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Variation in the Composition of Secondary Metabolites in *Flavocetraria* Lichens from Western Siberia

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Abstract—The composition and contents of secondary metabolites in *Flavocetraria* lichens from Eastern Siberia were analyzed using herbarium specimens. Based on the composition of identified metabolites, three *F. cucullata* chemotypes and two *F. nivalis* chemotypes were distinguished. Distinct geographic differentiation between the *F. cucullata* chemotypes was revealed, probably reflecting their adaptation to environmental conditions. The content of usnic acid in *F. cucullata* thalli was found to correlate with the latitude of growing region. This may be regarded as evidence for a protective role of this metabolite in lichens growing at high latitudes and exposed to excess solar irradiation during the polar day.

Keywords: lichens, genus *Flavocetraria*, secondary metabolites, chemotypes
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Unique secondary metabolites of lichens, collectively referred to as lichen substances, include both aromatic compounds (depsides, depsidones, dibenzofurans) and aliphatic compounds such as fatty acids and γ-lactones. About 1050 lichen substances are known today [1].

As a rule, the qualitative composition of these substances in lichen thalli is fairly stable and, together with morphological and anatomical features, is an important diagnostic character. However, cases of intraspecific biochemical variation are known where the initial lichen substances are substituted by other compounds structurally close to them. Thus, chemical races, or chemotypes are formed [2].

Several hypotheses have been advanced to explain the formation of different lichen chemotypes. One of them is that they are formed as an adaptation to environmental conditions [3, 4], and this has been confirmed in experiments on in vitro cultivation of *Xanthoparmelia* lichens under different conditions [5]. However, the formation of chemotypes in natural populations is not always associated with ecological conditions for the growth of lichens [6, 7].

The effect of ecological conditions for lichen growth on the accumulation of secondary metabolites has previously been studied in the Soviet Union and Russia. In particular, it has been shown that the dynamics of usnic acid contents in lichens growing in Leningrad oblast and Yakutia has a strict seasonal pattern [8, 9]. In the study directly dealing with chemical variation in lichens growing in Siberia and the Russian Far East, Randlane and Saag [10] described chemotypes of *Asahinea* lichens that were new for the study region but revealed no distinct correlation between the composition of secondary metabolites contained in the lichens and the location of their growing area.

The genus *Flavocetraria* comprises arctoalpine lichen species that are widespread in the tundra and northern forest zone of Eastern Siberia, where they account for up to 70–80% of the total lichen biomass [11]. It is probable that their distribution over a vast area and growth in different biocenoses provided for the development of adaptive reactions at the biochemical level to facilitate adaptation of these lichens to diverse growing conditions.

The purpose of this study was to analyze the composition and quantitative contents of secondary metabolites in *Flavocetraria cucullata* and *F. nivalis* lichens growing in the north of Eastern Siberia.

MATERIAL AND METHODS

Lichens of the genus *Flavocetraria* (78 specimens of *Fl. cucullata* (Bellardi) Kärnefelt & Thell and 58 specimens of *Fl. nivalis* (L.) Kärnefelt & Thell) collected in summer in Yakutia, Krasnoyarsk krai, and Trans-

baikalia over the period from 1832 to 2017 were taken from the herbarium collections of the Institute for Biological Problems of the Cryolithozone (SASY) and Komarov Botanical Institute (LE).

To isolate and identify lichen substances, 30-g aliquots of air-dry thalli were extracted with acetone in a Soxhlet apparatus for 24 h, and the extracts were evaporated to a volume of 15–20 mL in a rotary evaporator. Preparative isolation of individual substances was performed in the Reveleris flash chromatography system (Grace Davison Discovery Sciences, United States) with a 111 × 10-mm packed with C18 silica gel (particle size 40 μm, 12 g) using as eluents 0.1% glacial acetic acid solution in water (eluent A) and ethanol (eluent B). Elution with an increasing eluent B concentration (10–100%) at a flow rate of 15 mL/min was performed for 30 min. After removing the eluent, the dry residue from each fraction was purified by sequential recrystallization from chloroform and acetone.

The isolated substances were identified by spectral analysis. Infrared spectra were obtained with a Varian 7000 FT-IR Fourier transform infrared spectrometer (Varian Medical Systems, United States) using potassium bromide pellets (400–4000 cm^{-1}). The UV spectra of the substances dissolved in methanol were recorded with a Shimadzu UV-2600 spectrometer (Japan) at 190 to 350 nm. Molecular weights were determined using an Agilent 6538 UHD Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, United States) with dual electrospray ionization (ESI). Capillary voltage at positive or negative ESI was 3.5 kV; nebulizer gas pressure, 30 psi; dryer gas (nitrogen) temperature, 350°C; dryer gas flow rate, 7 L/min. Ions were recorded within a mass range of 100–1000 m/z. The isolated substances were identified by comparing their molecular weights and IR and UV spectra with those of known lichen metabolites [12].

The isolated and identified lichen substances were used as standards for determining their contents in the thalli. Samples for analysis consisted of the upper (younger) parts of air-dry thalli, no longer than 1 cm. A sample was ground up, and a 10-mg aliquot was extracted with two portions of acetone, 1 mL each for high performance liquid chromatography (HPLC) or 0.2 mL each for thin-layer chromatography (TLC). Extraction with constant stirring was performed at 20–25°C for 24 h.

The extracts were analyzed by TLC (only qualitatively) on Sorbfil plates (Russia) in a toluene : glacial acetic acid (170 : 30) system, and the adsorbed substances were revealed based on their absorption and fluorescence at 254 and 365 nm, respectively, in a Lenkhrom UV cabinet (Russia)

Qualitative and quantitative HPLC analysis was performed in a Milikhrom A-02 chromatograph (Russia) with a ProntoSIL 120-5-C18 AQ reverse-phase column (2 × 75 mm) using as eluents 0.1% glacial acetic acid solution in water (A) and acetonitrile (B). Gradient elution with B concentration increasing from 10–50% during 5 min and from 50 to 100% min during 20 min was performed at a flow rate of 100 μL/min, column temperature was 40°C. The eluted substances were detected at 210, 230, 240, 260, and 280 nm.

All measurements were made in three analytical replicates. Standard errors of the mean and Pearson's correlation coefficient were calculated with StatPlus v. 2007.

RESULTS AND DISCUSSION

Chromatographic analysis of herbarium samples of *F. cucullata* and *F. nivalis* lichens collected in different years in the north of Eastern Siberia showed that the main secondary metabolites in *F. cucullata* were as follows: aliphatic γ-lactone protolichesterinic acid (retention time R_t = 23.5 min, retention factor R_f = 37) and its structural isomers such as lichesterinic acid (R_t = 23.0 min, R_f = 43) and alloprotolichesterinic acid (R_t = 22.1 min, R_f = 30; found in the species for the first time); aromatic depside gyrophoric acid (R_t = 15.5 min, R_f = 24; found in the species for the first time); and dibenzofuran (–)-usnic acid (R_t = 19.5 min, R_f = 70). *Flavocetraria nivalis* lichens contained (–)-usnic acid, (–)-isousnic acid (R_t = 20.5 min, R_f = 71), and squamatic acid (R_t = 11.3 min, R_f = 25; found in the species for the first time). Protolichesterinic acid was not revealed in our samples of *F. nivalis,* although other authors noted its presence in samples from Finland [13].

The qualitative and quantitative composition of secondary metabolites in lichen samples showed no significant dependence on the time of their storage in the herbarium (Fig. 1). The coefficient of correlation between the contents of secondary metabolites and storage time were as follows: usnic acid in *F. cucullata,* r = 0.18 (p = 0.16); usnic acid in *F. nivalis,* r = 0.06 (p = 0.65); gyrophoric acid, r = 0.09 (p = 0.51); alloprotolichesterinic acid, r = 0.23 (p = 0.08); protolichesterinic acid, r = 0.01 (p = 0.99); lichesterinic acid, r = 0.06 (p = 0.84); squamatic acid, r = –0.23 (p = 0.17).

In *F. cucullata,* all the five chemical components together were found only in 52 samples (chemotype I); in the remaining 25 samples, we revealed the absence or only traces of alloprotolichesterinic acid (chemotype II, 16 samples) or of alloprotolichesterinic and gyrophoric acids (chemotype III, 9 samples).

The main morphological characters of *F. cucullata* thalli—height, thickness, and color—vary within a very wide range and, on average, hardly differ between the chemotypes. The thallus consists of a bunch of vertical lobes 3–10 cm high and 2–10 mm wide, its color ranges from whitish yellow to greenish, turning reddish brown at the base. The lobes are almost tubular, with recurved tips. Apothecia were absent in the samples studied.

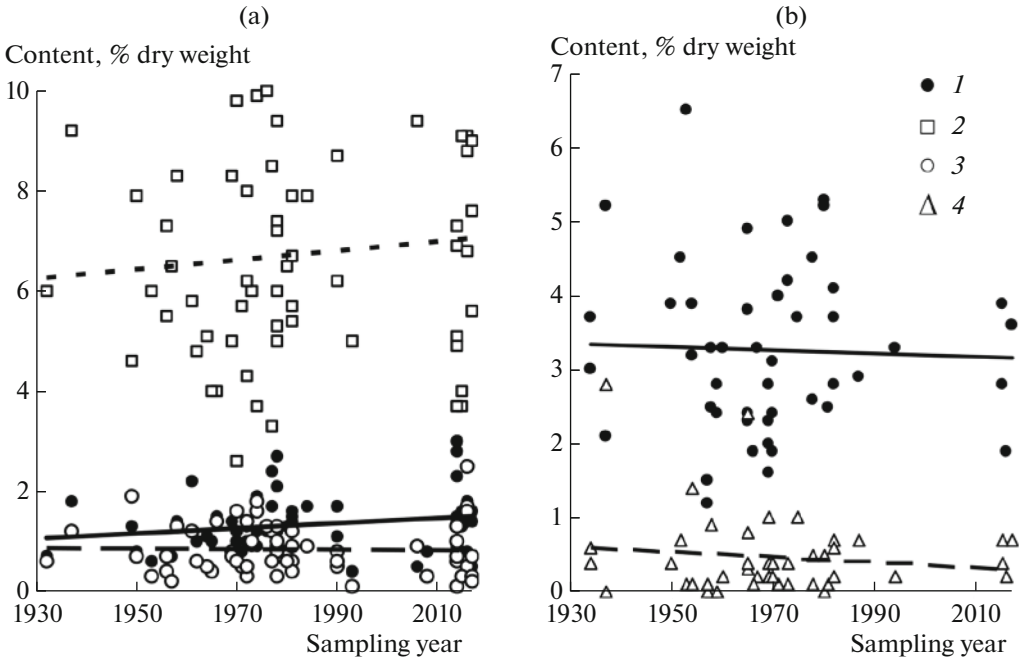

Fig. 1. Dependence of the contents of secondary metabolites in (a) *F. cucullata* and (b) *F. nivalis* lichens on the time of their storage in a herbarium: (*1*) usnic acid, (*2*) protolichesterinic acid, (*3*) gyrophoric acid, (*4*) squamatic acid.

Flavocentraria cucullata lichens of chemotype I occurred mainly in taiga forests, forest–tundras, floodplain marshy tundras, and on screes in the mountains with sharply continental subarctic climate (Fig. 2). Chemotype II prevailed in arctic and mountain tundras above 70° N, except for lichens from marshy tundras on the Yana Bay coast, where they occurred together with chemotype I. Lichens of chemotype III were confined to arctic deserts on the Novaya Zemlya Archipelago and De Long Islands.

The observed distribution of the study *Flavocetraria* species proved to be closely correlated with ecological conditions in their growing areas. For example, *F. cucullata* of chemotype III occurred only in arctic deserts, where biodiversity is low and the formation of a chemotype deficient in alloprotolichesterinic and gyrophoric acids could be accounted for by a low level of competition with other plants and lichens. It is known that one of the functions of lichen substances is to provide for the allelopathic interaction of lichens with other plant species [14, 15]. In particular, lichen acids can inhibit seed germination and the growth and development of seedlings in higher plants and have a similar effect on mosses [16].

The content of usnic acid in *F. cucullata* samples varied from 0.4 to 3.0%, averaging 1.3 ± 0.1%; of alloprotolichesterinic acid, from 0.1 to 2.6%; of protolichesterinic acid, from 3.0 to 9.0%; and of lichesterinic acid, from 0.3 to 1.5% dry weight. The content of usnic acid proved to directly correlate with the latitude of growing region ($r = 0.4$; $p < 0.01$) (Fig. 3), whereas no such correlation was revealed for lichesterinic, protolichesterinic, alloprotolichesterinic, and gyrophoric acids.

The above correlation between the content of usnic acid and the latitude of growing region is in agreement with previous data that the accumulation of this acid in *F. cucullata* depends on the length of the daylight period [17]. Among all secondary metabolites contained in this lichen, only usnic acid shows absorption in the near UV (UV-A) range incident on the ground surface [18]. Supposedly, usnic acid in *F. cucullata* lichens has a protective function under conditions of excess solar irradiation during the polar day (midnight sun) at high latitudes. This hypothesis is confirmed by data on the increased accumulation of usnic acid in *Cladonia* lichens under long-term exposure to UV radiation in the near range [19, 20].

In *F. nivalis,* 41 out of 53 samples contained usnic and squamatic acids (chemotype I), and other samples additionally contained isousnic acid (chemotype II). Both chemotypes grow in arctic and mountain tundras, forest–tundras, and arctic deserts, but we failed to reveal any geographic or ecological differentiation between them.

Morphologically, *F. nivalis* thalli do not differ between the phenotypes. They consist of cushions of vertical or sometimes horizontal lobes 2–7 cm long and 5–10 mm wide, flat or slightly grooved. Their color ranges from whitish yellow to dark yellow, turning brownish to dark brown at the base. Apothecia were not found in the specimens studied.

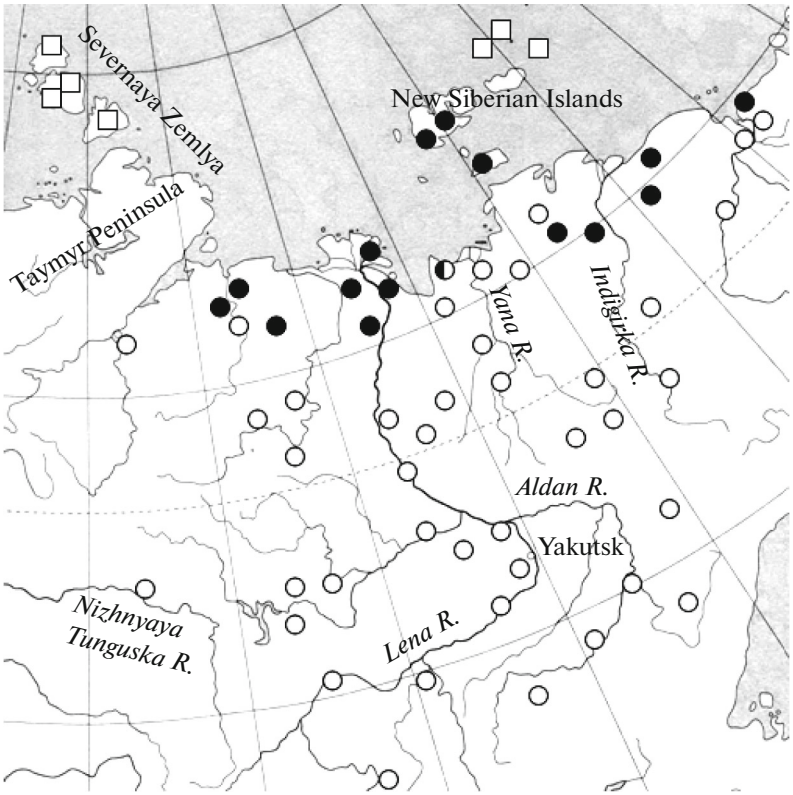

Fig. 2. Distribution of *F. cucullata* chemotypes: (○) chemotype I, (●) chemotype II, (□) chemotype III.

The content of usnic acid in *F. nivalis* specimens varied from 1.2 to 6.5% dry weight, averaging 3.3 ± 0.2%; of isousnic acid, from traces to 0.6%; of squamatic acid, from 0.1 до 1.4% (0.5 ± 0.1%). The contents of usnic and squamatic acids were not correlated with the geographic latitude of *F. nivalis* growing region. In the case of usnic acid, this can be explained by its high content (on average, 2.5 times higher than in *F. cucullata*. As shown previously [18], additional exposure of *F. nivalis* lichens to natural or artificial UV irradiation does not induce usnic acid biosynthesis. It may well be that the biological role of usnic acid in this lichen species is not limited to photoprotection but contributes to additional adaptive functions.

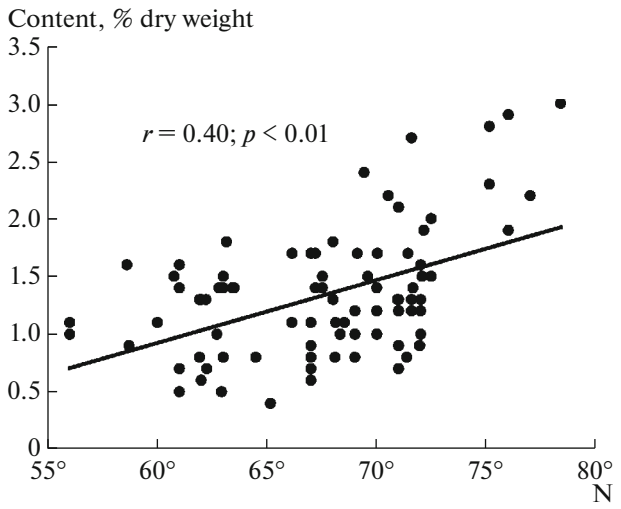

Fig. 3. Dependence of usnic acid content in *F. cucullata* on the geographic latitude of growing region.

Thus, we have revealed secondary metabolites that are new for *F. cucullata* (gyrophoric and alloprotolichesterinic acids) and for *F. nivalis* (squamatic and isousnic acids). The composition and contents of secondary metabolites in the lichens are not correlated with the period of their storage in the herbarium. Based on the composition of metabolites in *Flavocetraria* lichens growing in the north of Western Siberia, three chemotypes of *F. cucullata* and two chemotypes of *F. nivalis* have been distinguished. Distinct geographic differentiation is observed between the *F. cucullata* chemotypes, indicating that their formation may be a result of adaptation to environmental conditions. The content of usnic acid in *F. cucullata* thalli shows direct correlation with the latitude of the growing region, indicating a probable protective role of this metabolite in lichens growing at high latitudes and exposed to excess solar irradiation. The absence of such a correlation in *F. nivalis* lichens may be due to the high content of usnic acid as well as to additional functions (not related to photoprotection) that it may have.

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