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MYCOTOXINS AND USNIC ACID IN THE THALLI OF *HYPOGYMNIA PHYSODES* OF DIFFERENT AGES

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In the epiphytic lichen *Hypogymnia physodes* in the virginal (v2) and potentially generative (g3v) phases of ontogenetic development, mycotoxins and usnic acid were determined by the enzyme immunoassay. It is shown that both age groups contain a 12-component complex of mycotoxins previously established for this lichen during extensive mycotoxicological monitoring. Differences in the levels of accumulation of the most toxic metabolites of micromycetes and usnic acid for individuals in the early and chronologically more mature state were not revealed, but a statistically significant decrease in the contents of sterigmatocystin, mycophenolic acid, citrinin and alternariol has been shown. The features of the mycotoxin profile in a given habitat and the degree of variability in the quantitative content of these substances within a single biotope are discussed.

Key words: enzyme immunoassay, epiphytic lichens, *Hypogymnia physodes*, mycotoxins, ontogeny, usnic acid

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For lichens that have unique biochemical systems new metabolites were recently discovered. These metabolites are typical for free-living microscopic fungi and belonged to the group of mycotoxins (Burkin, Kononenko, 2010, 2013). Their composition was specific for lichens of different taxonomic affiliation (Burkin, Kononenko, 2014a). The ranges of variation of their contents, determined by the method of enzyme-linked immunosorbent assay (ELISA) in samples from the natural habitat with a variety of substrates and growth sites, as well as the collection time, proved to be extremely wide (Burkin, Kononenko, 2015a). This made it possible to assume the active participation of micromycetes-producers in the processes of lichen vital activity. For the experimental verification of the hypothesis, an extensive complex of mycological and biochemical studies is needed, including the identification of micromycetes responsible for the biosynthesis of these substances, finding out the sites of their localization in lichens and the role of micromycetes in the relationship between mycobiont, photobiont and communities of lichen-associated microorganisms, decoding the mechanisms regulating biosynthesis of mycotoxins, their intercellular and interstitial migration, accumulation and/or transformation, as well as the possibility of involving these processes in the main metabolic pathways.

This work is the first step in this direction and was undertaken to assess the degree of mycotoxin accumulation in lichens under natural fluctuation of external factors in a particular biotope and the phase change of their ontogenetic development. The elaboration of the highly sensitive and selective determination of usnic acid on the basis of ELISA (Burkin et al., 2013) made it possible to conduct a joint study of mycotoxins and one of the typical representatives of lichen substances.

As an object, *Hypogymnia physodes* (L.) Nyl. (*Parmeliaceae*) was chosen. It is a common epiphytic forest species, inhabiting almost the entire territory of Russia and often forming coalescing large clusters on the substrates. It has a high ecological plasticity, which is expressed by distinct reactions to the state of the environment : by changes of the structure of its populations (Mikhailova, 2007; Mikhailova, Vorobeichik, 1999) as well as by accumulation of anthropogenic pollutants (Mikhailova, Sharunova, 2008) and also by ratio of stable isotopes of carbon and nitrogen (Biazrov, 2012, 2013). The biological activity of its extractive substances is reported (Ranković et al., 2007), and neutral lipids, glycolipids, depsidones, dibenzofuran derivatives and among them usnic acid are described among their metabolites (Dembitskiy, Tolstikov, 2005). According to the number of mycotoxins contained in the talli, *H. physodes* takes a leading place among lichens : most of the 15 identified components had a regular occurrence (Burkin, Kononenko, 2013).

MATERIALS AND METHODS

Sampling was carried out in one day (23.04.2013) in green moss pine forest near Yoshkar-Ola (the Mari El Republic of Russian Federation) from ten closely located trees, intensively inhabited by distinct thalli of

H. physodes with clearly delineated boundaries. The approach described in the work (Suetina, Glotov, 2014) was used to determine the ontogenetic states of the lichen. On the basis of a combination of qualitative morphological features, two states were differentiated: virginal (v2) with the formation of a typical leafy rosette-shaped or indefinite-shaped thallus, and the appearance of soredias at the ends of the blades, and an old potentially generative (g3v), where in rosette-shaped thalli, the death of the central part begins with a change in color with gray on brown and black, and at the ends of the blades geleated sorales predominate.

From the trunk of each tree, twenty individuals were collected in the virginal (v2) state and five individuals in a potentially generative (g3v) state. After cleaning from the bark under the binocular, the thallus was placed in paper bags and stored as air-dried until analysis at room temperature for 2–3 weeks.

The g3v-thallus from each tree was analyzed separately. Three samples were prepared from the v2-thalli: the one from 80 specimens from four trees and two samples from 60 specimens each from three trees. The weight of each sample was 200–500 mg. Extraction was carried out with a mixture of acetonitrile and water in a volume ratio of 84 : 16 at a rate of 10 mL per 1 g of sample. After 10-fold dilution with a phosphate buffer solution and Tween 20, the extracts were used for indirect competitive ELISA. Mycotoxins – T-2 toxin (T-2), diacetoxycirpenol (DAS), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM), ergot alkaloids (EA), alternariol (AOL), roridin A (ROA), aflatoxin B_1 (AB_1), sterigmatocystin (STE), cyclopiazonic acid (CPA), emodin (EMO), ochratoxin A (OA), citrinin (CIT), mycophenolic acid (MPA), PR-toxin (PR), as well as usnic acid (UA) – were analyzed by certified immunoenzyme test systems (Burkin, Kononenko, 2011, Kononenko et al., 2012). The lower limit of quantitative measurements corresponded to 85% level of antibody binding. The R software package and a manual (Dospekhov, 1985) were used to process the results. Wilcoxon–Mann–Whitney non-parametric test was used for statistical analysis in R.

DISCUSSION

As follows from the data presented in the Table 1, in the samples of g3v-thalli for most mycotoxins, the accumulation was at the level of hundreds of ng/g, in STE and OA was lower (tens of ng/g), the highest values were detected for MPA, AOL, EMO – thousands of ng/g, and the content of UA corresponded to tens of thousands of ng/g. The values of mass concentrations of metabolites in samples from distinct trees were in narrow ranges, and the relative error of the sample mean, characterizing the degree of variability of the studied trait, did not exceed 10% for most of them, and only for EMO and UA was somewhat larger. This could indicate that such features of tree as its age, structure of the cortex, as well as factors related to the localization of the specimen (shading, moisturizing, etc.) did not have a significant effect on the measured values. Nevertheless, it is known that the growth rate as well as the anatomical and morphological structure of thallus of *Hypogymnia*, as well as in other epiphytes, depends on the location of the thallus (in the lumbar part or in the trunk zone), on the proximity to mosses or even on the inclination of the trunk (Mikhailova, 2007).

For v2-individuals, the same comparison could not be carried out, since the mass of 20 thalli collected from one tree was only 50–100 mg that was insufficient for analysis. Evaluation of the mycotoxin profile was performed on three prepared samples of 60 and 80 thalli, while the obtained extracts did not differ (in color intensity) from those prepared from 5 individuals in the g3v-state. The variation in the amounts of metabolites in v2-thalli although corresponded to a wider range (from 1.2 to 20.2%) remained still as weakly expressed (Table 1). It indicated that microclimatic factors do not influence the measured indices, regardless of the ontogenetic phase of lichen development.

The lichen has the same set of metabolites and the same ratio of accumulation levels in the virginal state as more matured thalli had: for most mycotoxins hundreds of ng/g, tens of ng/g for STE and OA, thousands for AOL, EMO, MPA and tens of thousands for UA (Table 1).

Generally on all the samples analyzed within this experiment the mean values of the content of mycotoxins and UA did not exceed the limits established during extensive monitoring of *H. physodes* in geographically distant sampling locations (Burkin et al., 2013; Burkin, Kononenko, 2014a, b). Some differences were observed only for rarely detected components. In a natural habitat with a variety of substrates and ranges, AB_1 was not found, and T-2, ROA and EA detected in 7–10% of samples in quantities close to the limits of detection (Burkin, Kononenko, 2014b). At local biotope selected for our experiment, T-2 was detected in only one sample (5 ng/g), ROA in 10 samples out of 13 in amounts 10–26 ng/g, and AB_1 in 9, but only in background contents (2–3 ng/g). The detection of nitrogen-containing EA was usually observed in lichens in anthropogenically disturbed areas, and in *H. physodes* these metabolites occurred at a frequency of 28% in the range of 10–60 ng/g and an average value of 22 ng/g (Burkin, Kononenko, 2014a). Under the conditions of this concrete ecotope, EA was found in all samples without exception in concentrations 3–6 ng/g.

In accordance with the data obtained, when the lichen development phases change from virginal to potentially generative, the content of UA and the most of components of the mycotoxin complex (OA, ZEN, DON, EMO, FUM, DAS, CPA, PR) remains practically unchanged, but for STE, MPA, CIT and AOL the tendency for decreasing was observed (Table 1). Nevertheless, the revealed changes were within the ranges of quantities established for chronologically even more adults, but ontogenetically uncharacterized thalli of

Table 1. The content of secondary metabolites (ng/g) in v2- and g3v-individuals *Hypogymnia physodes* on the pine trees from the same biotope (I) and in more mature individuals of unidentified ontogenetic state from different substrates and geographically distant areas (II)

Metabolites	I					II*	
	v2-state		g3v-state			Unidentified state	
	Mean (n = 3)	s_x,	Mean (n = 10)	min–max	s_x, %	Mean (n = 112)	min–max
STE	75 [a]	10.1	57 [b]	49–73	4.4	100	10–1500
OA	13 [a]	2.5	14 [a]	12–15	2.8	11	8–24
MPA	1810 [a]	6.8	1380 [b]	1010–1570	4.2	1040	40–7900
CIT	205 [a]	12.2	140 [b]	120–160	3.2	140	45–400
AOL	2990 [a]	13.6	1180 [b]	725–1700	9.0	520	100–3240
ZEN	120 [a]	5.1	105 [a]	88–130	4.2	180	40–6300
DON	200 [a]	14.1	180 [a]	120–265	6.7	240	100–700
EMO	1440 [a]	20.2	1250 [a]	789–2810	14.8	1470	150–10500
FUM	350 [a]	9.3	370 [a]	315–470	4.0	300	80–1350
DAS	475 [a]	6.1	495 [a]	435–615	3.0	480	210–1100
CPA	475 [a]	2.1	495 [a]	360–610	4.2	910	190–4100
PR	420 [a]	1.2	425 [a]	345–515	3.7	380	130–1550
UA	40000 [a]	18.8	25000 [a]	11000–43000	14.5	27000 (n = 9)	14000–65000

Note. STE – sterigmatocystin, OA – ochratoxin A, MPA – mycophenolic acid, CIT – citrinin, AOL – alternariol, ZEN – zearalenone, DON – deoxynivalenol, EMO – emodin, FUM – fumonisins, DAS – diacetoxycirpenol, CPA – cyclopiazonic acid, PR – PR-toxin, UA – usnic acid; n – number of analyzed samples, s_x – relative error of the mean; values in the same row with different superscripts (a, b) are significantly different ($P < 0.05$).
* Cited by references (Burkin et al., 2013; Burkin, Kononenko, 2014a).

H. physodes, whose mass approximately 5 times greater and the average values of the contents of STE, MPA and CIT were very close found for g3v-thalli. Perhaps the stabilization of equilibrium with the participation of these metabolites is completed precisely at this or a close phase of the development of the organism. The fact that in mature individuals the content of AOL was significantly lower than that of g3v thalli (Table 1) can be considered as evidence that fungi of the genus *Alternaria* producing this toxin are involved in many processes accompanying the age-related changes in the host organism. On the other hand, this comparison definitely indicates that when the lichen passes ontogenetic phases, which takes an extended time period, the profile of the analyzed substances remains generally stable for mycotoxins and UA, hence regulatory mechanisms that ensure a stable metabolic balance affect their own biogenesis pathways.

It is interesting to conduct an extensive study of metabolome in younger and more mature individuals of the same lichen with the appearance of signs of aging. However, the work with the senile state in epiphytic lichens will inevitably be complicated by the fact that dying may mean a loss from the substrate under the influence of natural factors. The virginal stage can be considered only as "conditionally early", since the actual age of thallus is quite sufficient for long-term contacts with microscopic fungi and the establishment of equilibrium relations with them. Nevertheless, this phase is already critical for applying of such a highly sensitive method as ELISA, since the sample preparation is extremely labour-consuming. The collection of the sample mass from even smaller individuals is practically impossible, since the v1 thallus is twice smaller than the v2, with a size of only 1–2 mm. Undoubted scientific interest is also the moment of the formation of the mycotoxin profile, possibly starting in soredias, from which thallus forms, but for its study other analytical techniques based on innovative developments are needed. Nevertheless, the sequence of ontogenetic phases available for experiments, from virginal to subsenile, is quite sufficient for an extended study of the physiological role of these substances. From this position, work with the same age groups of other epiphytic lichens, such as *Xanthoria parietina*, which is characterized by a small number of regularly occurring mycotoxins (Burkin, Kononenko, 2015a) or the ones who occupy an intermediate position in this regard, for example genera *Platismatia* or *Melanohalea* (Burkin, Kononenko, 2013) can have important informational value. The study of the dynamics of metabolic processes in lichens has a particular value in connection with the biological singularity of these symbiotic associates, but it remains problematic due to the record long life cycle. Thereby, it is advisable to use annual plants with short growth periods as experimental models. Recently for vegetative wild meadow grasses, significant changes were revealed in the content of mycotoxins during the

regular gatherings from June to September (Burkin, Kononenko, 2015b).

In recent years, a new direction of lichen research has been actively developing, devoted to the role of secondary metabolites in the internal regulatory mechanisms and external reactions. In epigeous lichens on fragments of thalli from zones of restrained and active growth, a noticeable accumulation of mycotoxins in the lower part of the thalli and an even greater content in the litter – the surface layer of the soil (Kononenko, Burkin, 2015) was revealed. However, an interpretation of the obtained results cannot be unambiguous due to the quiet possible contribution of toxin-forming micromycetes-destructors utilizing fragments of dying parts the lichens thallus.

Epiphytes are the most perspective objects for the study of metabolic migration, but experiments with them are just beginning. So, according to our first pilot several species (*Bryoria capillaris*, *B. chalybeiformis*, *B. fuscescens*, *Alectoria sarmentosa*, *Evernia prunastri*, *Usnea subfloridana*) contain mycotoxins and UA in comparable amounts not only at the tips of the branches (active growth zones) and the central part, but also in lignified parts of attachement to the substrate. For *Hypogymnia physodes* it has been shown that lichenic acids (conphysodalic, 4-o-methylphysodic and α-alectoronic) are present not only in the thallus, but also in the bark of spruce abundantly colonized by this lichen (Latkowska et al., 2015). Continuation of the search with such objects will allow us to expand understanding of the biochemical mechanisms that ensure the adaptation of lichens to the diversity of biocenoses and ecological situations.

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Микотоксины и усниновая кислота в талломах *Hypogymnia physodes* разного возраста

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С помощью иммуноферментного анализа изучено содержание микотоксинов и усниновой кислоты в слоевищах эпифитного лишайника *Hypogymnia physodes* в виргинильной (v2) и потенциально генеративной (g3v) фазах онтогенетического развития. Показано, что обе возрастные группы содержат 12-компонентный комплекс микотоксинов. Различия в уровнях накопления наиболее токсичных метаболитов микромицетов и усниновой кислоты в раннем и более зрелом состояниях слоевища не выявлено, однако показано статистически значимое снижение содержания стеригматоцистина, микофенольной кислоты, цитринина и альтернариола. Обсуждаются особенности состава микотоксинов и их количественного содержания в лишайниках из одного биотопа.

Ключевые слова: иммуноферментный анализ, микотоксины, онтогенез, усниновая кислота, эпифитные лишайники, *Hypogymnia physodes*