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Peculiarities of growth and lipoxygenase activity of wild fern *Dryopteris filix-mas* (L.) Schott

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We studied the peculiarities of growth, localization and dynamics of lipoxygenase activity in sporophyte organs of wild fern *Dryopteris filix-mas* (L.) Schott at various phases: intensive growth; sori ripening; spore release; summer vegetation, and vegetation termination. Morphometric analysis showed that the weight of one plant during sporophyte ontogenesis varied from 130 to 150 g. Frond length varied from 11 to 85 cm. Diameter of the overground part of rhizome was 9-10 cm. In the phase of spore release and during the summer vegetation we mentioned 7 fronds on plant, and in the phase of vegetation termination – 18. The plants had the biggest weight in phase of spore release. Following spore release and at the beginning of vegetation termination, the frond weight diminished almost by two times. An intensive elongation of fronds was observed prior to the start of spore release. When fronds unfold and grow the number of segments of the first order increase from 39 to 47 pairs. Frond length in the phase of summer vegetation increased from 4.3 to 11.2 cm (9 pairs), and then it gradually decreased from 10.2 to 0.1 cm (39 pairs) that resulted in the formation of a lanceolate lamina. In the phase of vegetation termination, the fern had 18 fronds, 12 of which remained green, while the rest dried and turned greyish-brown. The frond lamina contained 47 pairs of segments of the first order whose length increased from 4.5 to 10.5 cm, and then it diminished from 9.5 to 0.05 cm. The rhizome weight was in the range of 110.2–115.7 g in all phases except the phase of vegetation termination. The morphological analysis showed that plants had successfully passed all developmental phases, reached their normal dimensions, had a mass spore formation, no external signs of suppression and damages, and this was generally consistent with the highest level of life state assessment. Localization and dynamics of lipoxygenase (LOX) activity in organs of the sporophyte of *D. filix-mas* have been studied. We registered that fronds contained 13-LOX with $pH_{opt.}$ values of 7.2, rhizomes – 9-LOX ($pH_{opt.}$ 6.5). Peculiarities that were mentioned in localization and dynamics of LOX isoforms catalytic activity in the fern organs at various phases of development suggest that the enzyme might be involved in the regulation of lipid metabolism of growth processes that ensure plant adaptation to the environment.
Key words: *Dryopteris filix-mas* (L.) Schott; lipoxygenase; sporophyte; phenological phases

Introduction

To monitor environmental conditions and adapt to them, plants use a multi-component complex, a significant constituent of which is the lipoxygenase signaling. Lipoxygenase molecules (linoleate-oxygen oxydoreductase, EC 1.13.11.12, LOXs) contain non-heme iron (Ivanov et al., 2010; Borrego, Kolomiets, 2016; Babenko et al., 2017a) and catalyze stereo-specific peroxidation of polyunsaturated fatty acids (PUFA) that have at least one 1Z-4Z-pentadiene link (Joo, Oh, 2012). Formation of hydroperoxides of trans- and cis-conjugated dienes is a key reaction in the lipoxygenase cascade that initiates seven enzyme branches whose final products are biologically active metabolites – oxylipins (Feussner, Waster, 2002; Gao et al., 2008). These compounds are involved in the regulation of growth, development as well as formation of responses to environment signals and provide certain communication between living organism kingdoms (Christensen, Kolomiets, 2011; Borrego, Kolomiets, 2012, 2016; Babenko et al., 2014, 2017a; Savchenko et al., 2014; Wasternack, Song, 2016). Search for specific DNA sequences in the data base GenBank, Refseq, Uniprot, Ensembl made it possible to establish the presence of LOX in cyano- and proteobacteria, the simplest unicellular red and green sea algae, amoeba, fungi, mosses, angiosperms and animals (Ivanov et al., 2010; Babenko et al., 2017a). Although a biological role of LOX in lower plants remains unclear, the presence of LOX sequence in their DNA suggests that the genus of these enzymes is evolutionary ancient and emerged after the oxygen rise in Earth's atmosphere. LOX appeared among cyanobacteria that is the oldest life form (Andreou et al., 2010). In lower organisms, LOX occurs as hybrid enzymes in which the lipoxygenase domain may be associated with another catalytic domain a peroxidase one. A biological role of hybrid enzymes is not fully understood, but it was shown that they are involved in biosynthesis of signaling molecules of lipid nature (Koljak et al., 1997). Algae and mosses contain significant quantities of C_{20}-PUFAs (arachidonic and eicosapentaenoic) and C_{18}-

PUFAs (α-linolenic and linolenic) that are characteristic of animals, and which are typical lipoxygenase substrates in flower plants (Ponce de León et al., 2015). *Physcomitrella patens* were found to have LOX with pH_{opt} values of 7.0 and pH_{opt} values of 5.0 that produced (12S)-hydroperoxide with arachidonic and eicosapentaenoic acids and (13S)- hydroperoxide with α-linolenic acid (Anterola et al., 2009). Thus, in mosses C_{20} and C_{18}-PUFAs may be oxidized with LOX and converted to biologically active oxylipins (Anterola et al., 2009). Due to the presence of C_{20}-PUFAs the oxylipin spectrum in mosses was significantly larger comparative to flower plants. However, jasmonic acid (JA) – one of the most active oxylipins in flower plants and mosses, is not synthesized (Ponce de León et al., 2015).

According to the modern views on the systematics and phylogeny, *Polypodiophyta* are the most numerous group of vascular sporophytes that include 10 323 species united in 300 genera and 44 families (PPG, 2016). Competition with flower plants for resources turned out to be an evolutionary factor for ferns, which led to some diversification of ecological niches, species specialization, to increase in biological diversity and adaptation levels in many taxa (Page, 2002; Babenko et al., 2015a). New adaptation strategies contributed to spreading of these plants from humid to more drought ecosystems and even emergence of fern-xerophytes (Hietz, 2010). Presence of C_{20}- and C_{18}-polysaturated fatty acids in *Polypodiophyta* suggested that they contain lipoxygenases (Rozentsvet et al., 2011). Information on LOX and products of their metabolic pathways in *Polypodiophyta* is limited (Boland et al., 1995; Imbiscuso et al., 2009; Radhika et al., 2012; Babenko et al., 2016 a). So, *Polystichum aculeatum* (L.) Roth. fronds of an evergreen phenorhythmic type were revealed to have 13-LOX with $pH_{опт.}$values of 7.74, while rhizomes – 9-LOX with $pH_{opt.}$ values of 7.54 (Babenko et. al., 2017b). Like in flower plants, as a response to *Spodoptera littoralis* infection *Pteris vittata* was shown to synthesize H_2O_2 and emit volatile terpenoids (Imbiscuso et al., 2009). Given that lipoxygenase basic physiological functions include the involvement in the synthesis of signaling compounds, lipid peroxide oxidation and mobilization of lipid reserves during growth and development, while enzymes and products of lipoxygenase signaling play an important role in the formation of plant adaptation to effects of abiotic and biotic stressors, the aim of our work was to identify and study the pattern of localization and dynamics of lipoxygenase activity in the sporophyte organs of leptosporangiate fern of the Ukraine flora *Dryopteris filix-mas* (L.) Schott at various phenological phases of development.

Materials and methods

Studies were conducted with plants of the leptosporangiate homosporous fern *Dryopteris filix-mas* (L.) Schott. *D. filix-mas* is 50-100 cm long, it has a horizontal or skew, thick, short rhizome, on which petiole remains are densely located. In the form of a funnel-shaped bundle, 50-100 cm long fronds are on the rhizome top. Petioles are short, thick. As well as rachis they are densely covered with lanceolate scales. Laminas are elongated-elliptical, bipinnate. Parts of the first order are linear-lanceolate on short petiolules, deeply pinnate, with long, blunt, skewed parts of the second order. Sori are rounded, located in two rows along flanks of the middle rib on the frond abaxial surface, converging but not merged. Sori are covered with indusium, which shrivels when reniform spores are ripening.

D. filix-mas is distributed in the temperate zone of the northern hemisphere, it is a species common of the Ukraine flora, is found in forests, dump places in bushes and rock cracks (Tutin et al., 2010; Vasheka, Bezsmertna, 2012). It belongs to sciophytes, grows in shade of the overstory plants, which cover it from direct sunlight. According to its phenorhythm type *D. filix-mas* is a summer green plant (Vasheka, Brayon, 2000), vegetation of fronds of one generation lasts 200-225 days, in the end of autumn or in the beginning of winter when air temperature starts to drop fronds are dying off (Vasheka, Bezsmertna, 2012).

Phenological observations have been done according to the methodical recommendations developed specifically for pteridophytes by Kotukhov (1974) and modified by Vasheka (2004). Vegetation start was observed just from the moment of frond apices divergence, intensive coloring of footstalk in green and breaking-up of films that was followed by frond primordia expansion. Frond intensive growth was characterized by a rapid unfolding of leaf blades. Externally, the cessation of an intensive growth consisted in a full unfolding of blades, abrupt bending of the blade upper tip into the middle and darker coloring of the bent part. The cessation of fronds growth was characterized by straightening of their apices and after that, fronds acquired their typical appearance and form.

An important feature that characterizes the plant physiological state in certain growth conditions is spore formation. Sori emerged in the phase of an intensive growth beginning from the moment of unfolding of the first fronds pair that bear sori. The spores release phase (sporangia burst) was characterized by sporangium growing brown or yellow, their enclosure breaking and spores scattering. This phase was registered during mass sporangia bursting and visually observed when fronds were shaken off onto a white sheet of paper. Fronds started to die off in the mid of October and a complete destruction of their laminas was finished in the end of November. We studied plants that grew on the exposure plots of spore-bearing plants of academic O.V. Fomin Botanical Garden, Kyiv National University. Fronds and rhizomes were separated from plant samples. Samples to be analyzed were selected starting from April at intervals of one month until the mid of autumn according to the following phenological stages: intensive growth; sori ripening; spores release; summer vegetation; vegetation termination and overground part die off. Monthly average air temperature during the period of sampling was: April +12.4 °C, May +15.5 °C, June +20.6 °C, July +22.4 °C, August +21.1 °C, September +16.1 °C, October +6.5 °C. Natural soils on the cryptogamic plants exposition site of the O.V. Fomin Botanical Garden were represented by ashed, very loamy chernozem on forest loam soil having the following characteristics of the arable layer: pH_{KCl} – 5.7, total humus content – 4.1%, movable phosphorus and potassium compounds (according to Chirikov) – 138 mg/kg and 90 mg/kg of soil, respectively.

Morphometric studies were conducted in each phenological phase of development according to the method (Voronin et al., 2015).

To isolate LOX of the rhizome and fronds, plant samples were homogenized in cooled to +4 °C 0.1 M phosphate buffer (pH 6.3), which contained 2 mM phenylmethylsulfonyl fluoride, 0.04% sodium metabisulfite. Homogenate was centrifuged at 4 000 g for

30 minutes at +4 °C using "WPW-310" (Poland) centrifuge. The obtained supernatant was used to determine LOX activity. To construct curves of pH-dependence of lipoxygenase oxidation stationary rates of linoleic acid, 0,1 M sodium-acetate (pH 4.0–5.5), 0.1 M sodium-phosphate (pH 6–8) and borate 0.1 M (pH 8.0–9.5) buffer solutions were applied. A standard reaction mixture to determine LOX activity in the total volume of 2.5ml in the first case contained 100 μM of linoleic acid and 50μL of lubrol in 0.1 M Na – phosphate buffer (pH 6.5), in the second case – 100 μM of linoleic acid in 0.1 M Na – phosphate buffer (pH 7.2). Reaction was initiated by adding 50–100 μl of enzyme solution (protein concentration of 0.5–1.0 mg/ml) and at the constant temperature of 25±0.1 °C. Reaction was observed considering an increase in reaction mixture optical density at λ =235 nm that corresponds to a maximum absorption of conjugate diene chromophore in molecules of linoleic acid hydroperoxide whose molar extinction coefficient is 23000 M^{-1} cm^{-1} (Gibian, Vandenberg, 1987). Kinetic measurements were done using the spectrophotometer Specord M-40 ("Carl Zeiss Jen", Germany). Protein content was measured according to the method (Bradford, 1976).

Biological tests were repeated twice and analytical assessment thrice. When constructing kinetic dependence curves, we used average values of V_{st}, which were determined in three measurements (difference between values was not more than 5%). Findings were statistically processed using Student t-test, difference at $p \leq 0.05$ was regarded as statistically reliable.

Results and discussion

Morphometric analysis showed that the weight of one plant during sporophyte ontogenesis was in the range of 130–150g. Frond length varied from 11 to 85 cm. Diameter of the rhizome overground part was 9-10 cm. In the stage of spores' release and during the summer vegetation on one plant there were 7 fronds, and in the stage of vegetation completion termination – 18. The weight was the greatest in the phase of spore release. Following spore release and at the beginning of vegetation completion, the frond weight diminished almost twofold. An intensive elongation of fronds was observed prior to the start of spore release (table 1).

Table 1. Morphometric analysis of *D. filix-mas* fronds at different phenological phases of development, (mean and standard deviation, n = 6)

Stage	Weight, g	Length, cm
intensive growth	2.6+0.1	11.4+0.6
sori ripening	14.1+0.7	64.4+3.2
spores release	**19.3+1.0**	**84.1+4.2**
summer vegetation	10.4±0.5	82.0±4.1
vegetation termination	9.4±0.5	82.3±4.2

When fronds unfold and grow, the number of segments of the first order increased from 39 to 47 pairs. Frond length in the stage of summer vegetation increased from 4.3 to 11.2 cm (9 pairs), and then it gradually decreased from 10.2 to 0.1 cm (39 pairs) that resulted in the formation of a lanceolate lamina (Fig. 1). The frond abaxial surface had open greyish-brown sporangia. In the stage of vegetation completion, the fern had 18 fronds, 12 of which remained green, while the rest dried and turned greyish-brown. The frond lamina contained 47 pairs of segments of the first order whose length increased from 4.5 to 10.5 cm (11 pairs), and then it diminished from 9.5 to 0.05 cm (47 pairs)

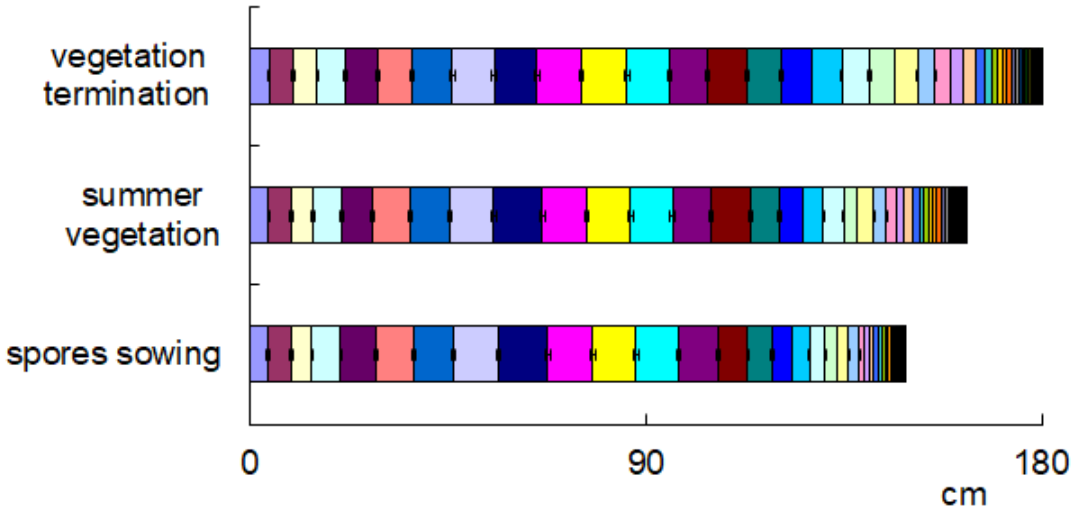

Fig. 1. The length of segments of the first order fronds of *D. filix-mas* at different phenological phases of development (X±SD, n=5).

The rhizome weight was in the range of 110.2–115.7g in all the stages of development except the phase of vegetation completion. An insignificant decrease in the weight of the rhizome during the final stage occurred due to growth cessation and wilting of fronds on its tip as well as due to the die-out of lateral roots (table 2).

Table 2. Morphometric analysis of *D. filix-mas* rhizome at different phenological phases of development (n = 6)

Stage	Weight, g	Length, cm
intensive growth	115.1+5.8	24.6+1.23
sori ripening	114.7+5.8	23.5+1.2
spores release	115.4+5.7	23.6+1.4
summer vegetation	110.2±5.5	22.4±1.1
vegetation termination	95.5±4.8	23.1±1.2

Thus, the morphological analysis showed that plants had successfully passed all developmental phases, reached their normal dimensions, had a mass spore formation, no external signs of suppression and damages, and this was generally consistent with the highest level of life state assessment.

Lipoxygenase activity was for the first time identified in organs of the *D. filix-mas* sporophyte. 13-LOX activity with $pH_{opt.}$ 7.2 was found in fronds and 9-LOX activity with $pH_{opt.}$ 6.5 – in the rhizome (Fig. 2). The analysis showed that during spring vegetation LOX activity in fronds increased reaching its maximum during spores' release phase that was in our opinion due to active metabolic processes during ripening (Fig. 3). Oxylipins – metabolites of the allene oxide synthase branch of the LOX pathway, jasmonic acid and its methyl ester, are known to promote fruit ripening in flower plants (Wasternack, Song, 2016; Babenko et al., 2015b, 2017a).

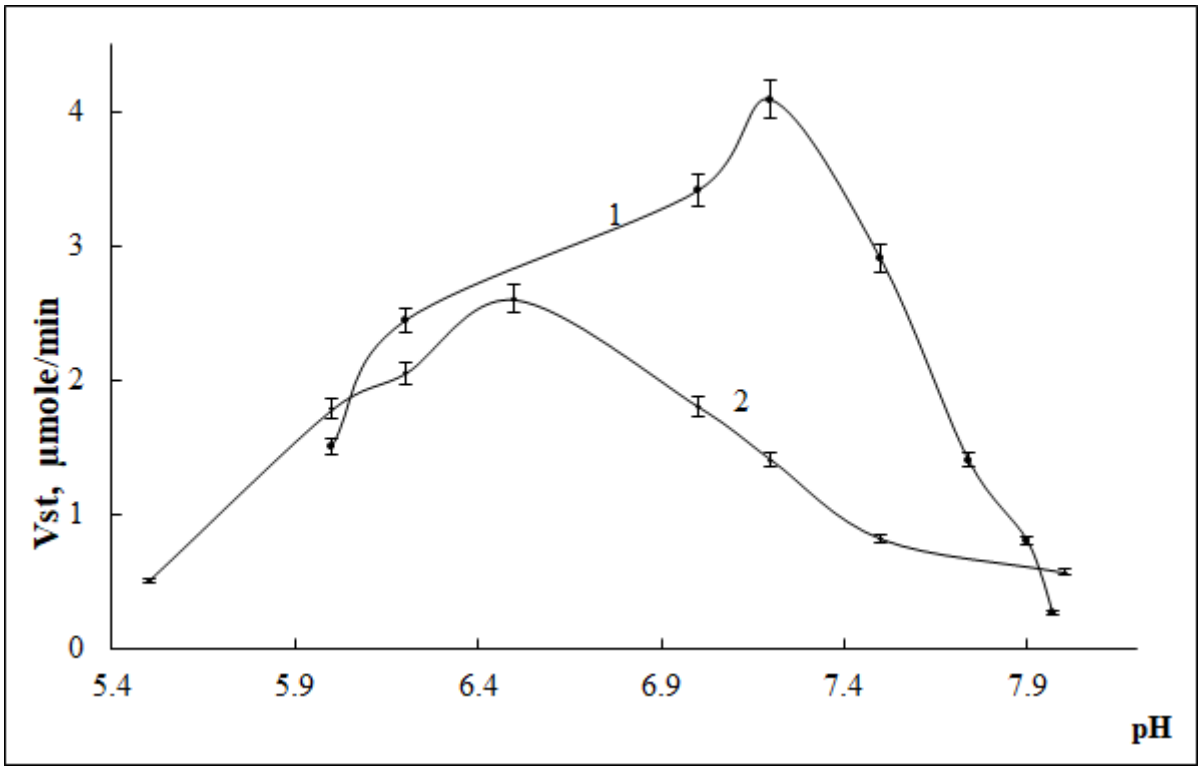

Fig. 2. Dependence of reaction stationary rate (Vst) of linoleic acid oxidation on incubation environment pH in *D. filix-mas* fronds (1) and rhizome (2)

Maximum of 13-LOX activity in fronds was at spore release phase. Following this phase, 13-LOX activity decreased twice and reached its minimum in the phase of vegetation termination. Changes in LOX-activity in the overground part of *D. filix-mas* sporophyte at the different phylogenetic phase and environmental conditions correspond to the data of other researches, who showed the relationship between LOX activity and photosynthesis. Thus, lipoxygenases defend the photosynthetic apparatus under stress conditions by involvement in the non-photochemical quenching of chlorophyll fluorescence through xanthophyll oxidation in the violet xanthine cycle (Chedea, Jisaka, 2013), and through activation of the octadecanoid defense signaling pathways (Schaller, 2001). At the same time, lipoxygenases can oxidize lipids of thylakoid membranes, and that contributes to the degradation of carotinoids and chlorophylls as well as negatively affects the efficiency of energy photochemical application (Radhika et al., 2012).

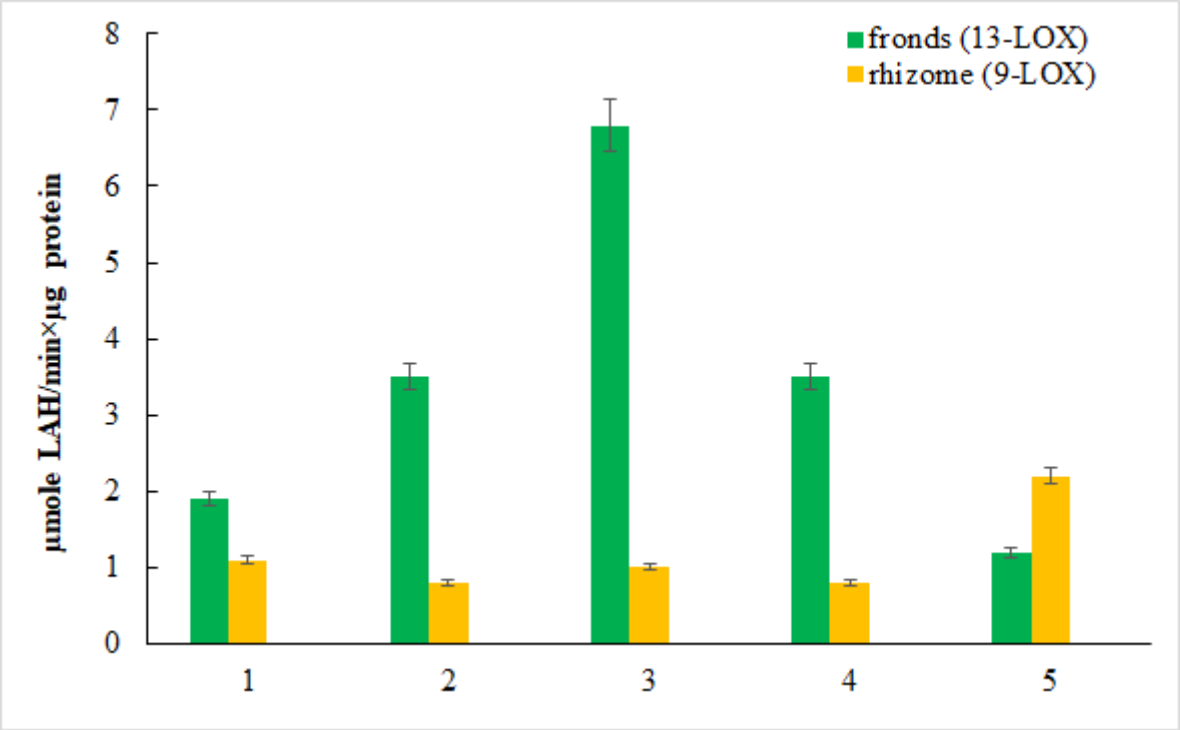

Fig. 3. Distribution of LOX activity in *D. filix-mas* organs in different phenological stages of sporophyte development: 1 – growth intensity; 2 – sori ripening); 3 – spores release; 4 – summer vegetation; 5 – vegetation termination. LAH – linoleic acid hydroperoxide.

The maximum of 9-LOX activity in the rhizome was recorded in the phase of vegetation termination. During the period of spring and summer vegetation until the autumn beginning, 9-LOX activity remained almost unchanged that seemed to relate to the transfer of metabolic activity center from the rhizome to the fern overground part (Fig. 3).

In our previous studies on organs of the vascular cryptogam *Equisetum arvense* L., we identified 13-LOX activity ($pH_{opt.}$ 7.2) in strobiles, internodes and leaves of generative shoots and 9-LOX activity ($pH_{opt.}$ 4.2) in rhizomes and strobiles. In vegetative shoots, on the contrary, 13-LOX activity was detected only in shoots and 9-LOX activity in the rhizome (Babenko et al., 2015c). 9-LOX activity is typical to higher plant root system where the enzyme is localized in cell cytosol. In higher plants, 9-hydroperoxides of PUFA are predecessors of compounds that stimulate synthesis of ketoles, which induce flowering, provide flower coloring, defense and apoptosis of leaves affected by pathogens, and regulate nodules formation (Babenko et al., 2017a). Changes in 9- and 13-LOX activity during *E. arvense* ontogenesis were like those observed in the water fern *Salvinia natans* L. and in the overground part of the evergreen fern *Polystichum aculeatum* (L.) Roth. (Babenko et. al., 2017b). Thus, in the rhizome of *E. arvense,* after spores are shattered (at the beginning of the generative shoot die-off) 9-LOX activity significantly increased while 13-LOX activity, vice versa, decreased. When vegetation of assimilated shoots in *E. arvense* ended, 9-LOX activity was high in rhizomes that corresponded to the formation of starchy nodules (Babenko et al., 2015c). In *S. natans,* a significant increase in 9-LOX activity occurred in the final stage of the sporocarps formation when the sporophyte vegetative part was dead. 9-LOX activity also somewhat increased in submerged fronds at the beginning of the sporocarps formation (Babenko et al., 2016a, 2016b). In *P. aculeatum,* the maximum of 9-LOX activity coincided with the phase of winter vegetation that is might be related to the transfer of the metabolic activity from the overground part to the rhizome (Babenko et al., 2017b). Thus, there is a certain relationship between changes in the activity and localization of the enzyme isoforms and generative organs formation and maturation.

However, it should be noted that the physiological functions of 9-LOX metabolites of PUFA in pteridophytes have not been clearly defined and the most studied are 13-LOX metabolites of C_{18}–PSFA – volatile organic compounds (VOC) (Imbiscuso et al., 2009, Radhika et al., 2012). VOC emission refers to indirect mechanisms of the plant defense from natural parasites (Arimura et al., 2005, Dicke, Baldwin, 2010). VOC, as a rule, function as "alarm signaling". The spectrum of produced VOC depends on a stressor and plant species. Terpenoids that are synthesized as a response to pathogenic effects are most widespread VOC (Halitschke et al., 2008). They are represented by a mixture of mono-, sesqui- and homoterpenes that are synthesized from isopentenyl - or dimethyl diphosphate through cytosol-localized mevalonate (MVA-pathway) or plastid-localized methylerythritol (MEP-pathway) (Arimura et al., 2005). So, *Pteridium aquilinum* fern fronds infection with pathogenic microorganisms *Pteridium aquilinum, Strongylogaster multifasciata* and *Spodoptera littoralis* as well as mechanical damages were associated with a low emission of VOC mixture that were predominantly composed of terpenoids (Radhika et al., 2012). An application of exogenous JA that had been found to be able to induce the synthesis of attractants in flower plants can stimulate the emission of similar substances (Chehab et al., 2010). An exogenous JA treatment of fronds led to an intensive emission of VOC mixture. A treatment with JA predecessors -12-oxo-phytodienoic acid and α-linoleic acids also caused VOC emission although as compared to JA it was less intensive. In angiosperms, terpenoids production resulting from JA treatment is blocked by fosmidomycin and mevinolin – inhibitors of MEP- and MVA-pathways. Therefore, like in angiosperms, the

production of VOC terpenoids in ferns involves JA-sensitive pathways, but their number is less and that indicates that these plants do not have a well- developed mechanism for an indirectly defense from pests and mechanical damages (Radhika et al., 2012). JA exogenous treatment of leaves of *Ginkgo biloba,* that is considered a descendant of one of the oldest groups of fossil seed ferns, resulted in the production of a significant number of VOC (Van Den Boom et al., 2004). However, like *P. aquilinum,* mechanically damaged *G. biloba* showed a low VOC emission (Van Den Boom et al., 2004, Radhika et al., 2012). It appears that the fern-like plants do not need any additional defense involving VOC. The presence of highly toxic indanones, cyanogenic glycosides and tannin in fern fronds may be sufficient to prevent plant mechanical damages inflicted by animals.

Conclusions

We identified two isoforms of lipoxygenase – 13-LOX and 9-LOX and their catalytic activity in organs of the leptosporangiate fern *D. filix-mas.* The pattern of this enzyme activity distribution in fronds and the rhizome at the various phenological stages of development was established. We suggested that the enzyme might be involved in the regulation of lipid metabolism of growth processes that ensure plant adaptation to the environment due to peculiarities, revealed in the localization and dynamics of the catalytic activity the LOX isoforms in fern organs,

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