa* metabolism is poorly understood.

Since fungi are widely used in various biotechnologies, the study of natural oxylipins using a universal model microorganism, the ascomycete *N. crassa*, in order to determine their functions and to search for the regulators of fungal differentiation is of urgent scientific interest. Light is one of the important natural regulators of *N. crassa* differentiation. The photoreceptor complex of *N. crassa* controls the biosynthesis of carotenoids in the mycelium, formation of the structures of asexual and sexual reproduction (conidia and protoperithecia), as well as phase shifting of the circadian clock, tropism of perithecia and reversion of their tips polarity, electrogenesis of cell membranes, and activity and molecular structures of some enzymes [22]. It is well known that blue light induces conidiation in the surface culture under carbon limitation conditions and protoperithecia formation under nitrogen limitation conditions [22].

The goal of the present work was to study the effects of hydroxy derivatives of natural polyunsaturated fatty acids, 3(*R*)-hydroxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (3-HETE) and 18-hydroxy-(9*Z*,12*Z*)-octadecadienoic acid (18-HODE), on the growth and the processes of asexual and sexual reproduction of *N. crassa*.

MATERIALS AND METHODS

Synthesis and extraction of oxylipins. The synthesis of the optically active 3(*R*)-hydroxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (3-HETE) and 18-hydroxy-(9*Z*,12*Z*)-octadecadienoic acid (18-HODE) of natural structure was carried out according to the previously described techniques based on acetylene synthesis strategy [16, 23]. Linoleic acid was extracted from sunflower oil using the modified method by Blight and Dyer [24]. The structures of all the compounds obtained were confirmed by the results of NMR- and mass spectrometry.

Subject of study and obtaining of the inoculum. The wild type strain 2218 of the ascomycete *N. crassa* was kindly provided to us by the Fungal Genetics Stock Center (FGSC, Kansas City, United States). The suspension of *N. crassa* conidia stored at –70°C was used as inoculum. The suspension was obtained by washing the spores off the surface of the *N. crassa* culture grown on agarized 2% Vogel's medium at 28°C for 6 days. Conidia were harvested by filtering through a gauze filter and glass wool. The concentration of spores in the suspension was 30 mg/mL.

Effect of oxylipins on the growth and aggregation of hyphae. The culture was grown on petri dishes (9 cm in diameter) with agarized Vogel's medium [25] on a cellophane disc. To obtain a fungal colony, cellophane membranes were stab-inoculated with portions of mycelium, and the plates were incubated in a thermostat at 28°C in the dark. After 18-h incubation, the tested compounds (1 mL, 5 μM) in the form of water–alcohol emulsions (at a final ethanol concentration of 1%) or the control solution (ethanol–water) were injected under the cellophane discs. After the incubation at 28°C (1 h) in the dark, changes in the growth rate (as compared to the control) were detected with a digital camera (Webbers, China) attached to the microscope eyepiece and transmitting the magnified image (×100) on a computer monitor. The measurements were made at 3-min intervals for 30 min. Aggregation of hyphae at the periphery of a single colony (20-h growth) was observed within 1 h after the addition of the tested compounds.

Effect of oxylipins and linoleic acid on carotenogenesis. The water suspension of *N. crassa* conidia (0.4 mL; 0.25 mg of spores) was applied on the surface of the cellophane–paper filter bilayer covering the agarized Vogel's medium and moistened with liquid medium of the same composition. After 24 h of cultivation at 28°C in the dark (at the beginning of the stationary phase), 1 mL of a water–ethanol emulsion containing various concentrations (5, 10, 20, and 50 μM) of the tested compounds was injected under the bilayer. The experiment was performed under red light (weak intensity). The culture was supplemented with linoleic acid or oxylipins, incubated in the dark at 28°C for 1 h, and illuminated for 4 h with LDC-36W lamps (Russia) at a light intensity of 15 W/m^2. The control plates were incubated in the dark at the same temperature as the illuminated ones. After the end of the exposure, the mycelium was harvested from the cellophane surface with a spatula. The excessive liquid was removed with filter paper.

Carotenoids were extracted from the mycelium with ethanol (3 mL) for 3 days. Absorption was determined on a spectrophotometer at 475 nm. The concentration of carotenoids was calculated as described earlier using the extinction coefficient $E_{cm}^{1\%} = 2500$ [26]. The light-dependent production of carotenoids was assessed by a difference between the contents of carotenes in the mycelium obtained in the light and in the dark.

Asexual reproduction was assessed by the amount of obtained conidia. The experiment was carried out as described above, but the amount of the inoculum was increased up to 1 mg of spores per petri dish, and the illumination was decreased to 2 h. The stationary cultures in petri dishes (experimental and the dark control) were then incubated at 28°C for 24 h. Conidia were washed off with water (5 mL per petri dish) and enumerated in a Goryaev count chamber. To elucidate

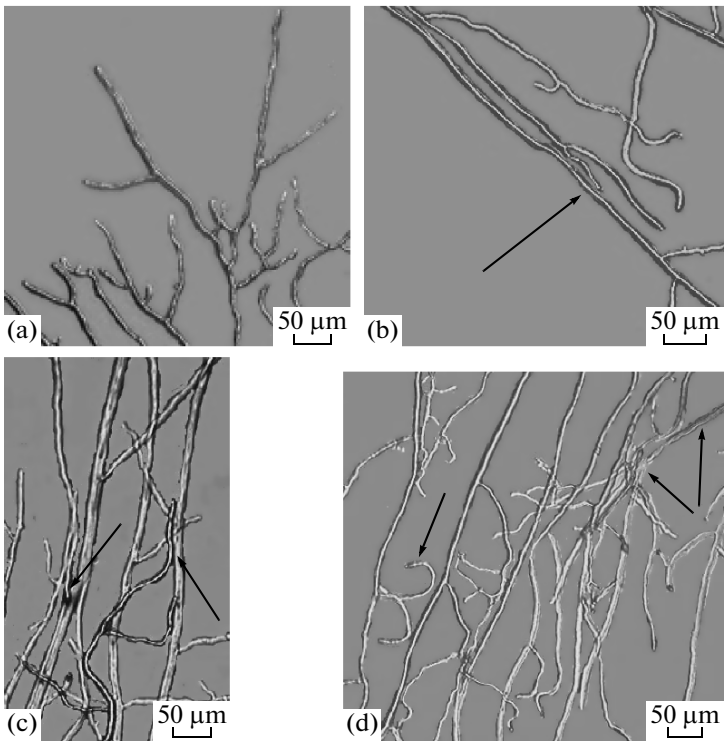

Fig. 1. Effect of linoleic acid, 3-HETE, and 18-HODE on *N. crassa* hyphae aggregation : control (a), linoleic acid (b), 3-HETE (c), and 18-HODE (d); 5 μM of the tested compound, 1 h. The arrows point to hyphae aggregations.

the effect of the tested compounds on the ability of conidia to germinate, the suspension was diluted 1 : 100, 1 : 1000, and 1 : 10000 and plated onto agarized Vogel's medium containing 1% of sorbose and 0.1% of glucose as a carbon source, and 4 mM of ammonium chloride as a nitrogen source [27]. The presence of sorbose allowed us to obtain well-isolated colonies. The number of germinated conidia was determined according to the number of obtained colonies after 2-day incubation in the dark at 28°C.

Sexual reproduction was assessed according to the number of protoperithecia formed in the dark or after the exposure of the *N. crassa* mycelium to light [27]. Aliquots (0.4 mL) of the water suspension of conidia (0.25 mg of spores) were applied to cellophane discs placed in the modified Vogel's medium containing 4 mM of NH_4Cl as a nitrogen source, 1% sorbose, and 0.1% of glucose as a carbon source. Protoperithecia formation was induced by the transfer of the mycelium grown on the cellophane discs in the dark at 23°C for 3 days to the same medium without nitrogen sources. After 24-h incubation on the medium without nitrogen source, 3-HETE, 18-HODE, or linoleic acid were injected under the cellophane discs. The tested compounds were injected under red light. After 1-h incubation, the cultures, except for the controls, were exposed to light (350–500 nm, 1 W/m^2) for 2 min. After 2-day incubation in the dark (23°C), the protoperithecia which formed on the mycelium were counted under a light microscope (×40), and the number of cells per cm^2 of the surface culture was determined.

The experiments were carried out in 3–9 repeats. The MS Excel 2000 software package was used for statistical analysis of the results obtained.

RESULTS AND DISCUSSION

Aggregation and growth. The addition of 3-HETE, 18-HODE, or linoleic acid (5 μM, 1 h) to the surface *N. crassa* culture induced hyphae aggregation and resulted in various morphological changes (swelling and flexing of hyphal tips) (Figs. 1b, 1c, and 1d). In the control samples treated with the water–ethanol solution, aggregation of hyphae was not observed (Fig. 1a). The growth rate of the tips of the leading hyphae in the presence of oxylipins or linoleic acid after 1-h treatment was 2 times lower than in the control samples. Aggregation of the hyphae under unfavorable conditions usually precedes the processes of fungal differentiation [22]. The fact that various 3-hydroxy derivatives of oxylipins enhance adhesion of sexual and asexual spores is known for yeasts and mucoraceous fungi [9].

Carotenogenesis. Photoinduction of carotenoid biosynthesis in the *N. crassa* mycelium is the most advanced model for the investigation of transduction of the light signal during the differentiation processes

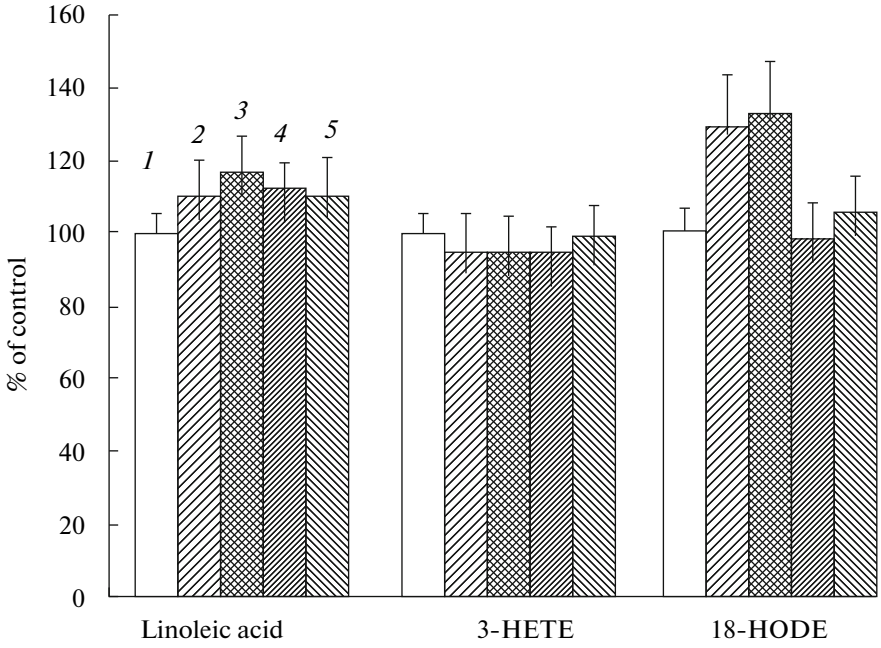

Fig. 2. Effect of linoleic acid, 3-HETE, and 18-HODE on the light-induced carotenogenesis of *N. crassa*: *1*, control (no added linoleic acid or oxylipins); *2*, 5 μM; *3*, 10 μM; *4*, 20 μM; *5*, 50 μM.

[22]. After 4-h exposure to light, the content of carotenoids in the control samples reached 0.41 ± 0.02 μg/mg of dry biomass. At concentrations ranging from 5 to 50 μM, 3-HETE and linoleic acid had no noticeable effect on carotenoid accumulation, whereas low 18-HODE concentrations (5 and 10 μM) increased the content of carotenoids by 30%. An increase in the 18-HODE concentrations up to 20–50 μM (inhibited) declined the light-induced carotenoid production down to the control values (Fig. 2). It may be concluded that oxylipins and linoleic acid had no pronounced effect on carotenogenesis

Sexual reproduction. In the control samples, blue light (350–500 nm) increased the formation of protoperithecia, precursors of the female structures, by 1.6–2.9 times (Tables 1 and 2).

In Tables 1 and 2, the data on the formation of protoperithecia in the dark and after exposure to light in the presence of various concentrations of 3-HETE and 18-HODE are shown. It is obvious that both oxylipins similarly affected the sexual cycle of *N. crassa*. In the dark, they induced a manifold increase in the amount of protoperithecia. The maximum effect was observed at concentrations ranging from 10 to 20 μM; however, the effect of 18-HODE was more significant. It should be noted that the effect of 3-HETE and 18-HODE on the formation of protoperithecia was so strong that it could be compared with light, a natural inducing factor. It is notable that, in the presence of both compounds at low concentrations (5 μM), the hydroxyacid–light synergism was observed; however, at high concentrations of oxylipins, their effect was less pronounced in the light, while it remained at the same level in the dark (Tables 1 and 2).

According to Kock et al., 3-hydroxy derivatives of oxylipins may be involved in the sexual process of gametangeogamy, as well as in the release of aggregated ascospores of *D. uninucleata* [9].

Interestingly, the effect of linoleic acid on the sexual reproduction was not detected in our experiments, even at concentrations of 20–50 μM. At the same time, in an earlier experiment with the mycelium covered with a filter with 1 mg of linoleic acid, the formation of *N. crassa* protoperithecia was intensified [19]. This may be attributed to the fact that the concentration of linoleic acid was much higher (by two orders of magnitude) than in our experiments.

Table 1. Effect of 3-HETE on the sexual cycle of *N. crassa*

Variant	Number of protoperithecia per cm^2 of the surface culture	
	Dark	Light
Control	178 ± 18	282 ± 28
+ 5 μM 3-HETE	340 ± 30	520 ± 12
+ 10 μM 3-HETE	402 ±28	350 ± 33
+ 20 μM 3-HETE	418 ± 28	407 ± 25
+ 50 μM 3-HETE	283 ± 15	243 ± 13

Table 2. Effect of 18-HODE on the sexual cycle of *N. crassa*

Variant	Number of protoperithecia per cm^2 of the surface culture	
	Dark	Light
Control	93 ± 7	267 ± 13
+5 μM 18-HODE	260 ± 15	428 ± 47
+10 μM 18-HODE	347 ± 40	398 ± 48
+20 μM 18-HODE	292 ± 43	295 ± 40
+50 μM 18-HODE	280 ± 18	250 ± 30

Asexual reproduction. Since in our studies of the sexual process of *N. crassa*, the effect of the oxylipins was most pronounced at 5 μM, these concentrations of 3-HETE and 18-HODE were used when studying their effects on the processes of asexual reproduction of the fungus. After exposure of the fungal mycelium to light, the total number of conidia in the control increased sharply (Tables 3 and 4). In the dark, the amount of conidia in the presence of 3-HETE was 3 times higher, while after light exposure, it was found to be 2 times lower (Table 3). By contrast, in the dark, in the presence of 18-HODE, the number of conidia was statistically the same as in the control; however, in the light, it was 1.5 times higher (Table 4).

The ability of conidia formed in the presence of 3-HETE to germinate in the dark was 2 times lower, whereas, after light exposure, it was 2 times higher than in the control. By contrast, 18-HODE had no significant effect on the ability of conidia to germinate (Table 3 and 4).

Hence, 18-HODE and 3-HETE had different effects on conidiation: 18-HODE and 3-HETE induced conidiation in the presence of light and in the dark, respectively. In other words, these compounds exerted different effects on conidiation of *N. crassa* in the dark and in the presence of light.

Importantly, oxylipins may act as hormone-like signals (autoregulators of the fungal metabolism) involved in the regulation of *N. crassa* growth [1–5, 29]. It was demonstrated that, in some representatives of the genus *Aspergillus*, the balance between the sexual and asexual reproduction is regulated by psi (precocious sexual inducer) factors represented by the oxidized metabolites of oleic (psiβ)), linoleic (psiα), and linolenic (psiγ) acids. The synthesis of the components of psi factors from linoleic and oleic acids is initiated by dioxygenases encoded by the *ppo* genes (psi factor producing oxygenase) [21, 30].

It is presently unclear which oxylipins are synthesized in *N. crassa* cells and participate in the regulation of growth. The presence of the enzymes responsible for the β- and ω-oxidation of lipids suggests the possible production of 3-hydroxy and 18-hydroxy derivatives of fatty acids; the presence of the *ppo* genes indicates the possibility of oxidation of the 8th and 10th atoms of the fatty acids [1, 17, 20, 21]. It should be also noted that the presence of the lipoxygenase gene [21] means that fatty acids may be oxidized via this pathway. The possibility of the endogenous regulation of *N. crassa* growth by oxylipins was confirmed by the data on the effect of unsaturated fatty acids on the formation of protoperithecia [19], as well as by changes in the fatty acid composition of this fungus during growth [31].

Differentiation of fungi is controlled by a complex of external signals, enabling them to survive under changing ambient conditions. Light plays an important role in the regulation of their sexual and asexual reproduction, which may occur via different pathways [22]. It was established that, in many fungi, the *velvet* gene is the global regulator of sexual and asexual reproduction depending on the presence of light [28]. In *A. nidulans*, VeA induces the formation of sexual structures; in *N. crassa*, its homologue Ve-1 controls conidiation [32]. The association between oxylipins and the *veA* genes has not been experimentally con-

Table 3. Effect of 3-HETE on conidiation and the ability of *N. crassa* spores to germinate

Variant	Number of conidia ($\times 10^4$ per 1 cm^2 of the surface culture)			
	Dark		Light	
	Number of conidia	Number of germinated conidia (2 days)	Number of conidia	Number of germinated conidia (2 days)
Control	12.7 ± 1.6	6.1 ± 1.4	226.5 ± 45.1	61.1 ± 6.7
+5 μM 3-HODE	41.3 ± 8.6	10.5 ± 3.2	125.3 ± 14.4	63.6 ± 11.5

Table 4. Effect of 18-HODE on conidiation and the ability of *N. crassa* spores to germinate

Variant	Number of conidia ($\times 10^4$ per 1 cm^2 of the surface culture)			
	Dark		Light	
	Number of conidia	Number of germinated conidia (2 days)	Number of conidia	Number of germinated conidia (2 days)
Control	14.6 ± 2.2	2.0 ± 0.3	52.9 ± 6.6	4.4 ± 1.1
+5 μM 18-HODE	15.3 ± 2.1	1.8 ± 0.4	81.5 ± 3.5	9.5 ± 1.6

firmed so far; however, Tsitsigiannis and Keller suggested [5] that there is a relationship between the genes *ppo* and *veA*.

Hence, in our experiments, oxylipins had a significant effect on the processes of sexual and asexual reproduction of *N. crassa* depending on the structures of the studied compounds. The results obtained indicate that the hydroxylated unsaturated fatty acids 3-HETE and 18-HODE may be involved in the light-dependent differentiation processes of *N. crassa*.

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