DOI https://doi.org/10.30525/978-9934-26-086-5-7

ENDOGENOUS AUXIN AND ABSCISIC ACID IN THE ORGANS OF THE *EQUISETUM HYEMALE* L. SPOROPHYTE IN ONTOGENESIS

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INTRODUCTION

Phytohormones play an important role in the regulation of various physiological and metabolic processes, the effects of which are determined by the concentration in organs and tissues and interhormonal interaction[1]. The presence of phytohormones in representatives of different taxa, the unification of their main structural elements indicate that these compounds arose in the early stages of evolution, but the emergence of separate classes of phytohormones was not simultaneous[2].

The most common auxin, the presence of which is found in plants of different systematic groups, is indole-3-acetic acid (IAA)[3]. The free active form of auxin together with the conjugates form an IAA pool. Bound forms support active auxin homeostasis and may perform a transport function[4]. Biosynthesis, conjugation, degradation / oxidation, and transport, as components of hormone metabolism, proceed in concert and modulate auxin gradients in plant[5,6]. IAA plays an important role in regulating plant growth and development. It activates cell division and

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elongation, stimulates the formation and supports the growth of lateral roots, is involved in protein biosynthesis, controls the development of vascular tissues, vegetative and reproductive organs, is involved in tropisms and apical dominance, the formation of stress resistance[7,8].

Abscisic acid (ABA) is a recognized stress hormone, and its stress-induced accumulation is considered as part of a protective mechanism aimed at slowing metabolism and adaptation to the effects of stressors[9]. The content and distribution of the hormone in the cells, tissues and organs of the plant are crucial in determining its effects[10]. The active isomer of *cis*-ABA is dominant in higher plants, and the main conjugate is glucose ester of ABA. This immobile form accumulates in vacuoles from where it is transported to other compartments of the cell and is a source of recovery of the pool of free form of hormone[11].

The division Equisetophyta is represented by one genus Equisetum, which has nine species in the flora of Ukraine. Typical representatives of horsetails include *Equisetum hyemale* L. – isosporous, perennial herbaceous plant with evergreen phenorhythm of development, up to 1 m tall. The species is distributed sporadically, in small aggregations throughout Ukraine, except the southern steppes and highlands. Grows in pine and mixed forests, ravines and dry meadows, forms dense thickets on the banks of forest streams[12]. A characteristic feature of this species is the simultaneous

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development of two types of shoots of different ages in one group (clone) of plants – annual reproductive and perennial vegetative. Reproductive shoots of the first year of sporophyte development contain strobiles at the apex, in the sporangia of which spores are formed. Instead, the vegetative shoots of the second year do not have strobiles and perform only photosynthetic and accumulative functions[13]. The aim of this work was to investigate the dynamics and distribution of endogenous phytohormones IAA and ABA in the organs of sporophyte of wintering horsetail (*Equisetum hyemale* L.) at different phenological phases of development.

1. Materials and methods of research

Reproductive shoots of the first and vegetative shoots of the second year of development with reduced leaves, strobiles and rhizomes of *E. hyemale*, which grew in the exhibition area of higher spore plants of the Academician O. V. Fomin Botanical Garden of Taras Shevchenko Kyiv National University (Kyiv, Ukraine). Plant material was selected during 2014–2015 in the reproductive and post-reproductive periods at six phenological phases of sporophyte development – pre-spring awakening (February), germination of reproductive shoots (April), intensive growth of reproductive shoots of the first year of development (June), summer vegetation, autumn sporulation (September) and dormancy (November). Along with the reproductive shoots of the first year of development, sterile (vegetative) shoots of the second year of development were present in all phases of the study. Perennial evergreen *E. hyemale* is characterized by the presence of one type of shoot, and sporulation occurs twice a year – in spring and autumn. Characteristics of the phases of horsetail development and morphometric studies are detailed in work[14].

Phytohormones were extracted three times with 80 % ethanol with the addition of antioxidant (0.002 % sodium diethyldithiocarbamate) for 24 hours. The combined extracts were evaporated to an aqueous residue and frozen at + 4°C. Isolation and purification of IAA and ABA were performed after acidification of aqueous residues of 2h HCl to pH 3.0 and centrifugation on a centrifuge K-24 company Janetski (Germany) at 15 000 rpm. for 30 minutes. Phytohormones were determined after acid-

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base re-extraction of the aqueous residue and purification by thin layer chromatography on plates Silufol UV-254, Merck (Germany) in a system of solvents chloroform, ethyl acetate, acetic acid in a volume ratio of 70: 30: 5[15].

Analysis of the qualitative and quantitative content of IAA and ABA was performed by high performance liquid chromatography – mass spectrometry (HPLC-MS) on Agilent 1200 LC liquid chromatograph with diode-matrix G1315B (DAD) and single-quadrupole mass-selective G6120A (MSD) detectors on Eclipse XDB-C18 column 4.6 × 250 mm with a particle size of 5 μm with a mobile phase rate of 0.5 ml/min in the solvent system, methanol water, acetic acid in a volume ratio of 40: 59.9: 0.1. Unlabeled IAA and ABA from Sigma (USA) were used to identify phytohormones. Recording of IAA spectrograms on DAD was performed in the UV region at a maximum absorption at a wavelength of 280 nm, followed by identification by mass spectrum equipped with a combined ionization source (MM-ES-APCI). IAA detection on a mass-selective detector was performed in SIM and Scan modes in Positive Polarity (registration of positively charged M^+ ions) in the mass range 100–300. Elution of ABA was performed at an analytical wavelength of detection of 254 nm. Analysis and processing of chromatograms was performed using Chem Station software version B.03.01 off line. The experiments were performed in triplicate biological and analytical replicates, and the results were statistically processed using the computer program Statistica 6.0. One-way analysis of variance was used, the differences between the mean values were calculated by the ANOVA criterion, which was considered probable by $P \leq 0.05$[16].

2. Results and discussion

2.1. Indole-3-acetic acid in the organs of the sporophyte *Equisetum hyemale* at different phenological stages of development

As a result of the researches it was established that in the rhizome in all phases, except for autumn sporulation, the conjugated form of IAA quantitatively prevailed (Fig. 1).

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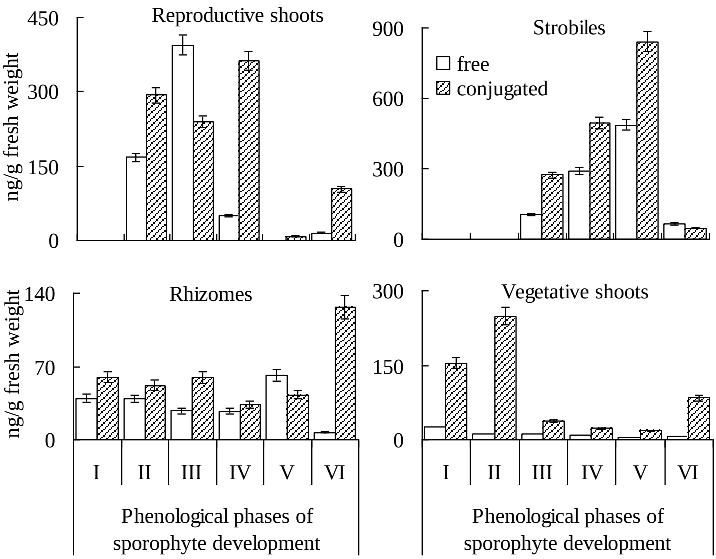

Fig. 1. Distribution of free and bound forms of IAA in the organs of *Equisetum hyemale* L. at different phenological phases of sporophyte development, ng/g FW. Designation of phases: I – pre-spring awakening, II – germination of the reproductive shoot of the I year of development, III – intensive growth of the reproductive shoot, IV – summer vegetation, V – autumn sporulation, VI – dormancy

The maximum accumulation was detected in the phase of winter dormancy (126.7 ± 6.3 ng/g FW), the minimum – during the spring sporulation (33.7 ± 1.7 ng/g FW). Instead, the amount of active free form of IAA during the development of horsetail in the spring-winter period gradually decreased and reached a minimum in the phase of dormancy – 7.1 ± 0.35 ng/g FW. The exception was the phase of autumn sporulation, when single elongated nodules began to form on the lateral roots, and the content of the active form of the hormone reached the level of 61.8 ± 3.09 ng/g FW.

During the growth of reproductive shoots of the first year of development there was a gradual increase in the content of free IAA to the summer growing season, varying from 167.1 ± 8.36 to 394.1 ± 19.71 ng/g FW, after which the hormone level significantly decreased – to trace amounts in the phase of autumn sporulation and to 14.7 ± 0.74 ng/g FW, in the phase of dormancy (Fig. 1). Conjugated IAA dominated all phases of development of reproductive shoots, except for summer vegetation, when its content was 362.7 ± 18.14 ng/g FW. In the autumn, during the maturation of spores in strobiles, the content of the immobile form of IAA was minimal (8.1 ± 0.41 ng/g FW). Significantly lower amounts of both forms of the hormone were found in vegetative shoots than in reproductive ones. The level of IAA gradually decreased, reaching a minimum in the phase of autumn sporulation – 4.5 ± 0.22 ng/g (free form) and 18.7 ± 0.93 ng/g FW (bound form) (Fig. 1). The ratio between the forms of the hormone during the growing season decreased mainly due to a decrease in the content of the inactive form. Thus, in the winter-spring period (phases I and II) the content of bound IAA was 84 and 95 % higher than the content of the active form of the hormone, while in the summer (phase III and IV) the period decreased to 69 and 57 %. In the phase of forced winter dormancy, conjugated IAA accumulated in vegetative shoots, the content of which was 91 % higher than the content of the free form of the hormone. It is likely that the accumulated bound form of IAA in the spring during the germination of horsetail becomes a source for the restoration of the active form of the hormone.

Autumn strobiles were characterized by an increase in the content of both forms of the hormone. The content of immobilized IAA was 72.7 % higher in the phase of autumn sporulation and 63.5 % and 71 % during the spring formation of strobiles and summer vegetation (Fig. 1). During the autumn ripening of spores, the amount of active and inactive forms of auxin was the highest and reached 487.5 ± 24.37 and 842.4 ± 42.12 ng/g FW, respectively. In the dormancy phase, when the spores from the sporangia erupted, the IAA level decreased significantly, and the ratio between the forms shifted towards the free form, the content of which was 1.4 times higher than the bound.

It has been established that during somatic and early embryogenesis two different ways of auxin synthesis are sequentially activated. The tryptophan-dependent pathway in the early stages of embryonic development is the source of the formation of a significant amount of the free form of IAA, which regulates cell division during embryo formation. This is followed by switching to the tryptophan-independent pathway of synthesis, which provides homeostatic control over the level of the free form of hormone[17]. Functional activity and ways of regulation of IAA

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levels in representatives of vascular spore plants and chorophytes are similar to those in seed plants[18,19]. IAA was identified in the rhizome and aboveground organs of the fern *Psilotum nudum* during and after sporulation[20]**.** In the water fern *Salvinia molesta*, the IAA content reached very high values and amounted to 393 pM/g DW[21]. The effects of natural and synthetic auxins and their inhibitors on embryogenesis and formation of axial organs of the fern *Marsilea vestita*[22]. The polar transport of auxins in representatives of mosses *Pellia epiphylla* and *Polytrichum ohioensis*[23] was studied. Auxin enhanced the differentiation of caulonema and rhizoids in mosses[24]. Sequencing of the *Physcomitrella patens* moss genome revealed a number of genes responsible for the synthesis of proteins involved in maintaining auxin homeostasis, which were similar to those of flowering plants[25]. Due to the increased synthesis of IAA on the abaxial surface of young weevils, almost all ferns have the so-called folded leaf folding (snail-like twisting). Gradually, the synthesis of auxin on both surfaces is leveled, and the wax is straightened [26]. Earlier we found that

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during the germination of reproductive shoots *Equisetum arvense* there is a direct relationship between the intensity of growth of aboveground and inverse – underground parts of horsetail and the content of endogenous IAA and ABA[27].

It has been established that in seed plants the main axes of the plant body are formed both in the early stages of embryogenesis and during the life cycle. During embryogenesis, the apical-basal and radial axes, cotyledons (embryonic leaves) and meristems of primary shoots and roots are formed. During postembryonic development, these meristems produce lateral organs along the growing axis of the main body and establish the proximal-distal axis[28]. Taking into account the obtained data on the dynamics and distribution of IAA in the sporophyte organs of wintering horsetail and taking into account the morphometric characteristics at the respective phases of vegetative development [12] it can be assumed that in the post-embryonic period auxin controls the development of repetitive morphological units, affects the division of interstitial meristematic cells, induces the growth of lateral roots and the formation of storage nodules and the formation of reproductive structures. Instead, the formation of axial structures occurs in the embryonic period. The effects of auxin are determined by the concentration of the active form in the tissues and are formed with the participation of auxin transporters[29].

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2.2. Abscisic acid in the organs of the sporophyte *Equisetum hyemale* at different phenological stages of development

During the growth and development of reproductive and vegetative shoots of the sporophyte, the content of ABA in the rhizomes was low, and the inactive form was dominant (Fig. 2).

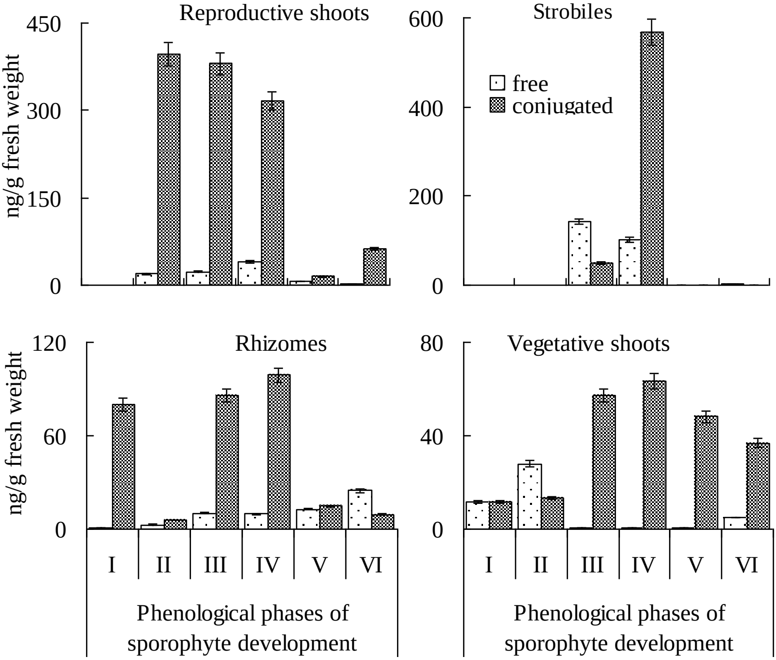

Fig. 2. Distribution of free and bound forms of ABA in the organs of *Equisetum hyemale* L. at different phenological phases of sporophyte development, ng/g FW. Designation of phases: I – pre-spring awakening, II – germination of the reproductive shoot of the I year of development, III – intensive growth of the reproductive shoot, IV – summer vegetation, V – autumn sporulation, VI – dormancy

The level of free ABA gradually increased from the phase of awakening (trace residues) to the phase of dormancy (24.6 ± 1.23 ng/g FW). Instead, changes in the content of the conjugated form were abrupt. High levels of bound ABA (80.1 ± 4.03 ng/g FW, 85.9 ± 4.31 and 98.8 ± 4.94 ng/g FW) accounted for I, III and IV phase of shoot growth.

During the periods of formation and development of strobiles (phases II and V), the amount of the hormone decreased tenfold.

The content of endogenous ABA in reproductive shoots was higher than in vegetative (Fig. 2). The shoots were dominated by a conjugated form of the hormone. The level of free ABA in reproductive shoots gradually increased until the summer growing season (39.4 ± 1.97 ng/g FW), while in autumn it decreased sharply. Instead, the content of bound form of ABA gradually decreased from 397.1 ± 19.87 ng/g FW (phase II) to 14.8 ± 0.74 ng/g FW (Phase V). The transition to a state of rest was accompanied by an increase in the level of inactive form by 4.2 times (62.3 ± 3.13 ng/g FW), which is 41.5 times higher than the content of the active form of the hormone. In vegetative shoots, the free form of the hormone in the amount of 11.5 ± 0.57 and 27.8 ± 1.39 ng/g FW, was found only during germination and growth of reproductive shoots of the first year of development. Subsequently, the content of ABA decreased, reaching trace values (Fig. 2). In contrast to the reproductive shoots in vegetative plants, the content of the conjugated form of the hormone was ten times lower. The level of the hormone initially gradually increased to 63.4 ± 3.17 ng/g FW (summer vegetation phase), and then – decreased to 36.9 ± 1.86 ng/g FW (dormancy phase). The concentration of endogenous ABA during the period of growth retardation (phase VI) in vegetative shoots was 1.7 times lower than in reproductive ones.

The strobiles in the summer (phase III) were dominated by the free form of ABA, exceeding the level of conjugated by 2.8 times. At the stage of development of reproductive structures (IV phase), the content of the free form of ABA reached a maximum (669.0 ± 33.45 ng/g FW), which was 60 % higher than the level of the bound form of the hormone. The onset of spores and the transition to dormancy occurred at trace values of the quantities of both forms of ABA (Fig. 2).

ABA is found in many vascular spore plants. ABA and its metabolites were identified in the cultivars of ferns *Cibotium glaucum* and *Dicksonia antarctica*[30]**.** ABA was found in spores and protonems of

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ferns *Anemia phyllitidis*[31] and *Lygodium japonicum*[32]. ABA has been identified in spores, fertile and sterile shoots of horsetail and in fertile shoots of forest and river horsetails. The hormone content in fertile shoots was higher than in sterile[33]. The changes we found earlier in the nature of accumulation and localization of ABA in the organs of horsetail testified to the involvement of the hormone in the regulation of the processes of transition from the vegetative to the reproductive phase of sporophyte development. The obtained results indirectly showed that the site of ABA synthesis in the reproductive phase is in the rhizome, while in the vegetative – in the aboveground organs of horsetail[34]. In the water fern *Marsilea quadrifolia*, endogenous ABA regulated the transition from aquatic to aerial existence, which manifested itself in the elongation of petioles and roots, changes in leaf morphology, increasing their surface area and shortening internodes. The expression of ABK-sensitive heterophilia genes (ABRH) was induced by natural *S*-(+)-ABA and synthetic *R*-(-)-ABA isomers of the hormone, with the effects of the latter being more pronounced[35].

The accumulation of ABA is usually associated with the formation of a response to stress[36]. The hormone regulates vegetative growth, induces the closure of the stomata and thickening of the cuticular wax, affects the

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structure of the root system, stimulates the synthesis of proteins and secondary metabolites[37]**.** ABK regulates the transition to a state of rest and exit from it[38]. The hormone stimulates the accumulation of reserve substances and regulates their storage in the seeds[39,40,] and also inhibits the appearance of vegetative leaves under normal growing conditions[41].

The obtained data on the dynamics and distribution of endogenous ABA in the sporophyte organs of wintering horsetail at different stages of development indirectly testified to the regulatory effects of the hormone. Thus, in the phase of intensive growth of reproductive shoots at low concentrations of ABA, an increase in the mass of the rhizome was recorded[42]. In the works of other researchers, it was reported that at high levels of ABA slowed the growth of primary roots of Arabidopsis and corn, while at low, on the contrary, was maintained [43,44.] The

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mechanism of inhibition of primary root growth involves genes of the protein phosphatase 2C (PP2C) family of the ABA coreceptor, which inhibit the signaling of hormone[45]. The dominance (65 %) of the free form of ABA over that bound in strobiles (phase III) corresponds to an increase in the mass and length of strobiles, the accumulation of spare substances in spores. In the phase of summer vegetation (IV phase) the content of the free form of ABA decreased by 5.5, after which the number of both forms of ABA reached the trace level. The probable site of ABA synthesis may be green photosynthetic internodes of reproductive shoots, from where the hormone is transported to the strobiles and rhizome. In general, the effects of endogenous ABA wintering horsetail are aimed at: inhibiting the growth of leaves of reproductive shoots, their rapid aging and decline in vegetative shoots of the second year of development; regulation of respiratory activity and maintenance of water balance, which allows horsetails to exist in a wide range of fluctuations of high and low seasonal temperatures and water shortages. Given the changes in the diameter and thickness of the internodes, especially the lower part of the shoots[46], we can assume that ABA is involved in this.

CONCLUSIONS

The maxima in the content of endogenous IAA and ABA in the sporophyte organs of wintering horsetail were during the periods of active growth and development of reproductive shoots and development and maturation of strobiles with spores. In the rhizome, the accumulation of endogenous IAA (mostly free active form) occurred during the formation of storage tubers, when the plants were preparing for the transition to dormancy. Levels of free form of ABA in the rhizome changed abruptly and reached a maximum in the phase of dormancy. The underground organs were dominated by the conjugated form of the hormone, the content of which increased until the summer growing season and decreased in the autumn. The lowest level of endogenous

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IAA and ABA was found in vegetative shoots of the second year of development, the strobiles of which were dry and non-viable, and the intensity of elongation of internodes and leaves throughout the growing season was low[47]. The deposition of the bound form of IAA in these shoots was observed in the phase of awakening and germination. Instead, the content of the bound form of ABA gradually increased, reaching a maximum in the phase of summer vegetation, and then decreased. Reproductive shoots of the first year, which are characterized by intensive growth processes, contained higher amounts of the free form of IAA, while the content of the active form of ABA was much lower. A specific feature of evergreen *E. hyemale* in comparison with *E. arvense*[48] was the predominance of the conjugated form of IAA and ABA in the sporophyte organs in almost all studied stages of development. The obtained results indirectly testified to the regulatory role of IAA and ABA in the transition from vegetative to reproductive phases of growth and development of sporophytic generation of wintering horsetail.

SUMMARY

The character of accumulation and localization of free and conjugated forms of indole-3-acetic (IAA) and abscisic (ABA) acids in the organs of *Equisetum hyemale* L. at different stages of sporophyte development was studied by high-performance liquid chromatography-mass spectrometry. Selection of wintering horsetail plants, which belongs to the higher vascular plants with evergreen phenorhythmotype, was carried out at the exhibition area of higher spore plants of the Academician O. V. Fomin Botanical Garden of Taras Shevchenko Kyiv National University (Kyiv, Ukraine). A specific feature of wintering horsetail was the dominance of conjugated forms of IAA and ABA during sporophyte development. The obtained results testified to the regulatory role of IAA and ABA in the coordination of growth and development of reproductive and vegetative organs of *E. hyemale*. The dynamics and distribution of IAA in the organs of the sporophyte of wintering horsetail suggest that auxin controls the development of repetitive morphological units, induces the growth of lateral roots and the formation of storage nodules on them. The

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nature of the accumulation of ABA indirectly indicates the involvement of the hormone in the activation of protective adaptation processes in the autumn growing season. Regularities of quantitative and qualitative changes revealed similarities in the functions of endogenous auxin and abscisic acid of wintering horsetail and higher vascular plants.

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