ENDOGENOUS PHYTOHORMONES BALANCE DURING PERENNIAL FERN *D. filix-mas* SPOROPHYTE GROWTH AND DEVELOPMENT

I.V. Kosakivska, L.V. Voytenko *, V.A. Vasjuk

M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, Ukraine

**Abstract.** We analyzed the endogenous phytohormones composition and dynamics in the fronds and rhizomes of perennial fern *Dryopteris filix-mas* using high-performance liquid chromatography in combination with mass spectrometry. Samples were selected at the following phenological phases: an intensive growth (30.04), sori ripening (29.05), spores release (17.06), summer vegetation (10.07) and vegetation termination (21.10). It was shown that the content of indole-3-acetic acid (IAA) and abscisic (ABA) acids in fronds was higher that in rhizomes. Conjugated IAA accumulated in rhizome during spring vegetation at the phase of intensive growth. In contrast, the active form of ABA was dominant in fronds. During the development of sori, spores maturation and their falling, the quantity of free IAA and ABA in fronds increased. At the end of the vegetation the content of IAA and ABA reached its maximum with the predominance of conjugated IAA in fronds and rhizomes, and free ABA – in fronds. The maximum content of free gibberellic acid (GA3) was in fronds at the phase of active metabolism, whereas the higher level of conjugated form was in rhizomes in the autumn vegetation phase during the sporophyte transition to dormancy. The obtained results indicated that support of the auxin homeostasis during the transition from intensive growth to the formation and maturation of spores in sori and at the end of the vegetation involved conjugation mechanisms, and in the period of mass falling of mature spores – metabolic strategies of biosynthesis / catabolism. The dynamics and localization of GA3 showed that hormone was involved in the regulation of spores development and maturation. The character of the ABA accumulation indirectly confirmed the involvement of the hormone in the activation of protective adaptation processes in the autumn vegetation.

**Keywords:** *Dryopteris filix-mas*, indole-3-acetic acid, abscisic acid, gibberellins, sporophyte, growth, development.

**Corresponding Author:** Lesya Voytenko, M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 01601, Tereshchenkivska st. 2, Kyiv, Ukraine, e-mail: lesyavoytenko@gmail.com

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**Abbreviations:**
- IAA – indole-3-acetic acid
- ABA – abscisic acid
- GA – gibberellic acid
- HPLC-MS – high performance liquid chromatography-mass-spectrometry

1. **Introduction**

Phytohormones are low molecular weight organic compounds, which in small quantities are synthesized in cells and are involved in the regulation of growth and development processes. With the help of physiological, biochemical, and genetic approaches, eight types of phytohormones have been identified, including auxins, gibberellins, cytokinins, abscisic acid, ethylene, jasmonic and salicylic acids, and brassinosteroid. The presence of phytohormones in the representatives of different taxa,
the uniformity of their main structural elements indicate that these compounds appeared at the earliest stages of evolution, but the emergence of individual classes of phytohormones was not simultaneous (Ross, Reid, 2013).

Auxines, gibberellins and abscisic acid belong to the classical phytohormones. Auxines control embryo-, organ- and morphogenesis, apical dominance, vascular differentiation, polarity of organs, development of the root system, seeds and fruits (Enders, Strader, 2015). Gibberellins, which have more than 130 forms, are involved in the regulation of seed germination, coordinate cells division and their extension, determine the sex and induce blossoming of flowering plants (Gantait et al., 2015). ABA is a recognized stress hormone, and stress-induced accumulation of ABA is considered as a component of the protective mechanism aimed at slowing down metabolism and adaptation to the influence of abiotic and biotic stresses (Voytenko & Kosakivska, 2016). Genome sequencing of different plants made it possible to trace the origin and development of phytohormones signaling systems (Simm et al., 2016). However, there is not enough information for conclusions about the evolution of phytohormones. The hormonal system of plants with different systematic position, studying the dynamics of hormonal balance during the life cycle, localization of free and conjugated forms in vegetative and generative organs, comparing such data with the speed and direction of growth processes needs further analysis. Vascular cryptogams are in this respect the least investigated group of plants. The extremely interesting and evolutionarily ancient are ferns. However, for today, most researches are aimed at studying the influence of exogenous phytohormones on their growth and development (Kosakivska et al., 2016). At the same time the research results presented in modern scientific literature on endogenous phytohormones in pteridophytes are fragmentary. Thus, the endogenous IAA was identified in Davallia trichomanoides (Croxdale, 1976), Asplenium nidus (Menéndez et al., 2011) and Salvinia molesta sporophytes (Arthur et al., 2007). GA was studied in the sporophytes of Cibotium glaucum and Dicktonia antarctica (Yamane et al., 1988). ABA was identified in spores and protonema of Anemia phyllitidis (Cheng, Schraudolf, 1974) and Lygodium japonicum (Yamane et al., 1980), and in the water fern Marsilea quadrifolia sporophyte (Lin et al., 2005). In previous studies, we analyzed the peculiarities of the accumulation and localization of endogenous IAA, GB and ABA in the sporophyte vegetative and generative organs of the water fern Salvinia natans (Vasyuk et al., 2016; Voytenko et al., 2016) and terrestrial fern Polystichum aculeatum (Voytenko & Kosakivska, 2017). Since the synergistic and antagonistic nature of the phytohormones interaction, as well as regulation of their biosynthesis and distribution in organs and tissues of plants play a crucial role in ontogenesis and adaptation, the purpose of this our work was to investigate the balance of IAA, ABA and GA, the patterns of their accumulation and localization in the vegetative and generative organs of Dryopteris filix-mas at different phonological phases of development.

2. Materials and methods

The study was conducted on the fern Dryopteris filix-mas (L.) Schott., growing on the cryptogam expositional site of the Botanical Garden of the Taras Shevchenko National University of Kyiv. The plant material was sampled during reproductive and post reproductive periods in 2015. According to the Hydrometeorological Information of the Boris Sresnevsky Central Geophysical Observatory an average temperature
during the summer was +21.6°C that exceeded the climatic norm by 2.9°C. During the summer, 68 mm of precipitation fell, which was 30% of the annual norm. Samples for analysis were taken in the next phases: intensive growth (30 April), sori ripening (29 May), spore release (17 June), summer vegetation (10 July) and vegetation termination (21 October). In the phase of intensive growth fronds had a snail-like shape. Fronds acquired a typical view in the phase of sori ripening, following their unfolding and straightening of the tops. The unfolded first couple of fronds contained immature young white sori formed in the rudimentary fronds. In the spore release phase, on the frond abaxial surface, there was a cluster of mature closed black-brown sori covered with indusia. In the phase of summer vegetation, sporangia acquired a brown color, their shells were broken, indusia curtailed, and spores began to massively pour off. In the phase of the vegetation termination, the destruction and drying of the fronds containing dried sporangia occurred. For revealing of the characteristic signs of sporophyte ontogeny in each age group at different phenological phases, 5-6 individuals were studied. The assessment of the vitality of the fern was determined according to (Voronin et al., 2015).

Phytohormones were extracted with 80% ethanol with the addition of antioxidant (0.002% sodium diethyl dithiocarbamate) (Kosakivska et al., 2014; The methodical recommendations, 1988). The isolation of IAA, ABA and gibberellic acid (GA₃) from an aliquot of the aqueous residue was carried out after freezing at −40°C and centrifugation in a Janetski (Germany) K-24 centrifuge at 15,000 rpm. for 15 minutes. ABA and IAA were determined after acidic-alkaline re-extraction of the aqueous residue and additional purification by thin-layer chromatography on Silufol UV-254 (Merck, Germany) plates in a chloroform solvent system: ethyl acetate: acetic acid (70: 30: 5), GC₃ - ethylacetate: chloroform : acetic acid (90: 60: 1). The final analysis of the qualitative composition and quantitative content of IAA, ABA and GA₃ was carried out using a liquid chromatograph on Agilent 1200 LC with diode-matrix detector G 1315 B (USA), column Eclipse XDB-C 18 4.6 x 250 mm, particle size of 5 μm and using mass selective detector with combined ionization source (MM-ES+APCI) model 6120. The chromatographic separation of IAA and ABA was done in the UV absorption region at an analytical detection wavelength of 280 and 254 nm, respectively, at a mobile phase speed of 0.5 ml / min. in the solvent system, methanol: ultra-pure water: acetic acid (40: 59.9: 0.1), GC₃ - in the solvent system acetonitrile: ultra pure water: acetic acid (30: 69.9: 0.1) in the UV absorption region at a wavelength of 210 nm.

Hormones were detected using a mass-selective detector in modes SIM (mass 264) and Scan (in the range of 100-300 bands) in Negative Polarity with a voltage of 70 V for fragmentors. For identification, unlabelled IAA (Sigma, USA), (±) cis-, trans-ABA (Sigma, USA), GA₃ (Sigma, USA) were used. Chromatograms were analyzed and processed using the Chem Station software version B.03.01 in off line mode. Experiments were conducted in triple biological and analytical repetitions. The results were statistically processed (P ≤ 0.05) using Microsoft Excel 2007 and Origin 6.0.

3. Results and discussion

Biometric studies. The perennial rhizome of D. filix-mas is short, thick, black and brown, obliquely upright, on the apex densely covered with elongated-elliptical, extended tips, light-bronze with darker base, with soft scales and residues of leaf
petioles (Fig. 1 A, B). The roots are adventitious. In young plants, the rhizome is located vertically, in the old – horizontally. On the underside rhizomes have 20-80cm long adventitious roots. At the apex of the rhizome there is a crown of large erect fronds with a bipinnate of plate, which make a funnel-like bundle (Fig. 1).

Fig. 1. Dryopteris filix-mas three years-old sporophyte with rhizome at the reproductive stage of development (A) and young snake-like fronds in phase of intensive growth (B)

Fig. 2. The general view of Dryopteris filix-mas frond in the sori ripening phase
Fronds consist of a short petiole, covered with brown scales and hairs, and a long plate. The plate is elongated, elliptic and narrowed to the base, biconvex, soft. Its adaxial surface has a light green color, abaxial one is green. The rachis is greenish-brown, with the medial veins covered with bronze-brown elongated scales (Fig. 2). Segments of the first order (feathers) on the truncated petioles are linear-lancet, sharp at the top, arranged alternately. Segments of the second order (feathers) are elongated, seated, with jagged edges, are rounded at the apex, in the basal part are narrowed. A frond lives 220-225 days (Vasheka & Bezsmertna, 2012).

On the abaxial surface of segments of the second order, rounded sori are formed and develop. They are located in two rows of 4-6 pairs along the edge of the middle vein of feathers, occupying 2/3 of fronds. Numerous sporangia are covered with round-shaped, filamentous, whole-coverings (indusia), attached to the radial groove. When spores have ripen and start falling down, indusia are loosely curtailed (Fig. 3).

Fig. 3. Young (A) and mature (B) covered with indusia and grouped in sori sporangia located on the abaxial surface of the *Dryopteris filix-mas* frond. Notation: sor. – sori, ind. – indusia

The spores have a kidney-shaped form with comb and truncated warts all over the surface (Fig. 4).
We found that the weight of one plant during ontogenesis was within 130-150 g, the length of fronds varied from 11 to 85 cm. The diameter of the above-ground part of the rhizome was 9-10 cm. In the phase of spore ripening, the fronds number was seven, while in the phase of vegetation termination – eighteen.

The largest weight of fronds was in the phase of spore release. After spores falling down and at the beginning of vegetation termination, the frond weight decreased almost twice and for a long time remained at one level. An intensive frond elongation occurred before the beginning of the spore release. Subsequently, no increment of frond length was observed (Fig. 5). As was shown before during frond unfolding and growth the number of segments of the first order increased from 39 to 47 pairs. In the phase of vegetation termination at the beginning of the above-ground part dying the number of fronds of one fern reached 18, only 2/3 of them remained green, other were dry and had a brown color. The frond plate contained 46 pairs of first-order segments (Babenko et al., 2018).

The weight of perennial rhizomes in *D. filix-mas* at all phenological phases of sporophyte development was in the range of 110.2 - 115.7 g. A slight decrease in the weight of the rhizome in the phase of the vegetation termination was due to the cessation of growth and wilting of the rhizomes located at the frond rhizome apex as well as lateral roots die-off (Fig. 5).

Consequently, the increase in the size and weight of *D. filix-mas* occurred due to the growth of fronds. After spores falling down the weight of fronds decreased. Dimensions and weight of rhizomes practically did not change. Biometric analysis revealed that the plants successfully passed all phases of development, reached normal size, damage were absent, which in general corresponded to the highest level of the plant state assessment.
Phytohormones balance. Accumulation and distribution of IAA. In the phase of intensive growth, the pool of endogenous IAA in the rhizome reached 134.5±6.7 ng/g of fresh weight, twice the hormone amount in fronds. During the sori ripening there was a slight decrease in the hormone content in fronds, but in the after ripening phase the level of endogenous IAA increased twice and amounted to 123.0±6.2 ng/g of fresh weight. At the same time, the IAA pool in the rhizome significantly decreased and remained low at the time of summer vegetation. The maximum amounts of IAA – 226.6±11.3 and 126.6±6.3 ng/g of fresh weight, respectively, were recorded at the final phase of sporophyte development in fronds and rhizomes. The conjugated form of the IAA was dominant in fronds at all phases of development, except the summer vegetation phase. The content of free IAA in fronds increased at the beginning of the spore release phase and reached the maximum (73.5±3.7 ng/g) during summer vegetation – the period of spores mass falling down (Fig. 6).

Fig. 5. Biometric studies of Dryopteris filix-mas fronds and rhizome. A – fresh weight, g; B – length, cm. X ± SD, n = 6

Fig. 6. IAA accumulation in the organs of Dryopteris filix-mas sporophyte at various phenological phases of development
The maximum accumulation of conjugated IAA was recorded in the rhizome in the intensive growth phase (90.1±4.5 ng/g of fresh weight) and at the end of the vegetation (116.4±5.8 ng/g of fresh weight). At the beginning of the spores falling down at the stage of summer vegetation the amount of conjugated form of the hormone decreased by half and reached the minimum value. The character of free IAA accumulation in rhizomes corresponded to the physiological state of the organ. Thus, in the phase of intensive growth a maximum was recorded, while in the final phase of sporophyte vegetation – a minimum in the contents of the free form of the hormone (Fig. 6).

As was shown by other researchers enzymes, receptors and protein-transporters, which involved in IAA biosynthesis, localized in chloroplasts, cytoplasm, endoplasmic reticulum membranes and mitochondria of apical meristem cells of the stem, as well as in young leaves cells, whereas the actual IAA and its metabolites were transported and stored in apoplast and vacuoles of stem, leaves and root cells (Ludwig-Müller, 2011; Robert, Friml, 2009; Woodward & Bartel, 2005). It has been established that AUX1 and PIN1, PIN2/AGR/EIR1 protein-transporters are involved in the basipetal transport of auxins from apex to root (Simm et al., 2016) The regulation of auxin homeostasis also occurs with the help of hormone transport inhibitors, which bind proteins that interact with the auxin exporter (Muday & DeLong, 2001; Enders & Strader, 2015).

After release, conjugated IAA, accumulated in the rhizome during the emergence of the snail-like shape fronds, can be involved in the processes of organ- and morphogenesis of fronds, which are formed at the apex of the rhizome, just as it occurs in higher plants (Scarpella et al., 2011). "Auxin spike" in the accumulation of free IAA, which was observed in fronds at the end of the spores ripening and reached the maximum during summer vegetation, indirectly confirms the involvement of the hormone in the growth regulation, the active course of which is indicated by the results of biometric studies (Fig. 5). The accumulation of the active form of IAA in mature spores was a similar phenomenon of the hormone accumulation by the higher plant seeds Glycine max, Sechium edule, Zea mays, Helianthus annuus and Acer saccharium (Sitnik et al., 2003). It was shown that regulation of morphogenesis involves concentration gradients that are formed during biosynthesis, conjugation and degradation of auxin (Normanly, 2010; Spiess et al., 2014), as well as through intercellular (Petrásek & Friml, 2009) and intracellular hormone distribution (Mravec et al., 2009). IAA gradients are thought to play a key role in plant aging (Ellis et al., 2005). Some increase in the content of the conjugated IAA in D. filix-mas organs observed during the vegetation can be attributed to a change in the ratio between the synthesis and conjugation of the hormone. Given that the auxin conjugates are associated with the development of higher plants (Cooke et al., 2002; Sztein et al., 1999, 2000), it can be assumed that the immobilized IAA from fronds is transported to the rhizome, where it is stored and then after release (hydrolysis) in spring may be involved in the growth and development of rudimentary fronds, the formation of lateral roots on the rhizomes and lateral roots growth.

Thus, the conjugated hormone was involved in maintaining auxin homeostasis in fronds during the growth and development of D. filix-mas sporophyte. The intense accumulation of free IAA in fronds during a massive spores falling down and in the rhizome during the formation of the rudimentary fronds coincided with the activation of physiological processes in the fern organs.
Abscisic acid. During intensive growth and summer vegetation phases, the pool of endogenous ABA in fronds reached 31.2±1.6 and 49.4±2.8 ng/g of fresh weight. During the sori ripening phase, the hormone amount decreased and the content of ABA reached a minimum of 2.2±0.1 ng/g of fresh weight in the spore release phase. The maximum in hormone amount 219.5±11.0 ng/g of fresh weight was observed in the phase of vegetation termination (Fig. 7). During the first two phenological phases of development, the pool of endogenous ABA in the rhizome was within 20 ng/g of fresh weight. The maximum in the accumulation of the hormone (30.6±1.5 ng/g of fresh weight) took place during the spore release phase, when on the surface of sori occurred funnel-shaped coagulation of indusia. During summer vegetation and especially in the phase of vegetation termination, the content of ABA in the rhizome decreased two and three times, respectively (Fig. 7).

The free form of ABA was dominant in fronds at all phenological phases of development. The maximum was recorded at the end of vegetation when the growth of the above-ground organs slowed and the weight and length of fronds decreased (Fig. 7; Fig. 5). During the spore release phase, the amount of free ABA reached the minimum. In the summer vegetation phase, the free form of the hormone increased twenty times. In all the phases of sporophyte development, fronds showed the low and trace amounts of the conjugated ABA (Fig. 7).

The free form of ABA also dominated in rhizomes in all phases of development except the last one, when the conjugated form of the hormone was dominant. Free form content remained practically unchanged during the first four phases of sporophyte development. The minimum of the conjugated form of ABA was found in the rhizome in the summer vegetation phase, and its maximum when the vegetation was completed. The highest level of endogenous ABA accumulation detected in the rhizome during the spore release phase that was accompanied by weight gain and an increase in the length of roots (Fig. 5, Fig. 7), indirectly indicated the involvement of the hormone in regulating the growth of the root system.

The obtained results suggest that the accumulation of free ABA in *D. filix-mas* fronds at the end of vegetation is associated with involvement of this hormone in
regulation of the aging process. The reason for such an assumption is the study of other authors who reported that ABA stimulated aging processes (Wang et al., 2012). However, we think that the hormone conjugated form, accumulated in the rhizome, generates a depot for further synthesize active form of ABA. From literary sources ABA conjugates are known to accumulate in the cell wall and vacuoles (Verslues & Zhu, 2005). From cytosol of root cells, with the involvement of ABA-transporters, they are transferred to the xylem parenchyma cells of the stem and released in vessels (López-Carbonell et al., 2009). Due to the hydrophilic properties, the conjugates move acropetally along the stem xylem. In leaf apoplasts they are cleaved with the formation of free forms and transported to the mesophile (Osakabe et al., 2014).

Thus, the active ABA dominated in fronds in all the phenological phases of D. filix-mas development. The maximum content of the free hormone in fronds following the first frost is the basis to assume that ABA is involved in the formation of protective adaptation processes under low temperatures. The dominance of the conjugated hormone in the rhizome of sporophyte at the end of vegetation is considered as ABA depositing.

Gibberellins. Domination of free GA$_3$ was observed for the whole period of fronds intensive growth and during the formation of spores (Fig. 8). At the end of vegetation, the amount of free GA$_3$ in fronds was low and that coincided with the beginning of these organs die-off. At the same time, the accumulation of conjugated forms of the hormone was detected in rhizomes (Fig. 8). In general, the content of endogenous GA$_3$ during the summer vegetation, when sori with spores were formed and developed on the frond abaxial surface, was significantly higher than that of endogenous IAA and ABA. The obtained results suggest that the key role in the formation and development of reproductive structures and spores in D. filix-mas belongs to gibberellins.

Fig. 8. GA$_3$ accumulation in the organs of Dryopteris filix-mas sporophyte at various phenological phases of development

It is known that plants of different systematic groups differ in the gibberellin spectrum, which is not static and varies in the process of ontogenesis, and the pattern of
the hormone action depends on its amount and is determined by the species of the plant and the phase of ontogenesis (Sitnik et al., 2003). In the early stages of development, gibberellins control meristematic growth and cell elongations, and in the later ones, they are involved in the formation of generative organs (Mutasa-Göttgens & Hedden, 2009). GA\textsubscript{1} and GA\textsubscript{3} are responsible for the active growth of young organs, whereas GA\textsubscript{5} – for being in dormancy (Phinney & Spray, 1982; Ross et al., 2000; Gaskin et al., 2001; Lewis et al., 2004). Free form of GA\textsubscript{9} affects the growth of wheat stems (Karnachuk et al., 2003), and exogenous GA\textsubscript{3} stimulated the elongation of eucalyptus internodes, which were in active growth state, but did not affect the internodes, whose growth was discontinued (Seo et al., 2006). The increase in the gibberellic acid content in the organs of \textit{D. filix-mas} corresponded to the dynamics of growth processes, and the high level of phytohormone at the stages when spores developed indirectly indicates that it is involved in the regulation of the reproductive development of ferns.

4. Conclusion

Our study showed that the content of IAA and ABA in fronds of fern \textit{Dryopteris filix-mas} was higher than in rhizomes. In the phase of intensive growth, the accumulation of conjugated IAA with domination in rhizome occurred. However, the active form of ABA dominated in fronds. During the sori development, maturation of spores and their falling (the phase of summer vegetation), the amount of free IAA and ABA increased. In the phase of vegetation termination, the IAA and ABA content reached the maximum with the predominance of conjugated IAA in fronds and rhizomes, and the free ABA – in the fronds. An increase in the content of free ABA in fronds after the first frost indicates that the hormone is involved in activation of protective processes. The maximum content of free GA\textsubscript{3} corresponded to the phases of active metabolic processes in fronds while in the vegetation termination phase during the sporophyte transition to dormancy, the maximum content of the hormone conjugated forms was in rhizomes. The high level of GA\textsubscript{3} at the stages when spores developed indirectly indicates that it is involved in the regulation of the reproductive development of ferns. The regularities of quantitative and qualitative changes revealed similarities in the functions of the main phytohormone classes in the fern \textit{D. filix-mas} and higher flowering plants.

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References


