Journal of Hazardous Materials 217–218 (2012) 429–438

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Changes in *Salix viminalis* L. cv. 'Cannabina' morphology and physiology in response to nickel ions – Hydroponic investigations

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ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 1 March 2012
Accepted 20 March 2012
Available online 28 March 2012

Keywords:
Nickel
Phytoaccumulation
Recultivation
Salix viminalis L.
Toxicity

ABSTRACT

The aim of the study was to assess the ability of 'basket willow' to bioaccumulate nickel with simultaneous analysis of biomass parameters and biomarkers of plants physiological reaction to the metal. Cuttings of *Salix viminalis* L. cv. 'Cannabina' were cultivated in Knop's medium containing nickel at 0–3 mM stabilized with quartz sand. Higher nickel contents were observed in *Salix* rods and roots (~15), and lower for leaves and shoots (~3 mg kg^{-1} DW) after 14 days at 3 mM Ni. The strongest inhibition was observed for root biomass, weaker for shoot, root and leaf elongation, and the weakest for the photosynthetic area (4, 24, 36, 55 and 70% of control, respectively). Soluble carbohydrates in leaves reached 340% of control at 3.0 mM Ni. Phenolics content increased four-fold at 3 mM Ni versus control, while salicylic acid content at 2.5 mM Ni was nearly 68 times higher than for Ni-untreated plants. The exudation of low molecular weight organic acids increased from ~40 to 130 μM kg^{-1} DW at 3 mM. The investigated cultivar exhibits sufficient resistance to nickel and can be cultivated at heavily contaminated sites. Nevertheless, strong inhibition of plant growth was observed resulting probably from Ni-induced disturbances in nutrient uptake accompanied by oxidative stress.

1. Introduction

The willow family, *Salicaceae*, includes three genera, i.e. poplar (*Populus*), chosenia (*Chosenia*), and willow (*Salix*), with over 300 known species. *Salix viminalis* L. – commonly called "an energy willow" – is a profitable short rotation coppice (SRC) species with a low greenhouse gas impact, relatively high yield of biomass (up to 32 tonnes ha^{-1} in one-year cycle) and high net energy production [1]. In recent years, many authors have indicated the possibility to use selected *Salix* taxa, including *S. viminalis* L., in phytoaccumulation and/or phytodegradation of contaminants from soils, sediments and effluent water due to the high tolerance to metals, inorganic salts and xenobiotics [2,3].

For the utilitarian purpose of phytoaccumulation, plants with an efficient capacity for metal sorption have been sought for the last two decades. The efficiency of metal accumulation in plants depends on numerous factors: the amount and chemical form of metal, presence of other elements causing synergistic or antagonistic interactions [4], soil/medium properties (redox potential, conductivity, organic carbon content, pH, granulometric composition) [5], and plant species/cultivar specific abilities [6]. The ability of metal biosorption is the result of complex defensive mechanisms activated within plant organs [7], combined with the exudation of specific molecules into the environment and the adaptive changes in plant structure and growth to withstand the adverse growing conditions [8].

The easiest method of estimating metal bioaccumulation efficiency of a plant species is to conduct a hydroponic experiment [9]. Although the results do not reflect the sorption abilities under environmental conditions, they provide the basic information on metal toxicity thresholds and uptake mechanisms of the species. In the present study, we investigated nickel accumulation and toxic effects on *S. viminalis* L. cv. 'Cannabina' – a cultivar with great potential in phytoremediation of metal contaminated sites and in biomass production as a source of renewable energy [10]. Nickel was chosen for the experiment as one of the metals with a slow, but gradual increase of its content in the environment due to human activity and increased industrial use (mainly metal mining and smelting, fossil fuel burning, chemical, food and electrical industry, etc.) [11]. Although nickel is an essential element for plants, its phytotoxicity thresholds and physiological contents in the majority of plant species are very low. Nickel has a great mobility from soil to topsoil parts of the plant compared with other metals; thus it may directly affect the photosynthetic tissue [12]. The toxic effects of nickel on plant growth and development are observed in a concentration range from 10 to 100 mg kg^{-1} dry weight of soil [13].

The aim of the study was to determine the ability of *S. viminalis* L. to accumulate nickel from a liquid medium and to correlate its

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http://dx.doi.org/10.1016/j.jhazmat.2012.03.056

content in willow organs with physiological as well as morphological parameters of the plant's response to nickel. Along with nickel accumulation in particular organs and biometric parameters of *Salix* growth, we investigated the roots' exudation of low molecular weight organic acids (LMWOAs) into the rhizosphere. Nowadays, the influence of LMWOAs on toxic elements' desorption from soil and phytoremediation rates is the subject of an ongoing debate. LMWOAs influence the solubility and/or mobility of the essential as well as harmful elements by acidification, chelation, precipitation and redox reactions in the rhizosphere [14]. Furthermore, we investigated the total content of phenolic compounds in *Salix* leaves. Phenolics are secondary metabolites widely distributed in plants. Effects on phenolics' biosynthesis and composition were found under biotic and abiotic stresses [15,16]. Their role in plant response to metals is well recognized, i.e. ameliorating them through chelation [17], and serving as antioxidants scavenging free radicals and reactive oxygen species (ROS) formed during oxidative stress. Among phenolics, salicylic acid (SA) was particularly assessed, being postulated as a biomarker of nickel-induced oxidative stress. Salicylic acid plays regulatory roles in plant resistance to pathogens and, probably, to other factors causing oxidative stress (ozone, xenobiotics, metals, etc.). SA alters hydrogen peroxide metabolism, leading to its intracellular accumulation and thus causing the oxidation of cell constituents, reduction of the efficiency of photosynthesis and eventually programmed cell death (PCD) [18]. Furthermore, we investigated the contents of soluble carbohydrates in *Salix* leaves. Sugars, being primary metabolites formed during photosynthesis, are a source of carbon and energy necessary for growth, lignification and biomass formation. Their contents determine the physiological and structural properties of plants and are dependent on many environmental factors including metal ions affecting their sink-source balance within a plant [19]. To conclude, a comprehensive evaluation of changes occurring in *S. viminalis* L. under the influence of nickel was planned to supplement earlier investigations on nickel toxicity to plants.

2. Materials and methods

2.1. Experimental design

One-year-old cuttings of *S. viminalis* L. cv. 'Cannabina' collected from three-year old rootstock without foliage were used in the experiment. Root formation of standardized rods (25 cm long, 15 mm in diameter) was induced under complete Knop's medium (pH = 5.51; 10 mL of 10% $Ca(NO_3)_2$, 2.5 mL of 10% KNO_3, 1.2 mL of 10% KCl, 10 mL of 2.5% KH_2PO_4, 5 mL of 5% $MgSO_4$ and 0.25 mL of 0.25% $FeCl_3$ diluted to 1 L with water). After 10 days, plants were selected according to the size of root systems to obtain a uniform group and transferred into Knop's medium (0.5 L) containing nickel salt $Ni(NO_3)_2 \times 6H_2O$ at 0, 0.5, 1, 1.5, 2, 2.5, 3 mM addition levels stabilized with steamed ultrapure quartz sand in hydroponic pots (one rod per pot and 4 plants per each Ni concentration). The 14-day experiment was performed in a climate chamber under controlled conditions (air temperature $21 \pm 1\,^{\circ}C$, relative humidity $79 \pm 1\%$), equipped with a fluorescent lamp (MASTER TL-D Secura 58W/830 1SL) providing a radiation (photon) flux of 235 $\mu E\, s^{-1}\, m^{-2}$ ($\mu mol\, s^{-1}\, m^{-2}$) at the top of the plant for 16 h a day.

2.2. Biometric analysis

For biometric analysis the total leaf surface area, mean length of shoots, roots and leaves and root biomass were measured at the beginning and at the end of the experiment to assess the increase of each parameter during the incubation. The total leaf area was measured with a DOCUPEN RC 800 portable scanner with ABBYY FineReader 6.0 Sprint and Adobe Photoshop 9.0 software.

2.3. Chemical analysis

2.3.1. Chemicals and standards

The LMWOA standards were purchased from SUPELCO with the certificated standard grade. Organic solvents were of HPLC grade, purchased from SIGMA. For pH adjustment concentrated acid (H_3PO_4, HCl, CH_3COOH) and other chemicals of analytical grade (SIGMA) were used. For Ni analysis analytical grade HNO_3 (65%), H_2O_2 (30%) and a standard Ni^{2+} solution of 1 000 mg dm^{-3} were purchased from FLUKA. All aqueous solutions were prepared with Milli-Q water.

2.3.2. Ni accumulation

Roots, rods, shoots and leaves of willow plants were washed with deionized water, dried in an electric drier at $105 \pm 5\,^{\circ}C$ for 72 h, and then ground for 3 min in a laboratory ball mill equipped with appropriate sieves to obtain the sample granulation of 0.25–0.35 mm. The material was mineralized with 65% HNO_3 and 30% H_2O_2 in a CEM Mars 5 Xpress microwave mineralization system. Ni concentration was analysed with flame atomic absorption spectrometry (FAAS) using a Varian SpectrAA 280FS spectrometer (Varian Inc., Mulgrave, Victoria, Australia) equipped with a Varian hollow-cathode lamp (HCL). Calibration curves were prepared before the analysis with five replicates per each Ni concentration. Results were validated on the basis of certified reference materials, i.e. NIST 1575a (pine needles), NCS DC 73350 (leaves of poplar) and NCS DC 73349 (bush branches and leaves), analysed in every fifth determination set.

The bioaccumulation factors (BAFs, expressed as L kg^{-1} of tissue) were calculated as the ratio of Ni concentration in willow organs (mg kg^{-1} of tissue, dry weight) to the metal concentration in the cultivation medium (mg L^{-1}) according to the USEPA regulations [20].

2.3.3. LMWOAs

Rhizosphere quartz sand samples were collected with a sample collector (100 mL in volume), mixed and dried at room temperature, and then 20 g of the samples were taken for extraction. The LMWOAs were extracted with 100 mL of water (pH = 2 acidified with concentrated HCl) in an orbital shaker at room temperature for 12 h. Extracts were filtered through Whatman No. 42 filters, and organic acids were extracted from the water solution three times with ethyl acetate (20 mL, 5 min). The solvent was reduced to a volume of 5 mL with a rotary evaporator at 40 °C and transferred into an amber glass vial. The residue was rinsed from the flask with 1 mL of distilled water and added to the vial. The solvent was evaporated at room temperature under a stream of nitrogen to obtain 1 mL of water solution. LMWOAs were analysed with a Waters Alliance 2695 Chromatograph coupled with a Waters 2996 Photodiode Array Detector (Waters Corp., Milford, MA, USA) at $\lambda = 220$ nm. Separations were performed on a Waters Atlantis C_{18} column (Waters Corp., Wexford, Ireland; 250 mm × 4.6 mm, 5 μm) at room temperature ($25 \pm 2\,^{\circ}C$) at a flow rate of 0.8 mL min^{-1}. The mobile phase consisted of 25 mM KH_2PO_4 adjusted to pH 2.5 with concentrated H_3PO_4 and methanol (95:5, v/v). A gradient elution was employed every fifth sample to final conditions of 5:95 (v/v) in 15 min to fully flush the column of hydrophobic compounds from previous injections [21].

2.3.4. Soluble carbohydrates

Salix leaves (approximately 0.5 g) were extracted for 60 min with 10 mL of ethanol/water mixture (80:20, v/v) at 80 °C [22].

Extracts were analysed with a Waters Alliance 2695 Chromatograph coupled with a Waters 2414 RI detector (Waters Corp., Milford, MA, USA). The acetonitrile/water mobile phase (75:25, v/v) was used at a flow rate of 1.0 mL min^{-1} to separate soluble sugars (fructose, glucose and sucrose) on a Supelcosil LC NH_2 column (Sigma-Aldrich, St. Louis, MO, USA).

2.3.5. Total phenolics

Salix leaves were ground in liquid nitrogen to a fine powder and approximately 0.5 g was taken for analyses. Phenolics were extracted twice with 10 mL of methanol, and after centrifugation, the content of total phenolics was measured using the Folin–Ciocalteu method [23] with gallic acid as a calibration standard. One mL of the extract was mixed with 0.1 mL of Folin–Ciocalteu reagent, and after 3 min, 1 mL of 10% Na_2CO_2 solution was added. After incubation (30 min) absorbance at λ = 765 nm was measured with a Cary 300 Bio UV-Vis (Varian Inc., Cary, NC, USA) spectrophotometer.

2.3.6. Salicylic acid

Salicylic acid was determined according to the methodology recommended by Yalpani et al. [24]. *Salix* leaves were ground in liquid nitrogen to a fine powder and approximately 0.5 g was taken for analyses. Salicylic acid was extracted twice with 3 mL of methanol, and after centrifugation, the supernatant was divided into two equal parts and the solvent was evaporated to dryness under a stream of nitrogen. A 5% solution of trichloroacetic acid (2.5 mL) was added to one part and free salicylic acid (SA) was extracted 3 times with 2.5 mL of organic mixture of ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v). To determine the total free and glucoside-bound salicylic acid (TSA), 40 units of β-glucosidase in 0.5 mL of NaAc/HAc buffer (0.1 M, pH 5.2) were added to the second part of the dry extract and incubated for 90 min at 37 °C. The reaction was terminated by the addition of 2 mL of 5% trichloroacetic acid and then salicylic acid was extracted as described above. After solvent evaporation, the dry residue was dissolved in the mobile phase (0.2 M KAc/HAc buffer, pH 5.0; 0.5 mM EDTA) and analysed with a Waters Alliance 2695 Chromatograph (Waters Corp., Milford, MA, USA) coupled with a Waters 2475 Multi-λ Fluorescence Detector. Chromatographic separations were made on a Spherisorb ODS2 Waters Company column (Waters Corp., Wexford, Ireland) (10 mm × 4.6 mm, 3 μm) with a flow rate of 1.5 mL min^{-1}. Detection parameters were as follows: λ_{Ex} = 295 nm and λ_{Em} = 405 nm.

2.4. Statistical analysis

Statistical analysis was performed with Statistica 8 software provided by StatSoft. The main and fixed effects of the two experimental factors, i.e. "Nickel addition level" to the cultivation solution and "*S. viminalis* L. organ" on nickel accumulation were tested with two-way analysis of variance (ANOVA) at α = 0.05 using the *F*-test. Significance of differences between means was tested with a post hoc Tukey's HSD test (detailed analysis presented in tables). The values of biometric parameters and physiological biomarkers of nickel impact on *Salix* were analysed simultaneously with multivariate analysis of variance (MANOVA) to test the differences between plants treated with different Ni concentrations. For MANOVA analysis, two independent tests were employed, i.e. Wilks' lambda (a commonly used test) and a Pillai's trace (a conservative one) followed by one-way ANOVAs with "Nickel addition level" as a single factor. Empirical *p*-values of one-way ANOVAs were presented in the tables (in the last row of each column). One-way ANOVA was followed by Tukey's HSD test to assess the significance of differences between treatments ("Nickel addition level") for each parameter separately. The results were shown within the tables as lower case superscripts. In addition, a Dunnett's test was performed to compare each treatment with the control. The results of the test were shown within the tables as asterisks at "% of control" value indicating the *p*-value for each treatment-to-control comparison.

A regression analysis was performed to determine the relationships between the parameters of morphological and physiological reaction of *S. viminalis* L. to nickel ions, as well as nickel accumulation in *Salix* organs (dependent variables) and nickel concentration in cultivation medium (independent variable). Analysis of significance for linear regressions was performed using the *F*-test at α = 0.05, and only significant regressions were presented in the table. In addition, polynomial regression, as well as nonlinear relationships (logarithmic, exponential) were tested. Only exponential regressions were found and dependences with R^2 greater than for linear ones were presented in the appropriate table. Furthermore, multivariate linear regressions displaying relationships between the parameters of morphological and physiological reaction of *S. viminalis* L. to nickel ions were tested for their significance at α = 0.05 (e.g. the simultaneous effect of soluble carbohydrates [glucose, fructose and sucrose] content in leaves on the rate of nickel accumulation in each *Salix* organ). Only significant regressions were presented in the table. The adjusted coefficient of determination (R^{2*}) was calculated to compare the relationships at different degrees of freedom.

3. Results

Nickel accumulation in *S. viminalis* L. depended significantly (p = 0.000) on the plant organ and the level of Ni addition to the solution (taking into consideration either the main or the fixed effects) (Table 1). The metal uptake was significantly higher for roots and wooden rods, and lower for the photosynthetic organs (leaves and shoots), i.e. ~7 and 1.8 mg kg^{-1} DW on average, respectively, and increased significantly from ~0.6 up to 9 mg kg^{-1} DW for the successive nickel concentrations in the cultivation medium (excluding 0.5 mM Ni comparing to untreated control). The effect of the interaction of the two experimental factors ("Nickel addition level × *S. viminalis* L. organ") was found significant (detailed analysis presented in Table 2). The highest nickel accumulation was observed at 3 mM Ni for roots and rods, and lower for shoots and leaves (~14 and 3.5 mg kg^{-1} DW, respectively).

According to the BAFs values, *S. viminalis* L. exhibited weak accumulation abilities towards nickel, however the absorption rate was clearly diverse for analysed organs (Table 3). The highest BAFs values (nevertheless below 1 L kg^{-1} DW) were found for willow rods and roots at 2.5 mM Ni (0.091 and 0.097, respectively), while for shoots and leaves the values reached 0.035 and 0.026 at 1 mM Ni, respectively.

A linear regression analysis revealed a significant dependence of nickel accumulation in *Salix* organs on its concentration in Knop's medium: R^2 = 0.9828, 0.9747, 0.9689 and 0.9136 for leaves, roots, rods and shoots, respectively (Table 4). However, for roots and rods, stronger exponential dependence was found, i.e. R^2 = 0.9871 and 0.9857, respectively, indicating stronger nickel sorption by the root system and wooden rods with the increase of its concentration in the solution (Table 5).

For all analysed parameters simultaneously, the MANOVA analysis was performed to assess the differentiating effect of the nickel addition level. The general hypothesis of no differences between treatments was rejected at p = 0.000 (Table 6) and this allowed us to perform the one-way ANOVAs for each parameter separately.

Biometric measurements indicated a decrease of plant biomass parameters for successive levels of nickel addition in the medium (Table 7). The highest inhibition rate was observed for root biomass

Table 1
Testing of general hypothesis in two-way analysis of variance (two-way ANOVA) for nickel accumulation in *Salix viminalis* L. organs at $\alpha = 0.05$ (df – degrees of freedom, SS – sum of squares, MS – medium square, F – statistical value, p – empirical significance level).

Source of variation	df	SS	MS	F	p
Nickel addition level (L)	6	985.5	164.3	1497	0.000
Salix viminalis L. organ (O)	3	762.6	254.2	2316	0.000
Interaction L × O	18	428.3	23.80	217	0.000
Error	84	9.220	0.110		
Total	111	2185.6			

Table 2
Nickel concentration in *Salix viminalis* L. organs and its change in relation to control plants.

Ni addition [mM]	Ni concentration in *Salix viminalis* L. [mg kg^{-1} DW]								
	Roots	% of control	Rod	% of control	Shoots	% of control	Leaves	% of control	Mean
0	$0.826^{mno} \pm 0.084$		$1.559^{klm} \pm 0.093$		$0.215^{no} \pm 0.035$		$0.134^{o} \pm 0.013$		$0.634^{F} \pm 0.594$
0.5	$1.082^{lmn} \pm 0.215$	130^{ns}	$1.609^{jklm} \pm 0.106$	103^{ns}	$0.372^{no} \pm 0.038$	173^{ns}	$0.391^{no} \pm 0.052$	292^{ns}	$0.863^{F} \pm 0.545$
1	$3.738^{ef} \pm 0.421$	453^{***}	$4.637^{e} \pm 0.305$	297^{***}	$1.901^{ijkl} \pm 0.145$	884^{***}	$1.354^{klm} \pm 0.126$	1010^{***}	$2.907^{E} \pm 1.399$
1.5	$6.144^{d} \pm 0.685$	744^{***}	$6.537^{d} \pm 0.129$	419^{***}	$2.237^{ijkl} \pm 0.319$	1040^{***}	$1.935^{hijk} \pm 0.136$	1444^{***}	$4.213^{D} \pm 2.232$
2	$8.636^{c} \pm 0.242$	1046^{***}	$9.166^{c} \pm 0.241$	588^{***}	$2.469^{ghij} \pm 0.403$	1148^{***}	$2.505^{ghij} \pm 0.300$	1869^{***}	$5.694^{C} \pm 3.330$
2.5	$12.415^{b} \pm 0.482$	1503^{***}	$12.623^{b} \pm 0.593$	789^{***}	$2.610^{ghi} \pm 0.355$	1214^{***}	$3.003^{fgh} \pm 0.136$	2241^{***}	$7.668^{B} \pm 5.038$
3	$14.654^{a} \pm 0.474$	1774^{***}	$14.816^{a} \pm 0.793$	950^{***}	$3.028^{fgh} \pm 0.283$	1408^{***}	$3.286^{fg} \pm 0.212$	2452^{***}	$8.946^{A} \pm 5.996$
Mean	$6.916^{A} \pm 5.212$		$7.150^{A} \pm 4.798$		$1.833^{B} \pm 1.070$		$1.801^{B} \pm 1.172$		

Mean values ($n = 4$) ± standard deviations. Identical capital/lowercase superscripts denote no significant ($p > 0.05$) differences between means for main effects ("Nickel addition level" or "*Salix viminalis* L. organ") and a fixed effect ("Nickel addition level × *Salix viminalis* L. organ"), respectively (according to a post hoc Tukey's HSD test following the ANOVA analysis). Additional asterisks indicate significant differences between nickel accumulation in control (0 mM Ni) and Ni-treated plants for each *Salix* organ separately according to a post hoc Dunnett's test (< > control; ns – not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

(4% of root biomass of the control plants), smaller for shoot, root and leaf elongation (24, 36 and 55%, respectively), and the smallest for the total leaf area (70%), each time for the highest Ni concentration (3 mM).

The negative linear regression was found significant for the total leaf area and the nickel concentration in the cultivation medium ($R^2 = 0.9694$) (Table 4), while for other biometric parameters negative exponential relationships with the nickel addition level were found, especially for root system parameters, i.e. root biomass and root length ($R^2 = 0.9815$ and 0.9681, respectively) (Table 5).

In the *S. viminalis* L. rhizosphere a high number of LMWOAs and a wide variation in concentrations of each individual acid were observed (Table 8). Ten LMWOAs were detected (malonic, maleic, malic, oxalic, acetic, fumaric, formic, citric, succinic and

Table 3
Efficiency of nickel uptake from cultivation solution and its accumulation in *Salix viminalis* L. organs presented in bioaccumulation factor (BAF) values.

Salix organ	Nickel addition level [mM]					
	0.5	1	1.5	2	2.5	3
Roots	0.061	0.081	0.078	0.085	0.091	0.088
Rod	0.110	0.108	0.101	0.088	0.097	0.093
Shoots	0.019	0.035	0.025	0.021	0.020	0.018
Leaves	0.019	0.026	0.023	0.022	0.021	0.018

Table 4
Analysis of significance for linear regressions displaying relationships between the parameters of morphological and physiological reaction of *Salix viminalis* L. to nickel ions (dependent variables) and nickel concentration in cultivation medium (independent variable) at $\alpha = 0.05$ (R^2 – coefficient of determination). Only significant regressions ($p \leq 0.05$) are presented in the table.

Dependent variables (Y)	Linear regression analysis (nickel addition level as an independent variable X)		
	R^2	p	Regression equation
Soluble carbohydrates content in *Salix* leaves			
Glucose	0.7975	0.0068	$Y = 3.28 + 22.85 \times X$
Fructose	0.8026	0.0067	$Y = 28.71 + 16.16 \times X$
Phenolic compounds content in *Salix* leaves			
Free salicylic acid	0.6373	0.0314	$Y = 1.15 + 6.30 \times X$
Biometric parameters			
Total leaf area	0.9694	0.0001	$Y = 183.03 - 16.83 \times X$
Shoot length	0.8149	0.0054	$Y = 8.17 - 1.95 \times X$
Root length	0.6707	0.0204	$Y = 7.65 - 1.68 \times X$
Root biomass	0.6004	0.0407	$Y = 6.70 - 2.57 \times X$
Nickel accumulation in *Salix* organs			
Roots	0.9747	0.0000	$Y = -0.61 + 4.96 \times X$
Rod	0.9689	0.0001	$Y = 0.22 + 4.66 \times X$
Shoots	0.9136	0.0008	$Y = 0.33 + 0.98 \times X$
Leaves	0.9828	0.0000	$Y = 0.11 + 1.11 \times X$

Table 5
Analysis of exponential regressions displaying relationships between the parameters of morphological and physiological reaction of *Salix viminalis* L. to nickel ions (dependent variables) and nickel concentration in cultivation medium (independent variable) (R^2 – coefficient of determination). Only significant regressions with R^2 greater than for linear dependence are presented in the table.

Dependent variables (Y)	Exponential regression analysis (nickel addition level as an independent variable X)	
	R^2	Regression equation
Soluble carbohydrates content in *Salix* leaves		
Glucose	0.9371	$Y = 10.40 + \exp(1.33 + 1.01 \times X)$
Fructose	0.9158	$Y = 31.85 + \exp(1.36 + 0.89 \times X)$
Sucrose	0.9740	$Y = 1.21 + \exp(-8.82 + 3.42 \times X)$
Phenolic compounds content in *Salix* leaves		
Total phenolics	0.9756	$Y = 12.80 + \exp(-8.20 + 3.88 \times X)$
LMWOAs in *Salix* rhizosphere		
Acetic acid	0.8251	$Y = 0.35 + \exp(-13.22 + 4.46 \times X)$
Oxalic acid	0.8212	$Y = 2.79 + \exp(-8.42 + 3.51 \times X)$
Total LMWOAs	0.8423	$Y = 3.27 + \exp(-8.47 + 3.58 \times X)$
Biometric parameters		
Leaf length	0.9819	$Y = 3.47 + \exp(0.92 - 4.19 \times X)$
Shoot length	0.8750	$Y = 2.28 + \exp(1.92 - 0.72 \times X)$
Root length	0.9681	$Y = 3.80 + \exp(1.78 - 2.07 \times X)$
Root biomass	0.9815	$Y = 1.08 + \exp(2.27 - 3.03 \times X)$
Nickel accumulation in *Salix* organs		
Roots	0.9871	$Y = -13.47 + \exp(2.60 + 0.25 \times X)$
Rod	0.9857	$Y = -8.92 + \exp(2.30 + 0.30 \times X)$

Table 6
Multivariate analysis of variance for the hypothesis of no overall "Nickel addition level" effect on biometric parameters and the level of selected biomarkers of nickel impact on *Salix viminalis* L. at $\alpha = 0.05$ (df – degrees of freedom [*er* – error, *ef* – hypothesized effect], F – statistical value, p – empirical significance level).

Test	Value	F	df_{er}	df_{ef}	p
Wilks's lambda	0	537.6	84	11.95	0.000
Pillai's trace	6	187.4	84	36	0.000

Table 7
The effect of nickel level in the cultivation medium on biomass parameters of *Salix viminalis* L. and their change in relation to control plants.

Ni addition [mM]	Leaves length		Total leaves area		Shoots length		Roots length		Roots biomass	
	[cm]	% of control	[cm^2]	% of control	[cm]	% of control	[cm]	% of control	[g]	% of control
0	$5.98^{a} \pm 0.18$		$184.2^{a} \pm 1.4$		$8.98^{a} \pm 0.12$		$9.86^{a} \pm 0.04$		$10.77^{a} \pm 0.08$	
0.5	$3.73^{b} \pm 0.06$	62***	$175.2^{b} \pm 3.7$	95**	$7.80^{b} \pm 0.24$	87***	$5.42^{b} \pm 0.03$	55***	$2.83^{b} \pm 0.03$	26***
1	$3.63^{bc} \pm 0.03$	61***	$161.3^{c} \pm 4.3$	88***	$4.57^{c} \pm 0.17$	51***	$5.32^{b} \pm 0.04$	54***	$2.16^{c} \pm 0.16$	20***
1.5	$3.62^{bcd} \pm 0.04$	61***	$157.2^{c\ d} \pm 1.7$	85***	$4.25^{c} \pm 0.10$	47***	$3.97^{c} \pm 0.09$	40***	$1.87^{d} \pm 0.10$	17***
2	$3.48^{cde} \pm 0.10$	58***	$153.8^{d} \pm 1.7$	84***	$4.65^{c} \pm 0.11$	52***	$3.91^{c} \pm 0.03$	40***	$0.96^{e} \pm 0.08$	9***
2.5	$3.39^{de} \pm 0.06$	57***	$143.5^{e} \pm 0.7$	78***	$4.35^{c} \pm 0.08$	48***	$3.86^{c} \pm 0.03$	39***	$0.84^{e} \pm 0.01$	8***
3	$3.27^{e} \pm 0.04$	55***	$129.3^{f} \pm 2.5$	70***	$2.15^{d} \pm 0.20$	24***	$3.52^{d} \pm 0.03$	36***	$0.47^{f} \pm 0.02$	4***
p-value	0.000		0.000		0.000		0.000		0.000	

Mean values ($n = 4$) ± standard deviations. Identical superscripts denote no significant ($p > 0.05$) differences between Ni concentrations for each parameter (within a column) according to a post hoc Tukey's HSD test. Additional asterisks indicate significant differences between control (0 mM Ni) and Ni treatments for mean value of each parameter according to a post hoc Dunnett's test (< > control; ns – not significant, $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$).

Table 8
The effect of nickel level in the cultivation medium on low molecular weight organic acids contents [μM kg^{-1} DW] in *Salix viminalis* L. rhizosphere.

Ni addition [mM]	Formic acid HCOOH	Malonic acid $CH_2(COOH)_2$	Acetic acid CH_3COOH	Citric acid $C_6H_8O_7$	Oxalic acid $(COOH)_2$	Lactic acid $C_3H_6O_3$	LMWOAs sum of ten acids
0	$0.248^{c} \pm 0.053$	$0.258^{b} \pm 0.034$	$2.372^{e} \pm 0.081$	$0.316^{c} \pm 0.020$	$34.682^{c} \pm 0.081$	$0.472^{c} \pm 0.061$	$38.343^{cd} \pm 0.086$
0.5	$0.340^{bc,ns} \pm 0.100$	$nd^{c,***}$	$4.455^{c,***} \pm 0.094$	$0.290^{c,ns} \pm 0.012$	$46.021^{b,***} \pm 0.472$	$0.145^{d,***} \pm 0.144$	$51.250^{b,***} \pm 0.404$
1	$0.467^{b,*} \pm 0.068$	$0.201^{b,ns} \pm 0.016$	$6.271^{b,***} \pm 0.113$	$0.556^{b,***} \pm 0.029$	$30.165^{d,***} \pm 1.889$	$0.702^{b,**} \pm 0.051$	$38.361^{c,ns} \pm 2.002$
1.5	$0.247^{c,ns} \pm 0.057$	$nd^{c,***}$	$nd^{f,***}$	$nd^{e,***}$	$0.074^{e,***} \pm 0.014$	$nd^{d,***}$	$0.753^{e,***} \pm 0.116$
2	$0.245^{c,ns} \pm 0.053$	$nd^{c,***}$	$4.794^{c,***} \pm 0.071$	$0.114^{d,***} \pm 0.031$	$29.143^{d,***} \pm 0.143$	$1.729^{a,***} \pm 0.078$	$36.026^{d,*} \pm 0.174$
2.5	$0.303^{c,ns} \pm 0.062$	$0.225^{b,ns} \pm 0.023$	$4.631^{c,***} \pm 0.098$	$0.514^{b,***} \pm 0.044$	$44.869^{b,***} \pm 0.432$	$0.723^{b,**} \pm 0.034$	$51.269^{b,***} \pm 0.387$
3	$1.712^{a,***} \pm 0.104$	$0.670^{a,***} \pm 0.049$	$15.083^{a,***} \pm 0.552$	$1.587^{a,***} \pm 0.050$	$109.033^{a,***} \pm 0.015$	$1.791^{a,***} \pm 0.092$	$129.872^{a,***} \pm 0.692$
p-value	0.023	0.011	0.000	0.000	0.000	0.000	0.004

Mean values ($n = 4$) ± standard deviations, nd – not detected. Identical superscripts denote no significant ($p > 0.05$) differences between Ni concentrations for each parameter (within a column) according to a post hoc Tukey's HSD test. Additional asterisks indicate significant differences between control (0 mM Ni) and Ni treatments for mean value of each parameter according to a post hoc Dunnett's test (< > control; ns – not significant, $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$).

Table 9
The effect of nickel level in the cultivation medium on carbohydrates (glucose, fructose and sucrose) contents in *Salix viminalis* L. leaves and their change in relation to control plants.

Ni addition [mM]	Glucose		Fructose		Sucrose	
	[mg g^{-1} DW]	% of control	[mg g^{-1} DW]	% of control	[mg g^{-1} DW]	% of control
0	$14.32^{f} \pm 0.87$		$37.56^{e} \pm 0.11$		$1.07^{d} \pm 0.10$	
0.5	$14.83^{f} \pm 0.06$	104^{ns}	$33.19^{f} \pm 0.77$	88^{***}	$0.60^{e} \pm 0.03$	56^{***}
1	$23.52^{e} \pm 0.23$	164^{***}	$44.89^{d} \pm 0.24$	120^{***}	$0.99^{d} \pm 0.09$	93^{ns}
1.5	$32.34^{c} \pm 0.28$	226^{***}	$50.17^{c} \pm 0.11$	134^{***}	$1.96^{c} \pm 0.11$	183^{***}
2	$25.76^{d} \pm 0.27$	180^{***}	$45.08^{d} \pm 0.37$	120^{***}	$2.00^{c} \pm 0.02$	187^{***}
2.5	$66.30^{b} \pm 0.47$	463^{***}	$74.11^{b} \pm 0.40$	197^{***}	$2.35^{b} \pm 0.03$	220^{***}
3	$85.91^{a} \pm 0.32$	600^{***}	$85.62^{a} \pm 0.11$	228^{***}	$8.91^{a} \pm 0.09$	833^{***}
p-value	0.000		0.000		0.000	

Mean values ($n = 4$) ± standard deviations. Identical superscripts denote no significant ($p > 0.05$) differences between Ni concentrations for each parameter (within a column) according to a post hoc Tukey's HSD test. Additional asterisks indicate significant differences between control (0 mM Ni) and Ni treatments for mean value of each parameter according to a post hoc Dunnett's test (< > control; ns – not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

lactic acids), and oxalic, acetic, formic and lactic acids were dominant. For untreated willow plants the total LMWOA concentration in the rhizosphere was nearly 4 μM kg^{-1} DW, while for plants treated with 3.0 mM of Ni it reached almost 13 μM kg^{-1} DW. The highest concentrations of a single organic acid were observed each time for the highest level of Ni addition to the medium. The exudation of total LMWOAs, as well as four dominant acids, increased from 0 to 1 mM Ni, rapidly decreased for 1.5 mM Ni, and again increased for 2 to 3 mM Ni. At 3 mM of Ni concentrations of formic, acetic, citric, lactic, oxalic and malonic acids were respectively about 7, 6, 5, 5, 3 and 2.5 times higher than for the control.

A significant exponential relationship was found for two of four dominant acids, i.e. oxalic and acetic, as well as for the total LMWOAs in *Salix* rhizosphere, and nickel concentration in the cultivation medium ($R^2 = 0.8212$, 0.8251 and 0.8423, respectively) (Table 5).

Changes in glucose, fructose and sucrose contents in *S. viminalis* L. leaves for successive Ni concentrations in the medium were found significant (Table 9). Contents of glucose in Ni-treated plants were higher than in control plants for each level of Ni addition (excluding 0.5 mM). The highest content of glucose was observed in the case of the highest Ni dose (3 mM), and was about six times higher than in untreated plants. At 2 mM Ni, a significant drop in glucose content was noted. Fructose content increased with Ni addition level up to 228% of the control, excluding 0.5 mM Ni concentration (88% of control). As in the case of glucose, a significant decrease of fructose content at 2 mM of Ni was observed. Sucrose content decreased for 0.5 and 1 mM Ni treatments when compared to untreated plants (56 and 93%, respectively), and then rapidly increased for successive levels of Ni addition, reaching over 800% of sucrose content observed for the control.

The regression analysis revealed a significant exponential dependence of glucose, fructose and sucrose contents in *Salix* leaves on nickel concentration in the solution ($R^2 = 0.9371$, 0.9158 and 0.9740, respectively) (Table 5).

Total phenolics content in willow leaves ranged from 10.56 up to 44.42 mg g^{-1} DW and was significantly related to the level of Ni addition (Table 10). Phenolics content at 0.5 mM Ni was nearly the same as in control plants and increased for successive Ni concentrations in the medium, excluding 2 mM treatment, when a significant drop was observed. The highest content of phenolics was observed in the case of the highest Ni concentration (3 mM), and was about 4 times higher than in control plants. The contents of free, as well as total salicylic acid (free and conjugated with glucose) in *Salix* leaves increased for 0.5, 1 and 1.5 mM Ni-treated plants, while 2 mM Ni addition caused a significant drop of salicylic acid contents. The highest contents were observed in the case of 2.5 mM Ni treatment, and were nearly 68 and 33 times higher versus control plants (free and total salicylic acid, respectively).

The regression analysis proved an exponential dependence of the content of total phenolics on the Ni addition level ($R^2 = 0.9756$) (Table 5), however a linear dependence was found between the content of free salicylic acid and nickel concentration in the cultivation medium ($R^2 = 0.6372$) (Table 4).

In addition, to analyse the relations between measured parameters of *Salix* response to nickel, a multiple regression analysis was performed (e.g. the relation between the accumulation of soluble carbohydrates [glucose, fructose and sucrose] and the total leaf area); significant relationships are listed in Table 11. Among soluble carbohydrates, sucrose accumulation was found to induce the exudation of LMWOAs, and linear regressions were found significant for formic, acetic, malonic, oxalic acids and the total concentration of LMWOAs ($R^2 = 0.6377$–0.8796). The decrease of biometric parameters of aerial *Salix* organs depended on fructose content in *Salix* leaves ($R^2 = 0.8090$ and 0.5464 for the total leaf area and shoot length, respectively). Simultaneously, nickel uptake by *Salix* was

Table 10
The effect of nickel level in the cultivation medium on salicylic acid and total phenolics contents in *Salix viminalis* L. leaves and their change in relation to control plants.

Ni addition [mM]	Total phenolics		Free salicylic acid (SA)		Total salicylic acid (TSA)	
	[mg GAE g^{-1} DW]	% of control	[μg g^{-1} DW]	% of control	[μg g^{-1} DW]	% of control
0	$11.14^{f} \pm 0.54$		$0.36^{f} \pm 0.03$		$2.44^{g} \pm 0.05$	
0.5	$10.56^{f} \pm 0.02$	95^{ns}	$2.27^{e} \pm 0.09$	631^{***}	$7.45^{f} \pm 0.08$	305^{***}
1	$13.98^{d} \pm 0.16$	126^{***}	$12.23^{c} \pm 0.11$	3397^{***}	$38.26^{d} \pm 0.11$	1568^{***}
1.5	$16.24^{c} \pm 0.06$	146^{***}	$11.59^{c} \pm 0.53$	3220^{***}	$71.91^{b} \pm 0.13$	2947^{***}
2	$12.88^{e} \pm 0.03$	116^{***}	$5.72^{d} \pm 0.08$	1589^{***}	$21.62^{e} \pm 0.18$	886^{***}
2.5	$17.28^{b} \pm 0.33$	155^{***}	$24.21^{a} \pm 0.11$	6725^{***}	$80.04^{a} \pm 0.22$	3280^{***}
3	$44.42^{a} \pm 0.01$	399^{***}	$16.89^{b} \pm 0.04$	4692^{***}	$61.31^{c} \pm 0.31$	2513^{***}
p-value	0.000		0.000		0.000	

Mean values ($n = 4$) ± standard deviations. Identical superscripts denote no significant ($p > 0.05$) differences between Ni concentrations for each parameter (within a column) according to a post hoc Tukey's HSD test. Additional asterisks indicate significant differences between control (0 mM Ni) and Ni treatments for mean value of each parameter according to a post hoc Dunnett's test (< > control; ns – not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

Table 11
Analysis of significance for simple and multiple linear regressions displaying relationships between the parameters of morphological and physiological reaction of *Salix viminalis* L. to nickel ions at $\alpha = 0.05$ (R^{2*} – adjusted coefficient of determination). Only significant regressions ($p \leq 0.05$) are presented in the table.

Dependent variables (Y)	Independent variables (X)	Multivariate linear regression coefficients and analysis of their significance		
		R^{2*}	p	Regression equation
	Soluble carbohydrates content in *Salix* leaves			
Total phenolics	Sucrose	0.9784	0.0000	$Y = 7.61 + 4.10 \times X$
Free salicylic acid	Fructose (X_1) Sucrose (X_2)	0.9169	0.0031	$Y = -19.74 + 0.70 \times X_1 - 2.68 \times X_2$
Malonic acid	Sucrose	0.6693	0.0153	$Y = 0.01 + 0.01 \times X$
Formic acid	Sucrose	0.8796	0.0011	$Y = 0.02 + 0.02 \times X$
Acetic acid	Sucrose	0.6868	0.0131	$Y = 0.18 + 0.14 \times X$
Oxalic acid	Sucrose	0.6377	0.0193	$Y = 1.73 + 0.97 \times X$
Citric acid	Sucrose	0.7372	0.0083	$Y = 0.01 + 0.97 \times X$
Total LMWOAs	Sucrose	0.6711	0.0149	$Y = 1.96 + 1.17 \times X$
Total leaf area	Fructose	0.8090	0.0037	$Y = 203.80 - 0.87 \times X$
Shoot length	Fructose	0.5464	0.0351	$Y = 10.25 - 0.09 \times X$
Ni accumulation				
Roots	Fructose	0.8406	0.0023	$Y = -6.90 + 0.26 \times X$
Rod	Fructose	0.8833	0.0010	$Y = -5.99 + 0.25 \times X$
Shoots	Fructose	0.6143	0.0227	$Y = -0.68 + 0.05 \times X$
Leaves	Fructose	0.7119	0.0105	$Y = -1.09 + 0.05 \times X$
	Phenolic compounds content in *Salix* leaves			
Glucose	Total phenolics (X_1) Free salicylic acid (X_2)	0.9359	0.0018	$Y = -6.17 + 1.44 \times X_1 + 1.67 \times X_2$
Fructose	Total phenolics (X_1) Free salicylic acid (X_2)	0.9440	0.0014	$Y = 22.24 + 0.94 \times X_1 + 1.29 \times X_2$
Sucrose	Total phenolics	0.9784	0.0000	$Y = -1.78 + 0.24 \times X$
Malonic acid	Total phenolics	0.7090	0.0108	$Y = -0.01 + 0.01 \times X$
Formic acid	Total phenolics	0.9640	0.0006	$Y = -0.02 + 0.01 \times X$
Acetic acid	Total phenolics (X_1) Total salicylic acid (X_2)	0.9189	0.0137	$Y = -0.02 + 0.04 \times X_1 - 0.02 \times X_2$
Oxalic acid	Total phenolics (X_1) Total salicylic acid (X_2)	0.9035	0.0177	$Y = 0.65 + 0.27 \times X_1 - 0.12 \times X_2$
Citric acid	Total phenolics	0.7978	0.0042	$Y = -0.03 + 0.01 \times X$
Total LMWOAs	Total phenolics (X_1) Total salicylic acid (X_2) Free salicylic acid (X_3)	0.9401	0.0087	$Y = 0.61 + 0.33 \times X_1 - 0.14 \times X_2 + 0.39 \times X_3$
	LMWOAs in *Salix* rhizosphere			
Glucose	Citric acid	0.5375	0.0370	$Y = 17.69 + 411.95 \times X$
Fructose	Citric acid	0.4943	0.0471	$Y = 39.35 + 281.70 \times X$
Sucrose	Citric acid	0.8796	0.0011	$Y = -0.03 + 50.81 \times X$
Total phenolics	Formic acid	0.9220	0.0004	$Y = 7.17 + 214.10 \times X$

also fructose-dependent. Stronger dependence was found for roots and rods ($R^2 = 0.8406$ and 0.8833, respectively), and weaker for photosynthetic organs ($R^2 = 0.6143$ and 0.7119 for shoots and leaves, respectively). The total phenolics content was strongly related to the sucrose content in willow leaves ($R^2 = 0.9784$); however, an antagonistic effect was found for fructose and sucrose in stimulating the accumulation of free salicylic acid. The content of total phenolics and salicylic acid simultaneously influenced the accumulation of monosaccharides (glucose and fructose) in *Salix* leaves ($R^2 = 0.9359$ and 0.9440, respectively).The exudation of LMWOAs was related to the content of total phenolics; however, in the case of dominant acids (acetic and oxalic), an antagonistic effect was found for the total phenolics and total salicylic acid content. In the case of the total LMWOAs concentration in *Salix* rhizosphere, additionally free salicylic acid was proved to positively influence their exudation by roots. Less significant relations were found for citric acid exudation and soluble carbohydrates content in willow leaves ($R^2 = 0.5375$ and 0.4943 for glucose and fructose respectively), with the strongest influence on sucrose accumulation ($R^2 = 0.8796$).

4. Discussion

Plants can withstand the presence of metals in the environment by employing diverse species-specific mechanisms of avoidance and tolerance [25]. When the concentration of a toxic element in tissue attains the phytotoxicity threshold, plants suffer severe disturbances in nutrient uptake, photosynthetic performance and gas exchange, leading to irreversible changes in their growth and development [26]. In our experiment, *S. viminalis* L. plants were exposed to the action of nickel (Ni^{2+}) in the cultivation medium, subjecting them to stress diversified by the level of metal addition.

Although nickel is thought to be quite a mobile element [27], it was found primarily in willow roots and rods, which may be a result of the short time of treatment (14 days) and/or a feature of the examined *Salix* taxon [9]. Simultaneously, nickel sorption in rods was observed at a comparable level as its accumulation in roots. A similar tendency was observed by Ali et al. [28] in the case of *S. acmophylla* Boiss. cultivated on Ni-supplemented soil. Nevertheless, nickel was also found in newly developed shoots and leaves, indicating the existence of impending Ni transport to the aboveground (aerial) willow organs. However, the BAF values below 1 indicated weak absorption abilities of investigated *Salix* cultivar towards nickel, as well as low rate of its translocation to shoots and leaves.

Although nickel is an essential metal in plant metabolism, serving as a cofactor of numerous metalloenzymes (e.g. glyoxalases, peptide deformylases, methyl-CoM reductase and ureases, a few superoxide dismutases and hydrogenases), it causes toxic effects to plants at relatively low doses. Chen et al. [29] proposed two distinct mechanisms of nickel toxicity to plants: (i) competitive interference with other essential metal ions, and (ii) induction of oxidative stress and apoptosis. As previously reported, nickel can decrease Fe

uptake and supply to aerial organs by competition, and cause nutrient deficiency followed by the retardation of germination, growth suppression and reduction of yield [27,30]. Similar to Ali et al. [28], we observed a negative effect of nickel ions on the growth of *S. viminalis* L., especially on the size of the root system (root biomass, 4% of control at 3 mM Ni), and strong inhibition of shoot elongation (24% of control at 3 mM Ni). In addition, our results confirm the previously reported significant influence of metal accumulation and the reduction of *Salix*, *Populus* and *Pinus* growth [30,31].

Metal uptake from soil solution can be modified by the exudation of low molecular weight organic acids by roots, which influence the solubility and/or mobility of the essential as well as harmful elements by acidification, chelation, precipitation and redox reactions in the rhizosphere [32]. Most studies on LMWOAs have been performed in field trials showing that interactions of organic acids with metals depend on soil type, and their deposition can be modified by root and soil structure, organic composition of the soil, pH, moisture and microbial activity, and often exhibits spatial variability at a given site and changes over time [7,33]. In pot experiments, quartz sand and Knop's solution were used to eliminate soil processes, and to assess the impact of nickel on LMWOAs exudation by *Salix* roots. As previously described, organic ligands not only enhance the solubility of trace metals, but also reduce their toxicity to plants [34]. Metals in ionic form are reported to be more toxic when compared to organically bound forms. The results presented here showed differences in concentrations of all 10 analysed LMWOAs in the *S. viminalis* L. rhizosphere depending on Ni addition level. Generally, LMWOAs exudation was significantly related to nickel concentration in the medium, with the exception of 1.5 mM Ni treatment. Probably it could be associated with the activation of another mechanism of plant tolerance to nickel. Nevertheless, the highest LMWOA concentrations in the root zone were found for 1, 2.5 and 3 mM Ni (~51, 51 and 130 μM kg^{-1} DW). The increase of LMWOA secretion can be explained by the reaction of *Salix* plants to stress, which activated mechanisms involving the tolerance of toxic elements, i.e. chelation and further transport via xylem followed by immobilization in the vacuole [35]. As previously reported, organic acids, such as citric, malic and malonic acids, bind Ni ions most efficiently [36,37]. However, in our studies, oxalic and acetic acids were found to be dominant in the *Salix* rhizosphere and strongly induced by nickel ions (~110 and 15 in total LMWOA content of ~130 μM kg^{-1} DW, respectively). This may indicate a plant defence reaction against nutrient deficiency, i.e. Fe, Mg, Ca ions, as the effect of the competitive inhibition of their uptake by nickel. The exudation of metal ligands, such as organic acids, into the medium may enhance the solubility of essential metals and improve their uptake by roots [37,38].

The first symptom of nickel toxicity is chlorosis caused by the exclusion of Ca from the Ca-binding site in the oxygen evolution complex, and replacing Mg^{2+} in the chlorophyll molecule, thus reducing the rate of photosynthesis via inhibition of the PSII electron transport chain [39]. In our experiment, we observed significant changes in contents of photoassimilates (glucose, fructose and sucrose) in leaves of nickel-treated *S. viminalis* L., probably resulting from disturbances in starch hydrolysis. These disturbances probably led to further changes in the rate of photosynthesis and in consequence in the sink/source balance within a plant. As previously described, the accumulation of soluble carbohydrates in leaves accelerated the decline in photosynthetic capacity and led to premature senescence [40,41]. The suppression of photosynthetic genes may be the effect of enhanced accumulation of glucose in leaves – a postulated signal molecule [41,42] – observed in the case of willow plants treated with Ni. The significant increase in sucrose contents in leaves of 1.5 to 3 mM Ni-treated willows (up to 833% of control) suggests Ni-caused inhibition of its transport from source to sink organs and/or the enhanced biosynthesis of reserved polysaccharides. Since carbohydrates are the main source of energy in plants, a decrease of fructose and sucrose at 0.5 mM and a significant drop of glucose and fructose contents at 2 versus 1.5 mM Ni treatment may be assumed as a consequence of an elevated energy demand of plants challenged with Ni-induced oxidative stress. In addition, at 2 mM Ni treatment probably other mechanisms of plant response to Ni, requiring more energy, were activated.

Furthermore, soluble carbohydrates are postulated to serve as signalling molecules controlling gene expression and developmental processes in plants [43,44]. However, due to their actions in many metabolic pathways, their particular role as signalling molecules is difficult to explain. Plants have the ability to monitor their levels as a result of changes in external environmental factors such as light or biotic and abiotic stressors. Usually, sugars are a source of carbon and energy metabolism as well as structural and storage molecules. Our research shows that low molecular weight sugars, mainly sucrose and fructose, may not only function as metabolites, but act as signal transducers in the phenomenon of oxidative stress induced by higher levels of nickel in the cultivation medium. It is difficult to separate the signalling role of sucrose, glucose and fructose because of easy sucrose hydrolysis to monosaccharides. Sucrose, being primarily a reserve material accumulated in plant roots, influences accumulation of sugars in leaves as a consequence of sucrose transport to sink tissues. External stress factors, such as metal ions, may result in sing/sours disturbances and probably a release of sucrose reserves. Furthermore, this could initiate the secretion of LMWOAs by roots as the first step of the defence mechanism against toxic metals. Additionally, soluble carbohydrates accumulated in leaves are proposed to stimulate the phenylpropanoid pathway and synthesis of phenolic compounds, including salicylic acid, which perpetuates the symptoms of oxidative stress and influences plants' metabolism in feedback reactions [45].

Nickel at high concentrations reduces the biosynthesis of numerous metalloenzymes (e.g. catalase, superoxide dismutase, peroxidases, glutathione reductase) by competitively reducing the uptake of Fe, Cu, Zn and Mn ions [46]. Since Ni treatment leads to enhanced indirect generation of reactive oxygen species via suppression of enzymatic antioxidant activity, it may cause the phenomenon of oxidative burst, and eventually self-oxidation of plant cells (i.e. cell death due to the ROS-mediated oxidation of cell membrane lipids, proteins and DNA). As previously described, the morphological changes in willow plants treated with nickel – a decrease of root length and biomass – are therefore probably a consequence of mitosis and cytokinesis suppression and indicate Ni-induced oxidative stress [47]. Furthermore, nickel ions probably triggered changes in physical and biochemical properties of cell membranes, affecting their permeability and ion fluxes. These changes may directly cause scarcity of water and lead to a decrease of shoot and leaf length [48].

In our studies, we confirmed the induction of phenolics biosynthesis in Ni-treated willows significantly depended on nickel concentration in cultivation medium, which may confirm their role in cell protection and detoxification mechanisms (acting as metal chelators and ROS scavengers), previously described by Michalak [49]. Localization of phenolics within plant cells depends on the metal-induced modifications of cell ultrastructure. Compartmentation and the formation of complexes with phenol derivatives in the vacuole is one of the mechanisms of plant resistance to metals [50]. However, we observed a significant drop of total phenolics content at 2 versus 1.5 mM Ni treatment. We assume that at this concentration nickel may cause a rearrangement of the *Salix* reaction. Since phenolics act not only as protective molecules, but also as structural components, a drop of their content may be a result of enhanced lignification of the cell wall for sufficient immobilization of metal ions [51].

Among phenolic compounds, salicylic acid content in *S. viminalis* L. leaves amounted to 80 $\mu g\, g^{-1}$ DW, and was each time higher versus untreated control plants. According to earlier reports, plants can accumulate salicylic acid even up to 75 $\mu g\, g^{-1}$ fresh weight, e.g. *Nicotiana tabacum* L. 6 h after inoculation with tobacco mosaic virus [52]. It is likely that distinct threshold concentrations of salicylic acid are required to induce programmed cell death (PCD) in plants challenged with diverse stress factors [53]. We observed high concentrations of salicylic acid glucoside, about 75% at 3 mM Ni of total salicylic acid (sum of free and glucosilic salicylic acid), which may confirm the storage mechanism of a locally and systemically active acidic form, probably for the purpose of further stress and cross-tolerance [54], and/or effective detoxification of salicylic acid, whose local concentration may exceed the phytotoxicity threshold [55]. It can be assumed that the enhanced biosynthesis of salicylic acid in leaves of Ni-treated willows confirms its involvement in PCD via the impact on hydrogen peroxide metabolism in response to toxic concentrations of nickel. According to Durner et al. [56], salicylic acid influences the cellular redox state and potentiates the generation of ROS in the protoplast, triggering suicidal cell death. In our experiment, the accumulation of free salicylic acid in *Salix* leaves showed an exponential dependence on the level of nickel addition. We assume, that the primarily observed increase of salicylic acid content in leaves (0.5–1.5 mM Ni) was probably the result of salicylic acid transport from roots, as a signal molecule informing distant plant organs about adverse growth conditions. After a significant drop at 2 versus 1.5 mM Ni addition, a second phase of salicylic acid accumulation was observed, most likely on account of enhanced nickel sorption and induction of its biosynthesis directly in leaves.

5. Conclusions

Taking into consideration the permanent increase of energy demand and progressive soil degradation, it is important to find energy crops characterized by both high biomass production and significant phytoremediation and/or recultivation potential. In our studies, "basket willow", i.e. *S. viminalis* L. cv. 'Cannabina', was cultivated hydroponically in Knop's medium containing up to 3 mM Ni. It can be assumed that this cultivar exhibits sufficient resistance to Ni ions together with a relatively low nickel uptake to the aboveground organs and can be cultivated even at heavily contaminated sites, primarily for the recultivation of post-industrial areas to restore soil microflora. Nevertheless, strong inhibition of plant growth was observed resulting from Ni-induced disturbances in nutrient uptake, and simultaneously occurring oxidative stress. This study demonstrated that *S. viminalis* L. could release, in a short period (14 days), relatively high amounts of LMWOAs into the cultivation solution in conditions of increasing concentrations of nickel. Since LMWOAs exhibit a capacity to promote solubilization of metallic elements, their exudation may enhance the availability of nutrients and reduce the phytotoxicity of unessential metals to the investigated *Salix* cultivar. The rate of LMWOAs exudation was probably regulated by changes in carbohydrates metabolism and phenolics accumulation in *Salix* leaves. Ni-treated *S. viminalis* L. plants accumulated soluble carbohydrates, i.e. glucose, fructose and sucrose, in leaves, probably due to disturbances in starch hydrolysis and changes in their transport, which in turn negatively affects photosynthesis efficiency. The induction of phenolics biosynthesis and their accumulation in leaves of *S. viminalis* L. treated with nickel significantly depended on nickel concentration in solution, suggesting activation of defence mechanisms in protoplast due to the protective function of these metabolites as antioxidants and metal chelators. Furthermore, the excessive accumulation of low molecular weight phenolic compounds may suggest the first stages of lignification for effective metal binding within the cell wall. Nickel accumulation caused the enhanced biosynthesis and accumulation of salicylic acid in *S. viminalis* L. leaves in free as well as in glucosidal forms, which proves the occurrence of Ni-induced oxidative stress followed by cell apoptosis.

Acknowledgements

The authors would like to kindly thank Prof. Zygmunt Kaczmarek from the Institute of Plant Genetics, Polish Academy of Sciences for his support in statistical analysis of the data throughout this paper. The authors wish to acknowledge the financial support of part of the studies by project grants N N305 045836 and N N305 372438 from the Polish Ministry of Science and Higher Education.

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