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Allelopathic effects of lichen metabolite usnic acid on growth and physiological responses of Norway spruce and Scots pine seedlings

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ABSTRACT

Lichens are globally widespread organisms playing an important role in diverse ecosystems. They produce secondary metabolites, unique compounds, which play many important ecological and biological roles, including their effects on other plants, through allelopathy. Usnic acid is one of the most frequent secondary compounds in thalli of lichens forming the layer on the surface of soils, interacting with the seedlings of conifers in the boreal forests. The main aim of this study was to investigate the growth, ploidy level, reactive oxygen species (ROS) production and element content in the Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) seedlings cultivated for 14 days using substrates containing addition (10 mg per cultivation tube) of (+) usnic acid (UA). We also investigated UA root content in these plants.

The root:shoot ratio (R:S) decreased in stressed pines by over 31%. The average root length diminished by 48% and the shoot length (to the cotyledon base) by 25%. For spruce, the R:S ratio decreased by more than 41%, the root length by 46% while the shoot length by only 9%. The UA treatment particularly increased the number of non-fully developed seedlings during the germination. The seed germination rate did not vary significantly when compared to control. No significant ploidy differences between control and treated seedlings were observed in neither of the species. Ploidy aberration in two *P. abies* seedlings was discovered. The amount of UA in the roots, including UA bound on their surfaces, in spruce varied from 3.6 to 325.5 μg g^{-1} DW and in pine roots from 15.6 to 252.3 μg g^{-1} DW. A significant decrease in total macroelement content in roots of both species was noted, particularly for P, K, Ca, Mg and S contents. Interestingly, the contents of stress markers, e.g. superoxide dismutase and peroxidase were not significantly changed when compared to controls.

1. Introduction

Lichens are important components of the boreal forest vegetation and subarctic ecosystems. Overall mat-forming lichens cover about 8% of the terrestrial surfaces on the Earth (Larson, 1987). In open coniferous forests in northern Canada, they cover up to 97% of the woodland surface and represent 20% of the total ecosystem biomass. *Cladonia* species belong to the most abundant lichens in these ecosystems (Auclair and Rencz, 1982). In the case of undisturbed conditions in late successional boreal or subarctic forest respectively, the lichen mat may grow to a thickness of 10–15 cm (Ahti, 1977). Lichens are characterised by their exceptionally varied secondary metabolite production. Most of the metabolites occur exclusively in these organisms. More than 1000 secondary lichen metabolites have been identified so far (Stocker-Wörgötter, 2008). Typical secondary metabolites of lichens are produced by the fungal partner of lichen symbiosis, and their main role is to regulate growth and metabolism of algae and/or cyanobacteria, which are symbiotic autotrophic partners (Bačkor et al., 2010; Bačkor et al., 2013). They have antimicrobial effects against certain bacteria and fungi occurring in humans and animals (Ranković and Mišić, 2008) and affect the growth and development of wood-decaying fungi as well as pathogenic and parasitic fungi associated with lichen thalli. Many of them also inhibit the growth of higher plants (Białońska and Dayan, 2005). Usnic acid (UA) is one of the most studied lichen secondary metabolites. The enantiomer most commonly found in nature is (+) UA (Latkowska et al., 2008).

One of the main functions of the lichen secondary metabolites is allelopathy. In general, they are substances released into the environment with the ability to affect photosynthesis, respiration, transpiration, nucleic acid and protein synthesis, membrane ion transport and permeability in the nearby plants (Molnar and Farkas, 2010).

The results relating to usnic acid effect on the isolated protoplasts, chloroplasts, and whole plants are often discrepant. These discrepancies might be explained by various factors affecting the transport of usnic

Abbreviations: dw, dry weight; PA, *Picea abies*; PS, *Pinus sylvestris*; SD, standard deviation; UA, usnic acid.

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acid in the plant such as degradation of UA in the apoplastic medium, trapping of the UA molecule at the cell wall level and also the limited permeability of the endodermis towards UA (Vavasseur et al., 1991).

The beginning of research dealing with the effect of lichen allelopathy on Gymnosperms goes back into the last century. Some authors (Brown and Mikola, 1974) mentioned negative lichen allelopathy effects on the germination and development of conifer trees, others (Stark and Hyvärinen, 2003; Kytöviita and Stark, 2009) documented neutral, no allelopathic effect of lichens on conifer trees. On the other hand, the negative influence was achieved when the plant species naturally not co-occurring with lichens were laboratory treated with UA. Such species include tomato (Latkowska et al., 2008), wheat and sunflower (Lascève and Gaugain, 1990), red clover (Nieves et al., 2011), lettuce (Romagni et al., 2000) and oak (Orus et al., 1981).

Cardarelli et al. (1997) demonstrated that usnic acid in low concentrations dramatically retards the protoplast proliferation in tobacco culture and for 90% of the cells UA was not toxic. Usnic acid has a strong effect on cell division in protonemata of *Physcomitrella patens* suggesting a strong impact on the early stages of bryophyte development. Furthermore, significant differences in cell lengths and widths were also noticed (Goga et al., 2016). UA thus has antimitotic activity and inhibits the growth of several cell cultures, including those of animals. However, this effect is not mediated by the action of UA on DNA. Its effect on the genome is mediated through gene expression modulation associated with oxidative stress and lipid metabolism (Luzina and Salakhutdinov, 2016). It is unknown whether UA can affect the ploidy level of single cells or even whole tissues. This is more plausible in the group of lower plants, such as *Bryophyta*, which are naturally endopolyploid. Conifers do not belong to endopolyploid plants.

Usnic acid is weakly soluble in water, but it leaches from lichen thalli by rainwater and dew and accumulates in the soil profile (Dawson et al., 1984). The highest solubility of UA is in organic solvents such as ethyl acetate, acetone, *n*-hexane, and ethanol (Ju-Qing et al., 2013). Phenolics leaching from the lichen *C. stellaris* (the most abundant in boreal forests) are a source of energy rather than allelopathic agents for soil microorganisms (Stark and Hyvärinen, 2003).

In this study, we examined the effect of (+) UA on the growth of Norway spruce and Scots pine seedlings. These conifer species and lichens naturally co-occur in boreal forests and young emerging plants are in direct contact with lichen secondary metabolites. The main aims of present study were to (I.) identify the morphological effect of (+) UA on the growth of *P. abies* and *P. sylvestris*, (II.) verify the hypothesis whether (a) the UA treatment acts selectively against plants with high DNA content or higher ploidy level during early seedling development or (b) UA generates mitotic abnormalities resulting in polyploid (aneuploid) cells in tissues, (III.) test the potential effects of UA on ROS production in conifer tissue, (IV.) evaluate UA content in the treated seedling roots and (V.) monitor overall macroelement content. For the implementation of these objectives, we analysed growth parameters, genome size, ROS production and UA content in conifer seedlings due to 14 days prolonged usnic acid exposition in the substrate. We also assessed macroelement composition in roots and shoots of seedlings due to excess of UA in the cultivation substrate.

2. Materials and methods

2.1. Seed cultivation

Seeds of Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) H. Karst.) were supplied by the state enterprise Forests of the Slovak Republic (Liptovský Mikuláš, Slovakia). The seeds were collected in the northern Slovakia and Kysucko-oravsky region, respectively. The seeds were grown in 50 ml polypropylene falcon tubes with a hole in the bottom for irrigation with distilled water from the bottom up. Agroperlite was used as the growing medium in the amount of 25 ml per tube. Five seeds per tube were sown, totally 200 seeds for each treatment. No mycorrhizal inoculation was added to provide direct contact of roots with UA. The seedlings were grown in the laboratory conditions with 14 h + 10 h (day–night) light period provided by Osram L36W/840 lamps with a photon flux density of 210 μmol m^{-2} s^{-1} and constant 20 °C temperature. Usnic acid treatment (UA, Aldrich 329967 5C) involved the addition of 10 mg UA dissolved in 5 ml acetone per tube. The addition of 5 ml acetone per tube served as the control treatment. Sowing took place 24 h after acetone evaporation in both treatments. Two-week-old seedlings of *P. sylvestris* and *P. abies* were used for the proposed measurements.

2.2. Morphometric analysis

The seedlings were carefully lifted from the perlite and subsequently photographed using a *Sony Alpha A200 10,2 MP Digital SLR Camera* with lens Minolta AF ZOOM 0.4 m/1.3 ft. Macro. Root length and shoot length (to the cotyledon base) were measured using the program Fiji ImageJ 2.0.0-rc-54/1.51 h. Overall, we evaluated 167 pine and 283 spruce seedlings.

2.3. Genome size measurement

Genome size was determined applying flow cytometry method in 10 selected seedlings per species and treatment (altogether 40 plants). We applied the two-step procedure with internal standardisation. *Vicia faba* cv. Inovec (26.9 pg DNA, Doležel et al., 1992) was used as an internal reference standard. Seeds of *V. faba* were acquired from the Institute of Experimental Botany, Olomouc, Czech Republic. Standard plants were grown in common laboratory conditions. Nuclei were isolated from the cotyledons of young spruce and pine seedlings and leaves of internal reference standard in Petri dish using chopping technique in 1 ml of cold General purpose buffer (0.5 mM spermin.4HCl, 30 mM sodium citrate, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0.5% (*v*/*v*) Triton X-100 pH 7.0, (Loureiro et al., 2007). The resulting nuclear suspension was filtered through a 42 μm nylon mesh filter. Consequently, samples were supplemented with propidium iodide (in the final concentration of 30 μg/ml) for DNA staining, RNase (30 μg/ml) and β-mercaptoethanol (2 μl per sample). The PI fluorescence signal was measured using *Partec CyFlow ML* (Partec Gmbh, Münster, Germany) *flow cytometer* (Institute of Biological and Ecological Sciences, P. J. Šafárik University in Košice, Slovakia) equipped with 532 nm (150 mW) green laser. FCM histograms were analysed using FlowJo ver. 10.1 (FlowJo LLC, Ashland, USA) software. Three replicates per sample were made in three different days. Usually, more than 2000 nuclei were recorded per sample. If one of the three measurements did not fill quality criteria of FCM measurements (e.g. sufficient number of recorded nuclei, CV values below 6.0%), the sample was re-analysed. Then the genome size mean value of samples was calculated from three replicates. Differences in genome size between control and experimental plants were tested applying t-test in Past 3.10 (a significance level of 0.05 was used).

2.4. ROS visualisation

The control and UA treated seedlings were stained with NBT (4-nitro blue tetrazolium chloride, SERVA 30550.02) and DAB (3,3′-diaminobenzidine, D8001 Sigma–Aldrich) after the previous morphometric analysis. DAB is oxidised by H_2O_2 in the presence of peroxidases and produces a reddish-brown precipitate. NBT reacts with O_2^- to form a dark blue to black insoluble formazan compound. Both are used to display the ROS in living tissues. The staining protocol was conducted as proposed at http://www.bio-protocol.org/e1108, excluding the chlorophyll removal in hot absolute ethanol. Subsequently, the seedlings were once again photographed using the same camera and stained surface measurements were carried out in the Fiji ImageJ program. Statistical analysis of the experimental data was used to evaluate the

significance ($P < .05$) of the differences between results from the control and experimental plants and was provided by the ANOVA single factor.

2.5. Element analysis

Accumulation of macroelements (N, P, K, Ca, Mg and S) in the samples was measured using a *JEOL JSM IT 300 scanning electron microscope* (SEM) equipped with an *EDAX system* (Ametek GmbH, Germany) for performing energy-dispersive X-ray microanalysis (EDX) (Sassmann et al., 2015). Plant material of *P. abies* and *P. sylvestris* (roots and shoots) were mounted on 0.5“ aluminium specimen stubs covered with *SEM-carbon foils* (PELCO TabsTM Carbon Conductive Tabs, Double Coated, Christine Gröpl, Austria). Samples for element analysis were dried and carbon coated with a 5–10 nm carbon layer (Leica; Carbon coater, MED 020) to prevent the surface charge. For specific spectra analyses, background subtraction and data collection the EDAX-TEAM software Version V4.3 (Ametek Material Analysis, USA) was used. For deconvolution of the spectra, corrections for interference between elements were applied according to the software. Element analyses were performed with the following constant SEM settings: acceleration voltage of 20 kV, working distance 11 mm (sample to the final lens), take-off angle 35.1, dead time 30% and measurement time of 50 s (Lsec 50) for each measurement. From each sample, multiple measurements ($n = 6–7$) were taken. Taking into account that samples showed uneven surface and texture, all element analyses were performed at a magnification of 500x for plant material. Attention was taken on the orientation of sample surface in respect to the Silicium Drift Detector (SDD), model Octane Plus Det, geometry was kept stable. Thereby, a possible blur of the measurements could be minimalized. All concentrations are shown as weight percent of the dry mass (dw %).

2.6. Usnic acid determination

For quantitative identification of usnic acid from root extract of *P. abies* and *P. sylvestris* seedlings, the semipreparative *HPLC with DAD detection* (Agilent Technologies 1260 Infinity device) and 7 μm *Kromasil SGX C18* column was used. Mobile phase A (5% acetonitrile + 1% (*v*/*v*) trifluoroacetic acid) and mobile phase B (80% acetonitrile) were in gradient program with a flow rate of 0.7 ml min^{-1}: 0 min 50% A and 50% B; 25 min 0% A and 100% B; 30 min 50% A and 50% B, detection was done at 254 nm. For quantitative analysis of root extract, usnic acid (Aldrich 329967 5C) was used as a standard. Six root extracts from each conifer species were analysed. Calibration curve was in the range from 0.05 mg/ml to 0.5 mg/ml.

3. Results

3.1. Morphometric analysis

The mean values of the root length and shoot length to the cotyledon base as well as root: shoot ratio of the control and treated pine and spruce seedlings are shown in Table 1. Photos of typical representatives of seedlings used for morphometric analysis are shown in Fig. 1. It is visible that the presence of UA in substrate caused root and shoot length decrease in both conifer species tested. However, the effect was more pronounced in the case of roots, which is evident from the determination of root/shoot ratios (Table 1). In addition, we also designated the mean number of cotyledons. These values were not affected by the presence of UA in the substrate, and on the other hand, we found significant differences between species (Table 1).

The UA treatment did not affect the germination rate. Among 200 sown seeds, 52%–57% pine seeds and 88%–89% spruce seeds germinated. On the other hand, UA particularly increased the number of non-fully developed seedlings during the germination. There were only 7% of non-fully developed pine seedlings in control treatment, while 18% in UA treatment. The greatest difference was observed among spruce seedlings. There were only 2.5% of non-fully developed plants in control, but up to 33% in UA treatment. It seems that UA delays seed germination and seedling establishment of conifers is much slower.

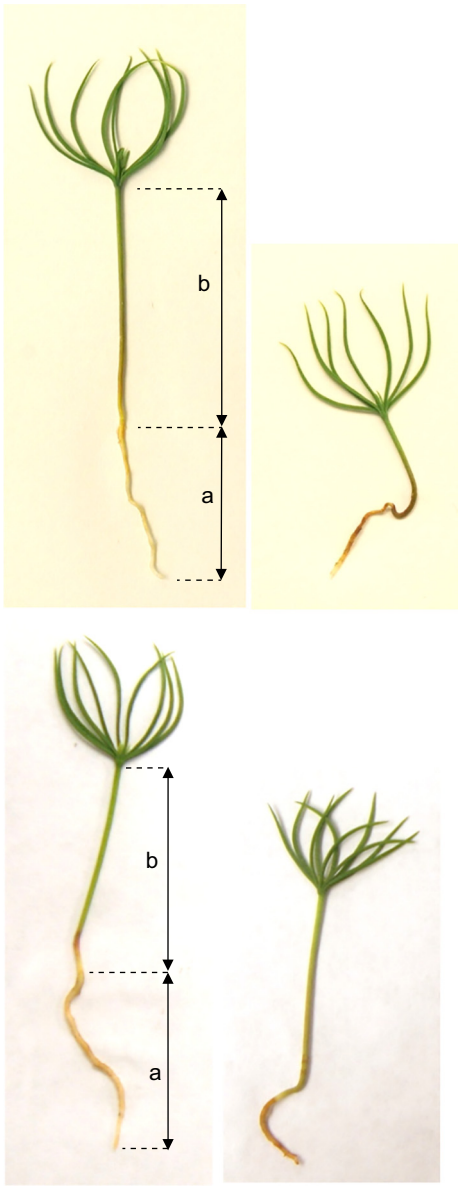

Fig. 1. Photos of representative seedlings used in experiments. Left, the control seedling with marked root length (a) and the shoot length to the cotyledon base (b). Right, UA treated seedling. Top = *P. sylvestris*, bottom = *P. abies*.

3.2. Genome size measurement

All 40 analysed seedlings were diploid except two analysed samples found in *P. abies*, from which one was found to be triploid and one tetraploid (Table 2). No signs of genome size differentiation between control and treated diploid seedlings were determined [*P. abies* ($n = 9$ per group): t-test, $t = -0.70477$, $p = .49109$; *P. sylvestris* ($n = 10$ per group): t-test, $t = 0.03340$, $p = .97373$; Table 2 and Fig. 2]. However, we found significant differences in genome size between both conifer species tested (Fig. 2).

3.3. ROS visualisation

DAB and NBT staining revealed, that presence of UA in substrate had no visible effect on the production of both, hydrogen peroxide as well as superoxide in tissues of *P. abies* and *P. sylvestris* seedlings (Fig. 3).

Table 1
Root length and shoot length to the cotyledon base in cm, cotyledon number and root:shoot ratio.

Treatment	Root			Shoot			Cotyledon			Root:shoot ratio		
	Avr	±SD	Min.–max.	Avr	±SD	Min.–max.	Avr	±SD	Min–max.	Avr	±SD	Min.–max.
PS	2.73	1.02	0.9–6.09	2.99	0.42	2.08–4.04	5.92	0.78	4–8	0.92	0.35	0.30–2.27
PS + UA	1.41	0.86	0.19–3.34	2.23	0.58	0.85–3.43	5.94	0.80	4–8	0.63	0.40	0.13–2.29
PA	2.16	0.56	1.01–4.14	3.04	0.48	2.05–4.63	8.53	0.94	6–12	0.73	0.24	0.34–1.96
PA + UA	1.16	0.60	0.17–3.03	2.76	0.45	0.95–3.97	8.33	0.98	6–11	0.43	0.22	0.06–1.10

Data are represented as average numbers (avr) ± SDs. Minimal and maximal values (min.-max.) are shown as well, $n = 100$ for PS, $n = 67$ for PS + UA, $n = 171$ for PA and $n = 112$ for PA + UA treatment. The significance values calculated from a two-factor Anova are $P < .001$ for all treatments except cotyledon number. PS = *P. sylvestris*, PA = *P. abies*, UA = usnic acid.

3.4. Element analysis

Content of selected elements is shown in Table 3. Nitrogen (N) content in the control and treated *P. abies* (PA) and *P. sylvestris* (PS) tissues were comparable. We observed a little decrease of N content in PA and PS-treated roots (3.73% and 8.2%) and a little increase in PA and PS-treated shoots (4% for both species). However, these changes were not strong enough to be significant.

Phosphorus (P) content was significantly lowered due to the presence of UA in the substrate. We found a significant decrease in the P content of both species of plant tissues. In PA roots P content decreased by 38.05% and in shoots by 17.54%. In PS roots P content decreased by 31.48% and in the shoots by 63.54%.

Potassium (K) content was significantly lowered due to the presence of UA in PA roots by 62.27%, but not significantly changed in the shoots due to the high variability of samples. K content was also significantly lower in PS roots and shoots by 50.92% and 74.55% respectively.

Presence of UA in substrate significantly decreased calcium (Ca) content in PA root by 69.87%, but did not significantly changed shoot levels. Ca content was significantly lowered in PS roots and shoots by 75.83% and 80.08% respectively.

Magnesium (Mg) content was significantly lower in PA roots and shoots due to UA presence in the substrate by 69.13% and 30.95% respectively. In PS roots, the Mg content was significantly lower by 27.69%, but relatively stable in shoots when compared to respective controls.

Sulphur (S) content was significantly lower in the tissues of both species when seedlings were exposed to UA excess. In PA roots S content decreased by 57.62% and shoots by 29.17%. In PS roots S content was lowered by 35.51% and in shoots by 66.67%.

3.5. Usnic acid content

The acetone extracts of the treated, unwashed, seedling roots of *P. abies* and *P. sylvestris* were analysed by HPLC. We found in all samples of UA treated roots detectable amounts of UA. UA content of the roots varied from 3.6 to 325.5 μg g^{-1} DW of spruce, and from 15.6 to 252.3 μg g^{-1} DW of pine roots.

Table 2
Genome size data (in pg) for 40 seedlings analysed in the study.

Ploidy level	Treatment	N	pg	SD
Picea abies				
2x	Control	9	39.62	0.22
2x	Usnic acid	9	39.71	0.30
2x	Together	18	39.66	0.26
3x[a]		1	57.00	NA
4x[b]		1	77.67	NA
Pinus sylvestris				
2x	Control	10	45.56	0.13
2x	Usnic acid	10	45.56	0.17
2x	Together	20	45.52	0.15

[a] Found in the control group.
[b] Found in the treated group, SD = standard deviation.

4. Discussion

Mat-forming lichens, including numerous species of lichen genus *Cladonia*, are an important component of terrestrial vegetation of boreal forests. One of the most typical representatives of genus *Cladonia* is *Cladonia stellaris*. Thalli of this lichen typically contain approximately 15 mg/g (*w*/*w*) of usnic acid based on its dry weight (Kytöviita and Stark, 2009). These authors used in their study 100 mg of pure UA per 284 g of soil, which served as a growing medium for cultivation of *P. sylvestris*. To soak properly 25 ml of perlite in narrow Falcon tube with 5 ml of acetone in the present study, we decided to apply 10 mg (+) UA, what is comparable availability of UA as in experiments of Kytöviita and Stark (2009).

The values of root and shoot length and their ratio of stressed plants were significantly lower for both species in comparison with the control plants ($P < .001$ for all values). The absence of mycorrhiza allowed roots to be in direct contact with usnic acid. UA-treated seedlings were visibly smaller. The decrease of plant growth due to excess of UA had been observed in previous studies. Tomatoes grown in a medium containing 40 μg/M (+) UA had significantly shorter shoots and roots and smaller leaf blades (Latkowska et al., 2008). Lascève and Gaugain (1990) observed root system dwarfism and deformation in sunflower and wheat seedlings as a result of application 50 μM UA concentration. These authors reported that UA negatively affected diverse plant development, although the studied species do not co-occur naturally with lichens.

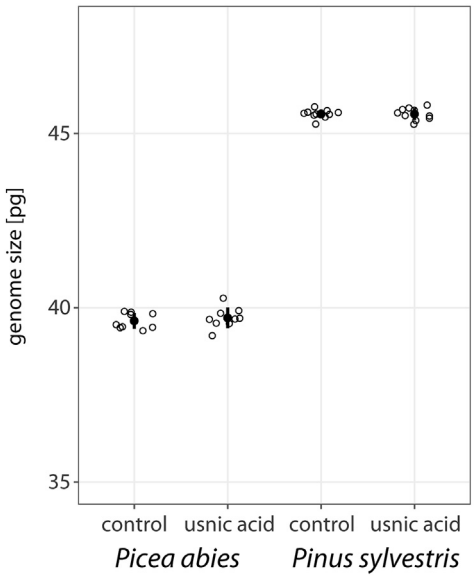

Fig. 2. Genome size variation in conifer seedlings (3x and 4x plants are not included). Comparisons are made between the control and treated groups of both *P. abies* and *P. sylvestris*. Mean values are depicted by full symbols, error bars represent standard deviations.

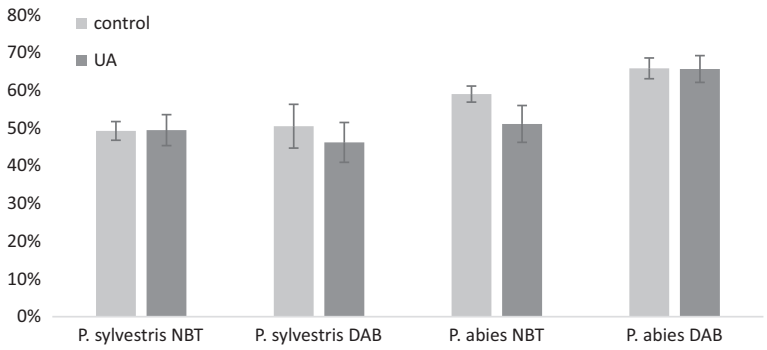

Fig. 3. Percentage of tissue coloration stained by NBT and DAB. Data are presented as means ± SDs. Value 100% is representing total plant area analysed. Numbers of replicates is as follows (from left to right): $n = 52$, $n = 35$, $n = 18$, $n = 15$, $n = 22$, $n = 20$, $n = 23$, $n = 20$. The significance values from a two-factor Anova are $P > .1$ for all treatments.

Conifers native to boreal forests are constantly exposed to secondary lichen metabolites and therefore it is possible that due to coevolution they are better adapted. Based on the above-mentioned facts we presume that the nutrient uptake and metabolism disruption might be the reason for the root growth reduction and, consequently, shoot growth reduction, which was reflected in the resulting R:S diminution in exposed plants. Cotyledons are formed during the embryogenesis when a zygote forms the embryo of a new plant (Möller and Weijer, 2009). The cotyledon number is thus predetermined and the UA cannot be expected to affect this process.

No significant differences in ploidy levels between control and treated seedlings in neither of the species were observed. The UA treatment does not act selectively against plants with high DNA content or higher ploidy level and does not generate mitotic abnormalities resulting in polyploid (aneuploid) cells in tissues. However, we discovered ploidy aberration in two *P. abies* seedlings. Among 10 randomly selected control individuals, 1 was triploid and among 10 treated individuals one was tetraploid. Visually they could not be morphologically distinguished. This rare phenomenon was previously noted by only a few authors e.g. Kiellander (1950) and Khoshoo (1959). To what extent polyploid spruces occur in the Slovak forests (and other countries) is also unknown. Kiellander (1950) suggests that the frequency of polyploid spruces is approximately 0.08‰. Polyploidy is not common among Gymnosperms. The ploidy level is determined already over the embryogenesis and the possibility of its change over the germination is highly improbable. Korshikov et al. (2012) suggests that the frequency of mitotic pathologies in root cells of spruce seedlings is not dependent on a locality. Moreover, UA does not affect DNA directly (Luzina and Salakhutdinov, 2016).

Plant cells produce reactive oxygen species (ROS) as a by-product of aerobic metabolism. Increased production of ROS in plants was observed under a wide range of unfavourable conditions, which can be generated by various abiotic and biotic factors (Bačkor et al., 2010). Accumulation of ROS is damaging to various cellular components and macromolecules. However, in this study, we did not find a significant increase of hydrogen peroxide or superoxide levels, as no significant differences in ROS production in tissues of control, or UA-treated seedlings of conifers were observed. These results are in accordance with results of Latkowska et al. (2008). They found that superoxide dismutase and peroxidase activities in UA treated tomato roots were only slightly affected, not more than 10% in comparison with the control.

The amount of UA from root acetone extracts was very variable, as we expected from the results of preliminary experiments. However, the main reason for these analyses was to confirm the direct availability of UA from the substrate to experimental plants. As a consequence of this fact, we observed a significant decrease in total plant macroelement content in treated roots of both species except for nitrogen. The similar trend was also confirmed in the above-ground part of tested conifers.

Reduced nutrient content in tissues of plants exposed to usnic acid was previously demonstrated (Lechowski et al., 2006). Some lichen secondary metabolites are capable of binding metals, including Al, Cu, Fe or Mg. Lichen substances frequently contain polar donor groups in ortho position which favour the complex formation of cations (Syers, 1969;

Table 3
Content of selected elements (dw %) ± SDs in control and usnic acid (UA) treated *P. abies* (PA) and *P. sylvestris* (PS) roots and shoots, $n = 6$ to 7.

		N (dw %)	P (dw %)	K (dw %)	Ca (dw %)	Mg (dw %)	S (dw %)
PA root	control	6.013 ± 0.34	0.439 ± 0.06	4.696 ± 0.99	0.691 ± 0.46	0.319 ± 0.14	0.350 ± 0.09
	UA	5.520 ± 0.66	0.272 ± 0.10*	1.772 ± 0.63***	0.208 ± 0.04*	0.098 ± 0.06**	0.148 ± 0.06***
	F value	2.33	11.21	38.46	9.20	19.07	25.41
	P value	0.158	0.007	< 0.001	0.013	0.002	< 0.001
PA shoot	control	4.397 ± 0.14	0.464 ± 0.05	1.776 ± 0.05	0.050 ± 0.10	0.120 ± 0.01	0.137 ± 0.01
	UA	4.571 ± 0.18	0.383 ± 0.07*	1.387 ± 0.81	0.090 ± 0.17	0.083 ± 0.02***	0.097 ± 0.02***
	F value	4.18	6.94	3.93	4.19	21.13	27.03
	P value	0.063	0.022	0.071	0.061	< 0.001	< 0.001
PS root	control	4.713 ± 0.12	0.386 ± 0.05	2.757 ± 0.27	0.473 ± 0.05	0.093 ± 0.02	0.153 ± 0.01
	UA	4.537 ± 0.39	0.264 ± 0.03***	1.353 ± 0.13***	0.114 ± 0.05***	0.067 ± 0.01*	0.099 ± 0.01***
	F value	1.33	33.04	145.35	188.06	8.45	76.00
	P value	0.272	< 0.001	< 0.001	< 0.001	0.013	< 0.001
PS shoot	control	5.366 ± 0.32	0.183 ± 0.02	0.314 ± 0.12	0.059 ± 0.02	0.061 ± 0.01	0.080 ± 0.01
	UA	5.567 ± 0.44	0.067 ± 0.02***	0.080 ± 0.01***	0.012 ± 0.00***	0.050 ± 0.01	0.027 ± 0.01***
	F value	0.32	152.11	90.62	21.55	1.36	192.20
	P value	0.585	< 0.001	< 0.001	< 0.001	0.270	< 0.001

* Significant difference with $P < .05$.
** Significant difference with $P < .005$.
*** Significant difference with $P < .001$.

Purvis et al., 1987). Lichen acids are mostly phenolics such as depsides and depsidones. (Haas and Purvis, 2006; Takani et al., 2002). Due to their metal-chelating abilities, these acids have been postulated to be major drivers of lichen promoted rock weathering (Haas and Purvis, 2006). Bačkor and Fahselt (2004) demonstrated that usnic acid may contain traces of potentially toxic metals. Crystals of UA, isolated from the lichen *Cladonia pleurota* growing on metal-rich soils were found to contain small, but detectable amounts of Cu, Ni, Fe and Al (up to 0.06% of the weight). However, reports on the possible role of UA in the chelation of macronutrients are still scant. Thus, it is possible that observed macroelements such as P, K, Ca, Mg, S were chelated by UA and subsequently, their plant-accessible amount predominantly in the root has been changed. Excess of UA to plants has numerous harmful effects (Lascève and Gaugain, 1990; Latkowska et al., 2008; Lechowski et al., 2006) including ion leakage, alternation of the plasma membrane structure and reducing nutrient content. These factors together with UA chelation can, therefore, trigger the observed change in element content.

We conclude that (+) usnic acid negatively affects selected morphometrics (root length, shoot length, root:shoot ratio) of pine and spruce seedlings. UA does not generate mitotic abnormalities resulting in polyploid (aneuploid) cells in tissues of conifers. Only two aberrated *Picea abies* seedlings (triploid and tetraploid) were discovered. Usnic acid and possibly other secondary metabolites from lichens typically show low solubility in water. However, it seems that the presence of UA may play a significant role in the boreal forest ecosystem and it can negatively affect seedling establishment. Further studies are needed to explain mechanisms of allelopathic action of UA to conifers, which are co-existing in boreal forests with mat-forming lichens.

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