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Effects of Heavy Metals on the Metabolome of *Pinus sylvestris* (Pinaceae)

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Abstract—The effects of Cu, Ni, and Cd on the *Pinus sylvestris* metabolome was studied in experimental conditions by gas chromatography–mass spectrometry (GC–MS). Structural changes in plant metabolite network became detectable on day 6 of exposure to the metals, 3–6 days earlier than visual signs of toxicity developed. Differences at the metabolome level arose earlier in a control group of plants, and specific effects of particular metals on the plant metabolome became distinct on day 9. Both nature and concentration of a metal equally contributed to the plant metabolome clustering. Plant responses (changes in concentrations of individual metabolites) to metal exposure substantially differed depending on the metal concentration (1 or 5 mM) and nature. The effects of Cd and Cu were generally similar, while the effect of Ni was often different. Dynamic changes visualized in plant metabolite matrix reflected the changes in its correlation structure, rather than depending on the set of particular compounds.

Keywords: *Pinus sylvestris*, heavy metals, metabolome, stress, toxicity, adaptation

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INTRODUCTION

Heavy metal pollution poses a significant threat to ecosystems. Unlike organic pollutants, heavy metals are nonbiodegradable and can move through the trophic chain via bioaccumulation (Singh et al., 2011). Heavy metals entering the environment in considerable amounts cause a manifold increase in stress to all ecosystem components and may consequently change the ecosystem structure (Kurilenko et al., 2004). Heavy metals are natural components of the Earth crust. Both natural processes and human activities may lead to a substantial increase in the concentrations of particular metals in the environment, including air, soil, and plants. Anthropogenic sources of metal emission include extraction of hydrocarbon fuels, incineration of wastes, nonferrous metal ore processing, and transport (Pacyna, Pacyna, 2001; Sawidis et al., 2011). The problem of heavy metal pollution is especially pressing in urban environments (Al-Khlaifat, Al-Khashman, 2007; Sawidis et al., 2011; Chen et al., 2016; Zhao et al., 2016). To develop efficient means to evaluate and to reduce increasing anthropogenic pollution of the environment, it is necessary to study the mechanisms of plant resistance to the toxic effect of heavy metals.

Cadmium, copper, and nickel are priority environmental pollutants. Cadmium is not essential for plant growth, but is easily assimilated in roots and transferred into shoots. Its minimal amounts present in the environment already pose a substantial threat to plants (Xie et al., 2014). In contrast to cadmium, copper and nickel are necessary for the normal growth and development of plants. They are toxic when their environmental concentrations are too high (Valko et al. 2006; Bhalerao et al., 2015; Hassan et al., 2019).

Assimilation and accumulation of heavy metals in excess amounts cause various morphological, physiological, and biochemical responses in plants; their manifestations include growth inhibition and distortion of photosynthesis, respiration, water metabolism, and mineral nutrition (Nazar et al., 2012; Titov et al., 2014; Bhalerao et al., 2015). Certain metal ions cause oxidative stress by producing reactive oxygen species (ROS), which alter cell metabolism and exert many toxic effects, such as lipid peroxidation, protein cleavage, and DNA damage (Pongrac et al., 2009; Kandziora-Ciupa et al., 2016). Thus, many metabolic processes are distorted in plants exposed to metal toxicity.

One of the two strategies of heavy metal resistance is utilized in plants depending on their taxonomic and ecological position: metal intake is prevented or limited in plant cells or metal detoxification occurs within

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the cell (Titov et al., 2014). Plants have been shown to possess intracellular systems that control ROS with the use of nonenzymatic antioxidants, such as glutathione, proline, ascorbic acid, carotenoids, and SH-rich nonprotein compounds, and enzymatic antioxidant systems (Pongrac et al. 2009; Kandziora-Ciupa, et al., 2016). Low-molecular-weight compounds play an important role in plant adaptation to heavy metals. Certain metabolites, such as organic acids, sugars, and polyols, can act as antioxidants, osmoprotectors, signal transduction molecules, or side products of stress-related biochemical alterations (Rizhsky et al., 2004; Shulaev et al., 2008; Xie et al., 2014).

Metabolome profiling has recently provided a potent tool to study the specifics of metabolic processes in plants (Guy et al., 2008; Fiehn, 2002; Kaplan et al., 2004). In contrast to targeted assays of particular compounds, metabolome profiling yields data on concentration changes in many organic molecules and makes it possible to study emergent behavior in stress for the plant metabolite network, which is a complex biological system.

The Scotch pine *Pinus sylvestris* L. is one of the main forest-forming conifers in the North of European Russia and is highly sensitive to atmospheric pollution and other anthropogenic factors. On evidence from many studies, the species is broadly used as a bioindicator of heavy metal pollution of the environment (Yarmishko 1997; Ivanov et al., 2013; Kandziora-Ciupa et al., 2016; Václavík et al., 2016). Pine needles with their thick epicuticular wax layer are of interest for a biomonitoring of air pollution because both passive and active substance absorption from the atmosphere is possible in tissues (Kandziora-Ciupa, et al., 2016).

The objective of this work was to study the general regular changes in *Pinus sylvestris* metabolite profile and the concentration changes in particular metabolites that arise in response to copper, nickel, and cadmium.

MATERIAL AND METHODS

Plant cultivation and exposure to heavy metals. Experiments were performed using *P. sylvestris* seeds collected in 2011 and provided by the Vyborg Forestry (Leningrad oblast). Seeds were germinated in vegetation tanks filled with 1 kg of sand, which was watered every second day with distilled water before sprout emergence and the Arnon nutrient solution diluted 1 : 2 afterwards. Seeding was performed at 50 seeds per tank, and 20 plants per tank were let to grow after sprout emergence. Plants were grown in controlled conditions with a 16-h light period at 21°C for one month. Then the same nutrient solution was used in a control variant and was supplemented with metal sulfates ($CuSO_4 \cdot 5H_2O$, $NiSO_4 \cdot 7H_2O$, or $CdSO_4 \cdot 8H_2O$) in test variants. The heavy metals were used in effective concentrations of 1 and 5 mM, which were selected in a series of preliminary experiments (Drozdova et al., 2014). The concentrations pertain to the metals rather than to their salts. The aboveground biomass accumulation rate was estimated gravimetrically. To determine the absolute dry biomass, biomass was dried to a constant weight. Samples to analyze the *P. sylvestris* metabolite profile and the Cu, Ni, and Cd contents in plants were collected on days 3, 6, and 9 of metal treatment until visual signs of toxicity developed.

Sample preparation was performed by common modern methods (Kim and Verpoorte, 2010). Plants were fixed with liquid nitrogen, extracted with methanol, and centrifuged at 4000 *g* for 10 min. The resulting extract was dried in an IKA rotary evaporator at 40°C, and the dry pellet was dissolved in pyridine. Trimethylsilyl (TMS) derivatives were obtained using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Su-pelco, United States). Silylation was carried out at 100°C for 15 min.

To determine the heavy metal contents, plant material was fixed at 105°C and dried in an oven at 70°C to a constant weight. Dry samples (2 g) were reduced to ash in an ash muffle at 450°C for 12 h. Ash was dissolved in 6 mL of a mixture of 1.5 M HCl and 3.71 M HNO_3, and deionized water was added to a final volume of 25 mL.

Sample testing. Metabolite contents in *P. sylvestris* plants were determined by gas chromatography–mass spectrometry (GC–MS), using a Maestro instrument (Interlab, Russia) with a 5975S mass-selective detector and a HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent, United States). The temperature was programmed to change linearly from 70 to 320°C at 6°C/min. The carrier gas flow rate was constant, 1 mL/min. Helium was used as a carrier gas. Mass spectrum scanning was performed in a range of 50–800 m/z at a frequency of 2 scans/s. Total ion flow chromatograms were recorded. Data were collected using Agilent ChemStation software.

The Cu, Ni, and Cd concentrations were measured with a Kvant-AFA atomic absorption spectrophotometer (Kortek, Russia).

Data processing. Mass-spectrometric data were processed and interpreted using the AMDIS program (http://www.amdis.net), the NIST 2011 database, and the mass-spectrometric database created at the Komarov Botanical Institute. Retention indices were determined using calibration with alkane standards. Semiquantitative interpretation of the metabolite profile was performed by the internal standard method with areas under total ion flow peaks, using the UniChrom program (www.unichrom.com). Tricosane was used as an internal standard and was added to samples at the step of dissolution in pyridine. It should be noted that quantitative data on the contents of particular metabolites are no more than estimates of their contents in plant extracts, but can be used to construct a metabo-

lite matrix, which is a formal result of profiling and is used to statistically model the phenomenon under study. Statistical analyses of the results were performed by multivariate statistical methods, using Microsoft Excel and MetaboAnalist.

RESULTS AND DISCUSSION

Visual signs of toxicity became detectable on days 10–12. The growth rate decreased, plants dried up, chlorosis and necrosis developed in needles, the main and lateral roots shortened. We have previously studied the specifics of the effects exerted by Ni, Cu, and Cd used at 1 and 5 mM on growth-related and morphological traits in *P. sylvestris* (Drozdova et al., 2014). The study showed that the stem and needle growth decelerated by 20% relative to a control on exposure to 1 mM Cd and by 30% on exposure to 5 mM Cd. Copper exerted almost no effect on stem growth at the lower concentration and decreased the stem growth by 30–35% at the higher concentration. Needle biomass accumulation was affected to a greater extent by Cu toxicity at both concentrations and was 35–40% lower than in the control. Nickel did not affect the growth of the stem and needles at the lower concentration, but decreased the stem growth by 5–10% and needle biomass by 20% when used at the higher concentration. This study generally confirmed our earlier findings. On day 9 of the experiment, the greatest decrease in dry aboveground biomass (by 30% relative to the control) was observed in *P. sylvestris* plants exposed to 5 mM Cd. Nickel did not considerably affect the plant biomass when used at 1 mM, but decreased the biomass by 25% when used at 5 mM. Copper used at 1 and 5 mM decreased the biomass accumulation by 20–23% relative to the control.

Metabolome profiling of plants identified compounds of the following classes: aliphatic carboxylic acids, including those involved in the Krebs cycle; amino acids; sugar acids; fatty acids; sugar alcohols; mono- and disaccharides; cyclic acids (shikimic, quinic, coumaric, and ferulic acids); flavonoids (catechins and isorhamnetin); terpenes (including abietic acid and phytol); and sterols. Some compounds were not identified, but were included in constructions of statistical models. The metabolome profiling results were represented as a heat map (Fig. 1).

As is seen from Fig. 1, concentrations of many metabolites were affected by the metals added to the nutrient solution. In addition, the plant metabolome profile changed dynamically during plant growth. A combination of the two types of changes altered the structure of the total metabolic network.

The state of the plant metabolite network was modeled by principal component analysis (PCA) and represented in the form of points in the phase space where the coordinates showed the number of molecules found in the sample at a particular time point for all structural types. Based on the PCA results, differences in metabolite network structure between control plants and plants growing in the presence of the metals were seen as a clustering of test sample metabolomes and became detectable on day 6 of exposure. No difference was observed on day 3 (Figs. 1, 2). On day 9, distinct separation in the space of principal components (PCs) was observed for the metabolomes of plants growing in the presence of particular metals; i.e., specific effects of the metals became detectable. Thus, differences in metabolome between the control and test plants became detectable earlier than differences between plants growing in the presence of different metals. The rate at which the plant response developed at the metabolome level did not directly depend on the Cu, Ni, and Cd concentrations.

As is seen from Fig. 1, concentrations of many compounds greatly varied even within the same test group. The variation results from individual specifics of the metabolome profile in plants. Individual differences certainly contribute to the structure of the plant metabolite network. Thus, the dynamic changes visualized in the metabolite matrix of plants most likely reflect the changes in its correlation structure, rather than changes in a set of particular compounds.

Statistical analyses make it possible to estimate the loads of variables, that is, the significance coefficients of particular metabolites. In the case of PCs 1 and 2, the highest significance coefficients were obtained for shikimic, quinic, and glyceric acids on day 6 of treatment with ether metal concentration. Fatty acids and succinate acquired greater significance at the higher metal concentration (5 mM) and longer exposure to the metals (Table 1). Metabolome changes induced by 5 mM metals on day 9 were additionally associated with certain amino acids (proline and serine), polyatomic alcohols (myo-inositol and ononitol), phytol, and catechin.

Quantitative analyses of particular compounds showed additionally that Cd and Cu often similarly affect metabolite accumulation, while Ni exerts a different effect. The effects of Cu and Cd were associated with nearly the same sets of metabolites, which primarily included quinic, shikimic, succinic, and fatty acids. Exposure to Ni changed metabolism of amino acids (proline and serine) and carbohydrates (glucose and galactose) to a greater effect that that of quinic and shikimic acids.

As mentioned above, a great concentration variation was often observed for many metabolites among samples of the same test group. Only a few compounds significantly differed in mean concentration between the test groups at a significance level of 95% ($p = 0.05$). The compounds included quinic, shikimic, and succinic acids and the amino acid proline. Changes in their concentrations in response to the metals are considered below.

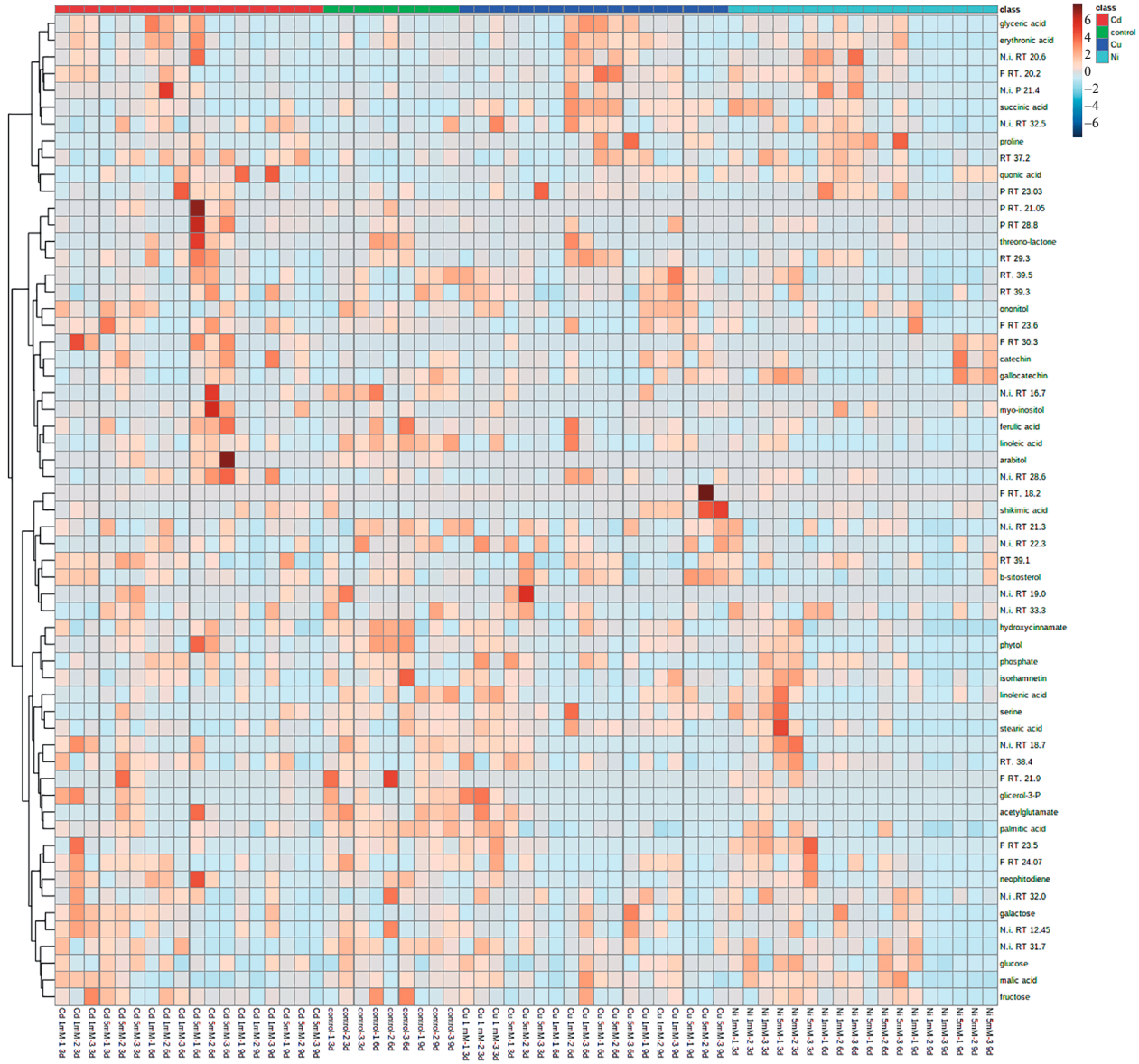

Fig. 1. Heat map of metabolites found in extracts of *P. sylvestris* plants exposed to the metals. Designations: N.i., not identified; RT, retention time; F, furanose; P, pyranose).

Figures 3 and 4 show how the quinic and shikimic acid concentrations changed in plants exposed to the metals. On day 3, the tissues concentrations of the acids tended to increase in plants exposed to 1 mM Cd or Cu and tended to decrease in plants exposed to 5 mM metals. Significant differences became detectable starting from day 6 of exposure to the metals.

The effect of Ni used at the lower concentration (1 mM) was virtually not associated with an increase in quinic and shikimic acid concentrations, in contrast to the effects of Cd and Cu. When Ni was used at the higher concentration, the acids showed higher accumulation on day 3 and decreased in content afterwards.

Shikimic and quinic acids are intermediates of the shikimate pathway, which is involved in biosynthesis of phenolic compounds. An elevated tissue amount of phenolic compounds has been observed in plants exposed to various stress factors according to the literature data. This is possible to consider as a general adaptive response to stress and as a strategy used to store carbon skeletons in conditions of limited plant growth. The mechanism whereby stress-induced biosynthesis of phenolic compounds provides for stress adaptation is still a matter of discussion. Phenolic

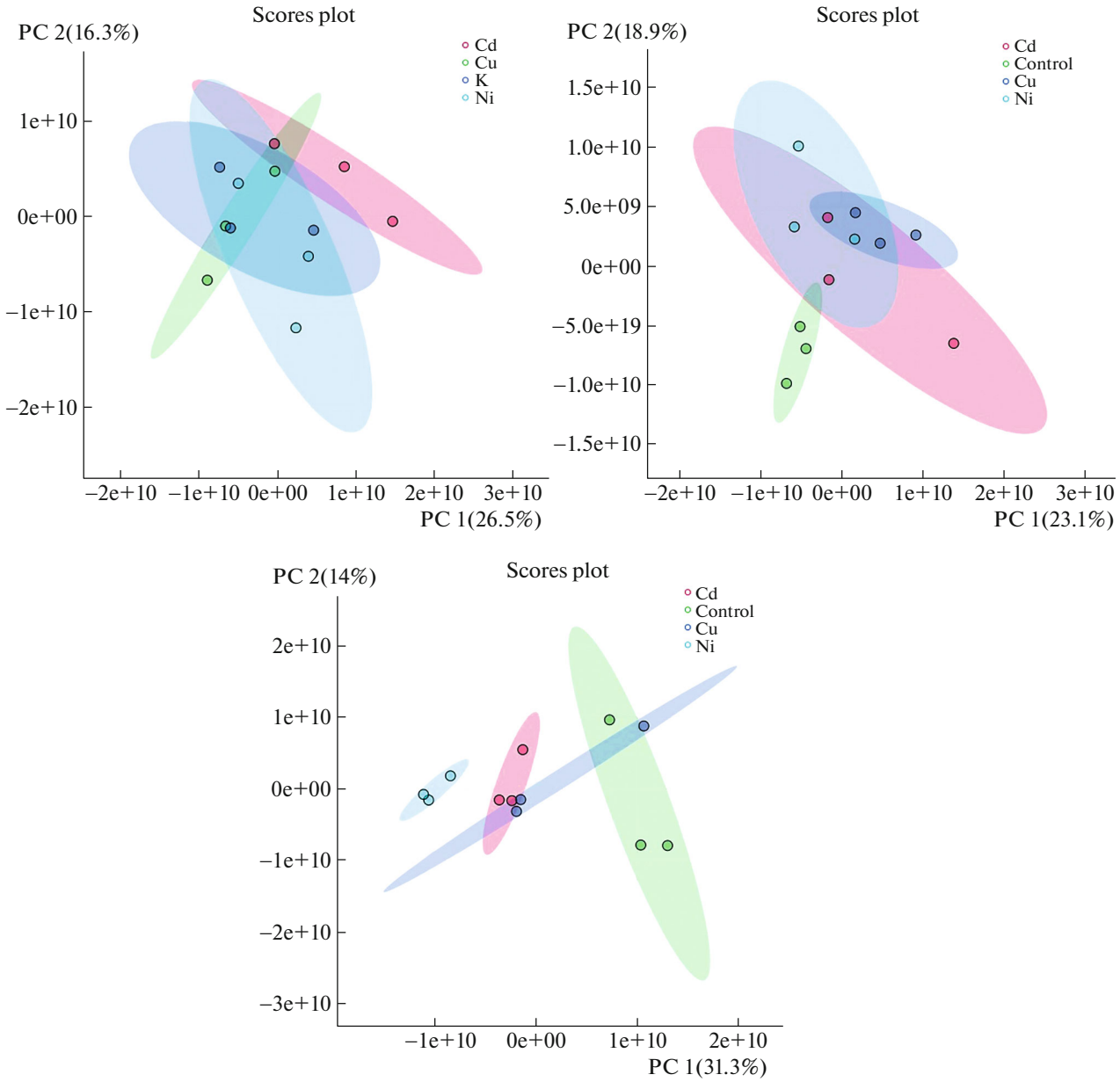

Fig. 2. PCA of *P. sylvestris* plants exposed to the heavy metals Cu, Ni, and Cd at a concentration of 1 mM. (a) Day 3; the resulting model explains 42.8% of the total variance (PC1, 26.5%; PC2, 16.3%). (b) Day 6t; the resulting model explains 42% of the total variance (PC1, 23.1%; PC2, 18.9%). (c) Day 9; the resulting model explains 45.3% of the total variance (PC1, 31.3%; PC2, 14%).

compounds presumably increase in amount when photosynthesis is inhibited to a lesser extent than the plant growth (Caretto et al., 2015).

The succinic acid content varied depending on the exposure duration, metal identity, and metal concentration (Fig. 5). Both Cd and Cu used at either concentration increased the succinate content relative to the control. An increase was observed on day 6 in plants exposed to Cu and on day 9 in plants exposed to Cd. Ni did not significantly affect the succinate accumulation in plants.

A greater accumulation of low-molecular-weight organic acids in response to heavy metals is a widespread phenomenon characteristic of plants (Xie et al., 2014; Osmolovskaya et al., 2018). Acids, such as citrate, malate, oxalate, malonate, aconitate, and tartrate, tightly bind and thus detoxify heavy metal ions (Anjum et al., 2015). A cadmium-induced increase in malate, citrate, and oxalate has been observed in *Amarantus cruentus* and *A. caudatus* leaves. The succinate content increases only in *A. cruentus* and decreases in *A. caudatus* (Osmolovskaya et al., 2019). Results of our

Table 1. The most significant compounds used to construct a statistical model of metabolomic differences

Day	Metal concentration	Compounds
6	1 mM	Shikimic acid, quinic acid, erythronic acid, glyceric acid, galactose
	5 mM	Shikimic acid, quinic acid, succinic acid, glyceric acid, arabitol, catechin, linolic acid, linoleic acid
9	1 mM	Shikimic acid, quinic acid, succinic acid, glyceric acid, erythronic acid, sitosterol, fatty acids (linolic acid, linoleic acid, palmitic acid)
	5 mM	Succinic acid, glucose, serine, proline, ononitol, catechin, myo-inositol, phytol, linoleic acid

experiment demonstrate changes in content only for free acids and cannot be used to speculate about the role of the acids in metal detoxification. Still the changes observed in succinate concentration in plants exposed to copper and cadmium indicate that carbon metabolism is specifically affected in *P. sylvestris* by the metals.

The amino acid proline accumulated in response to the metals starting from day 6. Cd and Cu increased the proline content when used at 5 mM; and Ni, at 1 mM (Fig. 6). An increase in proline in response to heavy metal exposure has been described in many plants (Balestrasse et al., 2005; Sharma, Dietz, 2006; Sun et al., 2007; Abdel-Latif, 2008), including *P. sylvestris* (Kandziora-Ciupa et al., 2016). Proline accounts for <5% of the total pool of free amino acids

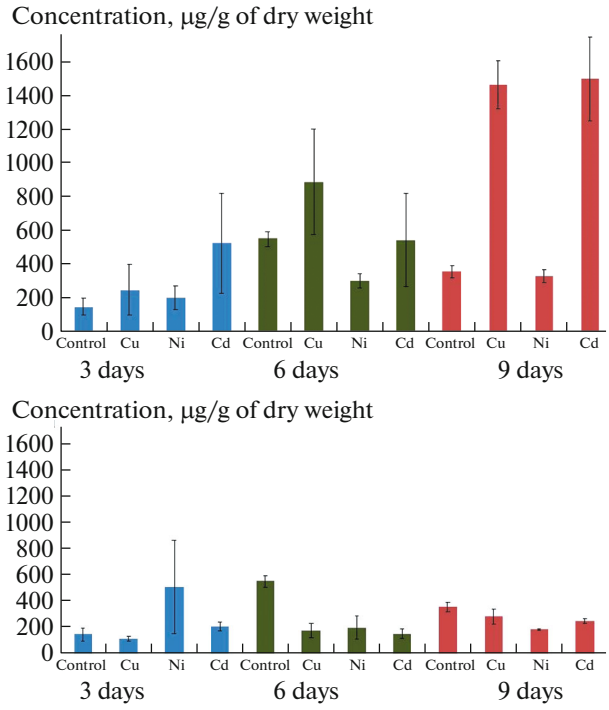

Fig. 3. Changes in shikimic acid content in *P. sylvestris* plants exposed to the metals at (a) 1 or (b) 5 mM.

in normal conditions and 80% of the total amino acid pool in stress (Kumar et al., 2010). Proline plays an important role in protecting cells from ROS-induced damage (Kartashov et al., 2008; Ivanov et al., 2013). It has been assumed that proline acts as a radical acceptor or contributes to metal ion chelation (Andrade et al. 2009).

When analyzing the concentration changes in other compounds (Fig. 1), we observed that free fatty acids (palmitic, linoleic, linoleic, and stearic acids) tended to decrease on exposure to the three metals at both concentrations (Fig. 1). Heavy metals are known to affect lipid and fatty acid metabolism (Morsy et al., 2012). Special techniques are necessary to employ in order to study the changes in lipid and fatty acid metabolism in detail.

Polyol contents substantially changed in response to the metals. Arabitol was detected only in the control plants and some of the plants exposed to 5 mM Cd. Ononitol and myo-inositol contents increased on exposure to all metals, but not until day 9 of exposure. A metal-induced increase in myo-inositol has already been described in certain plants (Osmolovskaya et al., 2019) and is most likely associated with a general distortion of plant carbohydrate metabolism.

Distinct patterns of concentration changes in response to the heavy metals were not observed for many other compounds, including unidentified ones.

Changes in plant metabolism were directly associated with metal intake and accumulation in plants (Table 2). The accumulation dynamics differed between the metals used at 5 mM. The most dramatic increase in Cd and Cu concentrations in the vegetative parts of plants was observed on day 6 of exposure. The Cu concentration showed a more than fivefold increase as compared with day 3, and the Cd concentration increased by a factor of 20. On day 9, the Cu and Cd concentrations were only 1.5–2.5 times higher than on day 6. In contrast, Ni accumulated more intensely to day 9 than to day 6. The lowest concentration on day 9 of the experiment was observed for Cu and the highest one, for Cd.

When used at 1 mM, the metals accumulated in plant tissues at a more steady rate and to far lower amounts. Accumulation in the vegetative parts of

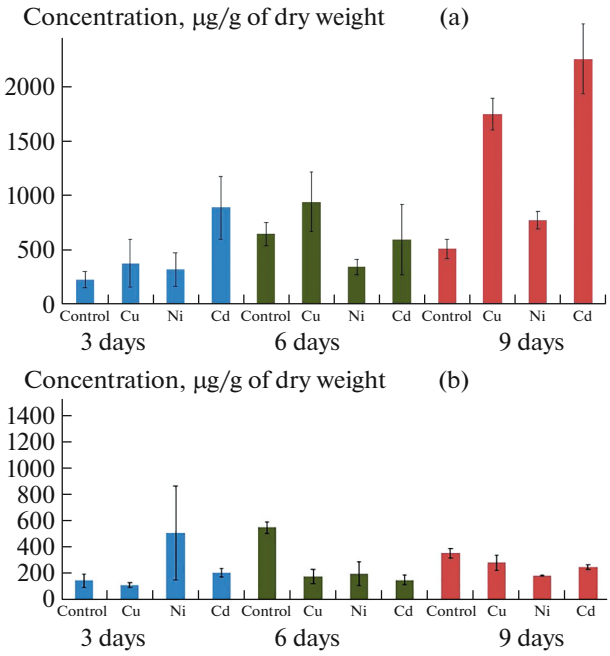

Fig. 4. Changes in quinic acid content in *P. sylvestris* plants exposed to the metals at (a) 1 or (b) 5 mM.

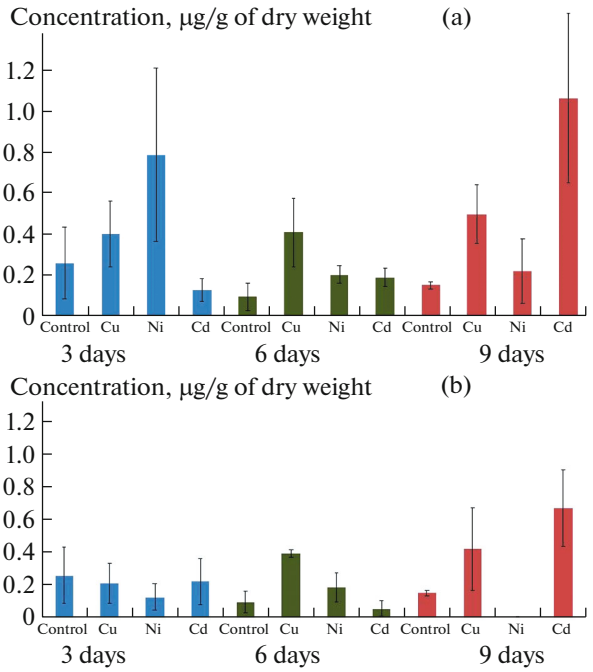

Fig. 5. Changes in succinic acid content in *P. sylvestris* plants exposed to the metals at (a) 1 or (b) 5 mM.

plants was the most intense in the case of Ni and the least intense in the case of Cu at this concentration.

Our results demonstrate that GC–MS and subsequent processing of the metabolite matrix by multivariate statistical methods makes it possible to detect a substantial part of the changes that occur in the plant metabolome in response to heavy metals. The dynamic changes visualized in the plant metabolite matrix reflected mostly the changes in the correlation structure of the metabolite network rather than the changes in metabolism of particular compounds. The formation of metabolome responses was detectable on day 6 of exposure to the metals at both higher and lower metal concentrations and seemed to lack a direct dependence on the metal intake in plants. Visual signs of toxicity did not become detectable until days 10–12. Thus, a metabolome analysis reports the biochemical changes that occur 4–6 days earlier than visual signs of toxicity. The control groups of plants was the first to separate, and specific effects of particular metals on the plant metabolome became detectable on day 9. Thus, the primary metabolomic changes that arose on exposure to the metals were most likely general stress reactions and were followed by adaptive changes in metabolism in response a particular stress factor.

Similar metabolic pathways were probably affected by the metals, leading to a common pattern of changes in the concentrations of certain metabolites. However, certain specifics should be noted in the metabolome response of plants to the toxic effects of particular heavy metals. The effects of Cu and Cd were similar and included changes in the concentrations of quinic, shikimic, and succinic acids and proline, while the effect of Ni differed appreciably. The dynamics of Ni

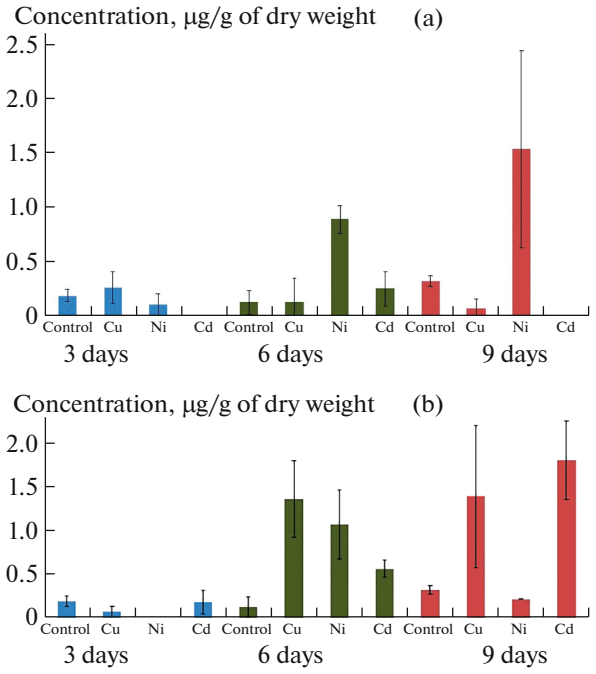

Fig. 6. Changes in proline content in *P. sylvestris* plants exposed to the metals at (a) 1 or (b) 5 mM.

Table 2. Heavy metal contents in *P. sylvestris* seedlings, mg/kg dry weight

Experimental variant	Exposure day		
	3	6	9
	Ni		
Control	1.95 ± 0.08	3.31 ± 0.16	3.41 ± 0.19
Ni 1 mM	18.5 ± 0.51	72.2 ± 1.8	96.5 ± 2.89
Ni 5 mM	190 ± 5.72	558 ± 13.9	3168 ± 110
	Cu		
Control	5.26 ± 0.05	4.90 ± 0.09	5.25 ± 1.10
Cu 1 mM	6.26 ± 0.25	12.9 ± 0.51	25.5 ± 0.52
Cu 5 mM	170 ± 0.52	1058 ± 31.1	1879 ± 56.3
	Cd		
Control	<0.05	<0.05	<0.05
Cd 1 mM	4.32 ± 0.04	30.2 ± 0.60	60.5 ± 12.2
Cd 5 mM	80.4 ± 16.1	1827 ± 54.8	3911 ± 83.4

intake in plant tissues also has its specifics; i.e., Ni used at the higher concentration slower accumulated in plant tissues as compared with Cu and Cd. Both Cu and Cd accumulated to substantial values as early as day 6 of exposure to exert their toxic effects. In the case of 5 mM Ni, similar concentrations were not reached until day 9.

A comparison of dose-dependent specifics of the metal effects showed that the identity and concentration of a metal equally contribute to the plant metabolome clustering. Plant responses to the metals (that is, changes in the concentrations of particular metabolites) substantially differed depending on the metal concentration (1 or 5 mM). The differences may indirectly indicate that adaptive processes take place on exposure to 1 mM metals, while toxic reactions develop in plants exposed to a higher (5 mM) metal concentration.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

This article does not contain any studies involving animals or human subjects performed by any of the authors.

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