Carpathian Journal of Earth and Environmental Sciences February 2025 Vol. 20, No. 1, p. 183 – 196; DOI:10.26471/cjees/2025/020/324

THE ROLE OF PHYTOHORMONES IN PHYTOREMEDIATION USING REED

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Abstract: Salinity is a global problem and salinity areas are constantly increasing because of poor-quality irrigation systems containing high concentration of dissolved salts, salt penetration, and water pollution. This study deals with the phytoremediation of salts from mine water using phytohormones auxin 1-naphthylacetic acid (NAA) and cytokinin (m-topolin) at a concentration 10^{-5} mol/L and common reed (Phragmites australis). The reed plants collected at the Lazy Mine were tested for their ability to accumulate salts from the surrounding mine waters. The experiment was carried out under controlled and controllable conditions in a phytotron. The hypothesis to be tested was a positive effect of phytohormones on the ability of the common reed to better accumulate salts from mine wastewater. The results show that the application of phytohormones in the long-range period, especially auxin, at a concentration of 10^{-5} mol/L could have a positive effect on the ability of reeds to accumulate salts from mine waters.

Keywords: common reed; phytoremediation; mine water; phytohormones; salt stress

1. INTRODUCTION

Salinity is a global problem and salinity areas are constantly increasing due to light rains, poor-quality irrigation systems with a high content of dissolved salts, salt penetration, and water pollution. In addition, the mechanism of plant stress tolerance is a very complex phenomenon (Mishra & Tanna, 2017). High salinity and insufficient water intake are important environmental factors affecting plant growth and productivity with consequent ionic toxicity, osmotic stress, and nutrient imbalances (Atta et al., 2023).

Salinity is a main environmental issue, dominant in arid, semiarid, and coastal areas, which are heavily affected by precipitation of soluble salt in soils and water systems, due to both natural and anthropogenic activities. High concentration of soluble salts in water and soil is well known to inhibit plant growth and development, directly affecting crop productivity and directly leading to land degradation. It also affects non-agricultural lands and urban landscapes through subsidence, corrosion. Groundwater pollution also affects urban landscapes. Moreover, it impacts negatively human health (Etikala et al., 2021; Mohanavelu et al., 2021).

However, some organisms have developed a unique biochemical, physiological and/or morphological mechanism that allows them to grow

and develop under extreme salt conditions. Plants with these characteristics are known as halophytes (Pongrac et al., 2013). Halophytes, plants that grow and propagate in environments where the salt concentration is about 200 mM NaCl or more, represent about 1 % of the world's flora. Some halophytes show optimal growth with salinity. Others grow optimally in low salinity conditions, but tolerate higher salt concentrations (Flowers & Colmer, 2015; Flowers et al., 2015; Abobatta, 2020; Chen & Wang, 2024).

Phytohormones are low molecular weight substances, produced naturally by plants, which are responsible for normal plant development such as root and shoot growth, flowering or fruit set and drop. They can act in tissues distant from the site of synthesis, even in trace quantities. The first five discovered hormone groups (auxins, cytokinins, gibberellins, abscisic acid and ethylene) have been joined more recently by polyamines, brassinosteroids, jasmonic acid, salicyl acid, and peptides that more or less fit the classic definition (Davies, 2010). Although primary metabolism supplies the energy and building blocks of plant life, plant hormones are irreplaceable in controlling the way plants grow, develop and react to both biotic and abiotic forms of stress. Hormones regulate the growth rate of individual tissues, organs and integrate them to produce a viable and fertile plant (Davies, 2010).

This study aimed to verify the phytoremediation capabilities of common reed's *(Phragmites australis)* root system and its stimulation with an exogenous supply of auxin IAA and/or cytokinin meta-topolin at a concentration of 10^{-5} mol/L. Auxin conjugates are believed to play major role in homeostasis as storage forms for the active plant hormone indole-3-acetic acid (IAA). In its free form, IAA comprises only around 25 % of the total amount of auxin, depending on the tissue and plant species studied. The main forms of the IAA conjugate are usually low molecular weight esters or amides, but there is increasing evidence for the occurrence of peptide and protein auxin conjugates (Ludwig-Müller, 2011). Stimulation of root formation is one of the significant biological activity of auxins. In many cases (including organ development), auxins act synergistically or antagonistically with other plant hormone species, mainly cytokinins. It depends on the context and their respective levels (Jing & Strader, 2019). The study of the interconnection and complexity of hormones, as well as nutrients, environmental stimuli, and stress signals that are involved, for example, in root development, is still in the early stages. Significant problems remain to be solved in understanding the dynamics of these networks (Jing & Strader, 2019).

Cytokinins (CK) are an important group of plant hormones, promoting, in the presence of auxin, cell division in both roots and shoots. They also affect apical dominance, axillary bud growth, chlophyll stability and senescence (Davies, 2010).

Topolins are natural aromatic molecules that have been developed as an effective alternative for 6-benzylaminopurine (BAP) and other canonical cytokinins used in in vitro culture of plants. Among them, 6-(3-hydroxybenzylamino) purine (meta-topolin), is the most active and its use in plant tissue culture has increased rapidly (Davies, 2010). During the last few decades, there have been numerous publications reporting the effectiveness of meta-topolin in micropropagation process as well as fighting against various physiological disorders, promoting rooting and acclimatization of *in-vitro* raised plants.

Phytoremediation is an innovative and alternative method that leverages plants inherent abilities to absorb, detoxify, and accumulate pollutants, effectively cleansing soil, water, and air. This approach has gained significant global interest for its potential to address environmental contamination (Khan et al., 2014; Park & Oh, 2023).

As an environmentally conscious and sustainable strategy, phytoremediation harnesses the natural power of plants to mitigate the harmful effects of pollutants on ecosystems. This method not only offers environmental benefits, such as solar-driven technology, but also provides economic advantages, making it a promising solution for achieving sustainability and resilience goals in modern societies (Nissim et al., 2023). Moreover, phytoremediation employs various plant species to minimize, remove, or immobilize soil contaminants, including heavy metals (Avkopashvili et al., 2022). Although effective and cost-efficient, this *in situ* method may require a longer timeframe to deliver the desired results (Lacatusu et al., 2012).

Common reed is a plant often used for sewage treatment of vegetation roots due to its ability to massively grow underground parts (roots and rhizomes) up to a depth of 60 - 70 cm (in favorable conditions up to 1.5 m). The above-ground part reaches a height of up to 4 m (in warmer areas and with a good supply of nutrients up to 6 m). Common reed propagates by rhizomes, grows very fast, and is very invasive. It tends to grow outside the sewage field, leading to clogged drainage, and in combination with other plant species it may displace them. It is relatively resistant to fluctuation in pH, organic and inorganic pollution, and climatic conditions. It is one of the most widely used species of wetland plants for

water phytoremediation, due to its absorption capacity and tolerance to the contaminated environment (Bonanno & Giudice, 2010; Brodská, 2018).

In this study, the primary objective was to investigate whether *Phragmites australis*, when stimulated with the phytohormones auxin and cytokinin, can accumulate specific saline water indicators. Phytohormones were applied to enhance root system efficiency, promoting the distribution of salts within the biomass. The process was monitored through analyses of salinity indicators in saline mine waters (e.g., chlorides, sulphates, dissolved inorganic salts, and trace elements such as sodium, potassium, calcium, and magnesium), as well as pH and conductivity measurements. Additionally, the study involved detailed biomass analyses, including phytohormones (auxins and cytokinins) and selected trace elements, to assess the role of hormonal stimulation.

2. MATERIALS AND METHODS

2.1. Plant material

The common reed (Phragmites australis) used in this study was taken from the Lazy mine (Czech Republic), specifically from the place where the wastewater from the mine enters the sedimentation tanks (GPS: 49°50‘3.238“, 18°26’43.805“). As verified by experiments, this reed is able to tolerate high salt concentrations at this location (Brodská, 2018).

2.2. Mine water and sediment sampling

Mine water samples were taken from the wastewater flow that entered the wastewater reservoir plant. The sampling was carried out at regular monthly intervals by following Standard ČSN EN ISO 5667-1 - CSN 75 7051 (ČSN, 2007). For sampling, we used 5-liter polyethylene samplers. The samples were delivered to the laboratory immediately after collection.

Analysis of the sediment collected in the Lazy Mine was performed once, on the same day and at the same sampling point as mixed wastewater and plants. The ČSN EN ISO 17402 - 836500 standard was followed during sampling (ČSN, 2012).

2.3. Plant adaptation to phytotron conditions and experiment implementation

The common reed clusters were set in six standard plastic pots (Ø 20 × 22 cm). Sludge sediment was used as a substrate. The reed samples this prepared were placed in a phytotron type Wiss Gallenkamp where they were adapted to the new environment for 90 days at 22 °C, 12 hours of day light / 12 hours of dark, and 60 % humidity, light intensity 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ Throughout the adaptation, the plants were watered 3 times a week with 250 mL of mixed mine water.

During the experiment, the pots with tufts of reeds were divided into three plastic plates without drainage (90 × 40 × 10 cm). Four pots with 24 bunches of reeds were placed on each plate. The plates were properly marked with the letters A (NAA, left plate), B (m-topolin, middle plate), and C (DMSO, right plate). The calculated aliquots of both phytohormones for watering plates A and B were dissolved in 5 mL of DMSO transferred to the solution, and made up to a volume of 1 L with mixed wastewater. At the same time, two pots with bunches of reeds, which were intended as control samples, were placed in the phytotron. The bunches of reeds in those pots were watered only with mine water.

The plants placed on the plate A were watered by wastewater with the addition of auxin, specifically 1-naphthyl acetic acid (NAA). The plants on the plate B were placed in mixed wastewater with the addition of cytokinin, specifically m-topolin. Stimulation of plant root growth by auxin is usually induced by a concentration in the range of 10^{-5} mol/L (Davies, 2010). Also in this case, the concentration of 10^{-5} mol/L was chosen. The same concentration of 10^{-5} mol/L was also chosen to stimulate the upper part of the plant by the cytokinin used.

The plants on the plate C, treated with DMSO, were used only as a control sample for phytohormones determination. The mixed waste (mine) water did not contain auxin or cytokinin (data not shown). The water used for the plate C contained only the dimethyl sulfoxide solvent (hereafter DMSO) at a concentration of 5 %. This solvent was chosen because of good solubility of both plant hormones.

The plants were regularly watered with 250 mL of mixed wastewater 3 times a week. On the day of application of auxin and cytokinin, regular monthly monitoring of pH, conductivity, SO_4^{2-}, Cl^-, Na^+, K^+, Ca^{2+}, and Mg^{2+} was started in mine water used and it was carried out for one year. For all analyses, the leaves and roots of the plants and sediment were taken from all three plates.

2.4. Determination of phytohormones

2.4.1. Determination of auxins

To analyze endogenous auxins, reed leaves were taken from the control tray B. A sample of 10 mg of reed leaf was analyzed in triplicate. The samples were

homogenized in a mortar in liquid nitrogen (-196 °C) and accurately distributed into 2 mL microtubes. The samples were purified using C8 cartridges (Bond Elut, 500 mg, 3 mL; Varian), [$^{13}C_6$]IAA, [13C6]oxIAA, [13C6]IAAsp and [13C6]IAGlu (5 pmol of each) were added as internal standards. The samples were evaporated to dry and dissolved in 30 µL of 10 % MeOH. 10 µL of the sample was injected and separated on chromatography column KINETEX 1.7 µm C18, 50 × 2.1 mm from Phenomenex (Torrance, USA) by UHPLC-MS/MS (Acquity UPLC®; Waters, Milford, MA, USA) (Novák et al., 2008).

2.4.2. Determination of cytokinins

To determine endogenous phytohormones, reed samples (20 mg) were taken in triplicates from all three trays on the day of phytohormone administration and on day 7 after phytohormone treatment. The leaf and root samples were homogenized in a mortar in liquid nitrogen (-196 °C) and accurately distributed into 2 mL micro tubes. The samples were extracted in 1 mL of modified Bieleski buffer (60 % MeOH, 10 % HCOOH and 30 % H_2O) containing a mix of 23 labelled cytokinin standards for control of the purification steps and to valid determination. Purification was performed using combination of solid phase extraction (SPE) columns of reverse phase C18 (100 mg) and MCX cartridges (30 mg) and extended by immunoaffinity chromatography (IAC) based on wide-range of CK-specific monoclonal antibodies, which relies on antibody-antigen interaction to form an immunocomplex (Ab-Ag) (Hauserová et al., 2005). The samples thus purified were evaporated to dry and dissolved in 30 µL of 10 % MeOH.

In the reed samples, 47 cytokinins and their metabolites were determined using UHPLC-(ESI+) MS/MS. Harvested leaves and roots were washed, dried with filter paper and immediately frozen in liquid nitrogen. Frozen samples were homogenized, divided into triplicates and subsequently extracted, purified C18 / SCX SPE followed by immunopurification, and measured by tandem mass spectrometry. Mean values (±SD) from triplicate measurements of three biological replicates are shown.

Separation and quantification of CK metabolites were performed by ultra-high performance liquid chromatography (Acquity UPLC®; Waters, Milford, MA, USA) coupled to a XevoTM TQ MSTM (Waters, Milford, MA, USA) triple quadruple mass spectrometer equipped with an electrospray interface (ESI+). The separation was carried out on a reverse phase column (Aquity UPLC, BEH C18, 1.7 *m*m, 2.1 × 50 mm, Waters) and analytes were eluted within a 5-minute linear gradient consisting of MeOH and 15 mM $HCOONH_4$, adjusted to pH 4.0 at a flow rate of 0.5 mL/min, ratio from 10:90 to 100:0 and a column temperature of 40 °C. The quantification of endogenous CK was achieved by multiple reaction monitoring (MRM) of $[M^+H]^+$ and appropriate product ion. Conditions (dwell time, cone voltage, and collision energy in the collision cell) corresponding to the exact diagnostic transition were optimized for each cytokinin. MassLynx software was used for quantification with the method of comparing the endogenous CK ratio and the ratio of labelled standards used with known concentration (Novák et al., 2008).

2.5. Determination of selected indicators in wastewater

The sampling followed the valid standards ČSN EN ISO 5667-1 - ČSN 75 7051 (ČSN, 2007). Determination of chlorides was carried out according to ČSN 75 7422 (ČSN, 2015). Chloride measurements were performed using a HACH DR/2000 spectrophotometer (Hach Company, USA) at a wavelength of λ 455 nm. The determination of sulphates was carried out using the turbidimetric method for determination of sulphates with barium chloride (Rossum & Villaruzz, 1961). Sulphate measurements were also performed using a HACH DR/2000 spectrophotometer (Hach Company, USA) at a wavelength of λ 450 nm. Determination of sodium and potassium was carried out according to ČSN ISO 9964 (ČSN, 1996) by flame emission spectrometry (AES) using an atomic spectrometer ∑ fy GBC. The emission intensity of these elements is measured in an acetylene-air flame, potassium emission is measured at a wavelength of 770 nm, sodium emission at a wavelength of 589 nm. Determination of calcium and magnesium was carried out according to ČSN ISO 7980 (ČSN, 1995) by atomic absorption spectrometry (AAS) using an atomic spectrometer ∑ fy GBC. The emission intensity of these elements is measured in an acetylene-nitrous oxide flame, magnesium emission is measured at a wavelength of 285 nm, calcium emission at a wavelength of 422 nm. Determination of dissolved inorganic salts was carried out according to ČSN 75 7347 (ČSN, 2009). Conductivity was measured according to ČSN EN 2788 - Water quality - determination of electrical conductivity. The pH determination was carried out according to ČSN ISO 10523 (ČSN, 2010).

2.6. Determination of selected indicators in sediment and plant material

Sediment sampling was conducted in accordance with the applicable standard ČSN EN ISO 5667-12 (757051) (ČSN, 1997).

The mineralization of the samples was carried out by closed microwave decomposition using the pseudotail mineralization method according to ČSN EN ISO 15587-2 (ČSN, 2003), BERGHOF mineralization device. The reagents used were nitric acid (65 %) and hydrochloric acid (35 %). Determination of sodium and potassium was carried out according to ČSN ISO 9964 (ČSN, 1996), part 3 by flame emission spectrometry (AES) using an atomic spectrometer ∑ fy GBC. The emission intensity of these elements is measured in an acetylene-air flame, potassium emission is measured at a wavelength of 770 nm, sodium emission at a wavelength of 589 nm. Determination of calcium and magnesium was carried out according to ČSN ISO 7980 (ČSN, 1995) by atomic absorption spectrometry (AAS) using an atomic spectrometer ∑ fy GBC. The emission intensity of these elements is measured in an acetylene-nitrous oxide flame, magnesium emission is measured at a wavelength of 285 nm, calcium emission at a wavelength of 422 nm.

3. RESULTS

3.1. Evaluation of analyses of selected mixed mine water parameters

The values of pH, conductivity, dissolved inorganic salts, chlorides, sodium, potassium, calcium, magnesium, sulphates in the mixed mine water taken at the Lazy mine were delivered to the laboratory. The processing of the results of the above indicators is given in Table 1.

Conductivity in the mixed mine water samples at the Lazy mine ranged from 5.51 to 6.42 mS/cm, peaking on December 5, 2016, with increased concentrations of soluble inorganic salts and all ions observed on this date (Table 1).

3.2. Evaluation of analyses of selected sediment parameters

Figure 1 shows the average values of ions in the sediment collected at Lazy mine on May 3, 2016: Ca^{2+} concentration was 48.37 mg/kg, Mg^{2+} 31.25 mg/kg, Na^{+} 20.00 mg/kg and K^{+} 19.33 mg/kg. Plants were grown in this sediment during the experiment.

3.3. Evaluation of analyses of selected metal cations in plant material

Values of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} were measured in leaves, stems, and roots of plants collected at the Lazy mine and one month after adaptation with mine water.

From the results of Figure 2 it is clear that the plants prospered during adaptation in the phytotron.

All monitored parameters accumulated evenly. The accumulation of calcium, magnesium, sodium, and potassium occurred primarily in the leaves of plants, then in the stems and roots of plants, where the values of some parameters differ only slightly.

Two types of phytohormones, auxins and cytokinins, were used. The tested plants were taken from the saline waters of the Ostrava-Karvina area. They were placed in a phytotron and part of them was treated with a solution with auxins, part with cytokinins and part with solvent only. Reeds treated with phytohormones have been shown to be able to bioaccumulate higher concentrations than untreated reeds.

The Figure 2 shows analyzed samples of roots, leaves and stems of selected indicators (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}). It can be seen from the figure that higher concentrations of selected indicators were achieved in plants treated with phytohormones. Higher concentrations were accumulated in the leaves and stems of the plants. Conversely, the lowest concentrations were accumulated in the roots of untreated plants.

The concentration of Ca^{2+} in the leaves and stems of the plants treated with cytokinin m-topolin at the beginning of the experiment was 14.7 mg/kg, at the end of the experiment 96.2 mg/kg. The Ca^{2+} concentration increased by 81.5 kg mg/kg. In the roots of the plants treated with the cytokinin m-topolin, the concentration of Ca^{2+} increased from the initial 9.7 mg/kg to the final 39.9 mg/kg, i.e. 30.2 mg/kg.

In the plants treated with auxin NAA, the concentration of Ca^{2+} in the leaves and stems of the plants increased from 17.6 mg/kg at the beginning of the experiment to 97.4 mg/kg at the end of the experiment, i.e. by 79.8 mg/kg. In the roots of the plants treated with auxin NAA, the concentration of

Table 1. Sampling date and analysis results for mixed mine water collected at the Lazy mine, intended for plant irrigation, including Sodium adsorption ratio (SAR).

Date of collection	Ca^{2+} [mg/L]	Mg^{2+} [mg/L]	Na^{+} [mg/L]	K^{+} [mg/L]	Cl^{-} [mg/L]	SO_4^{2-} [mg/l]	DIS [mg/L]	pH	Conductivity [mS/m]	SAR
03.10.2016	212.51	150.5	4607	113	7690	311	11 000	7.72	5.51	118.9
05.12.2016	263.16	205.2	6195	152	10 500	595	15 000	7.8	6.42	138.9
28.02.2017	231.85	70.9	3523	51.9	8000	214	12 000	7.8	5.91	103.8
27.03.2017	278.85	195.7	4886	121.8	8490	223	12 000	7.6	5.99	109.9

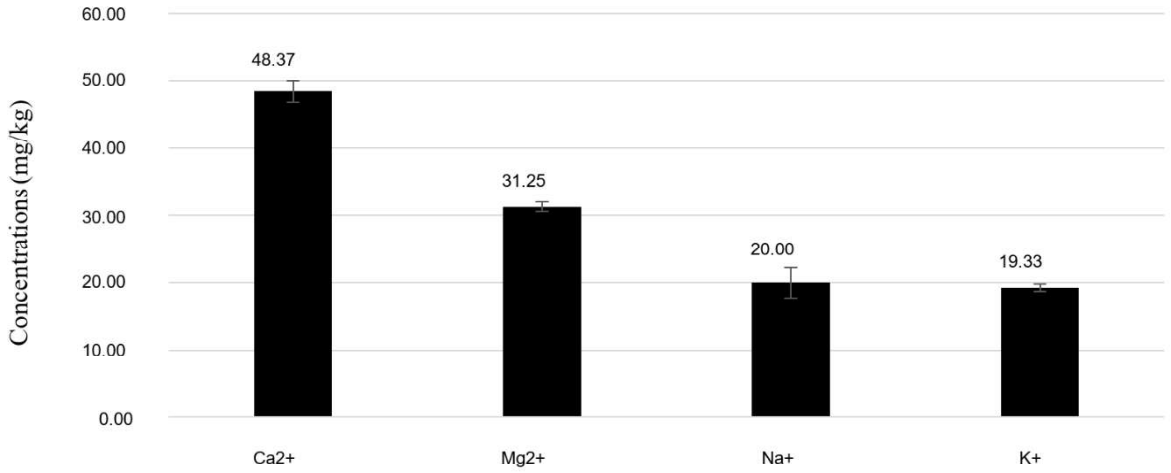

Figure 1. Comparison of the concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions in the Lazy mine sediment on May 3, 2016.

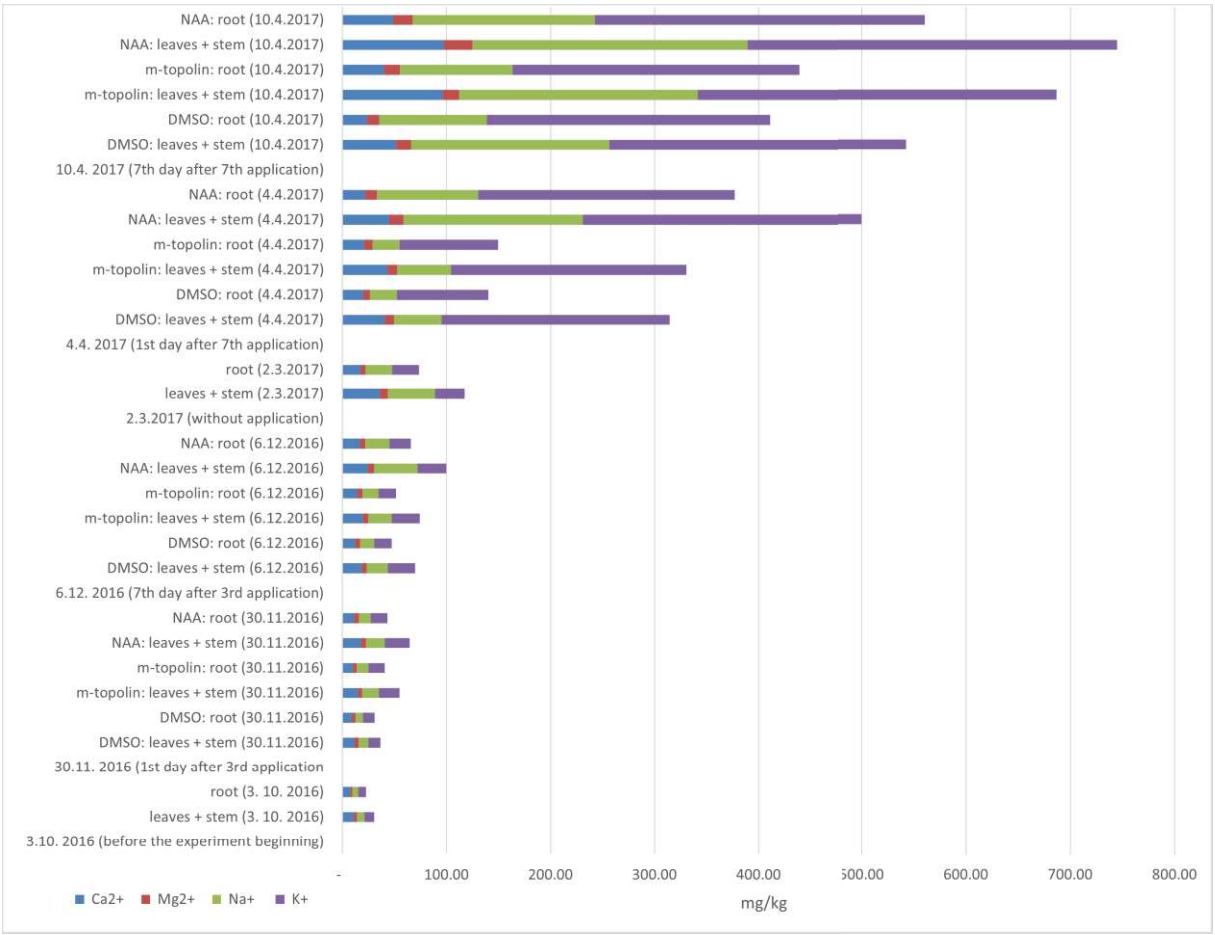

Figure 2. Comparison concentration of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions in plant material.

Ca^{2+} increased from the initial 10.98 mg/kg to the final 48.32 mg/kg, i.e. by 37.34 mg/kg.

The concentration of Mg^{2+} in the leaves and stems of the plants treated with cytokinin m-topolin was 4.17 mg/kg at the beginning of the experiment, at the end of the experiment the concentration increased to 15.88 mg/kg. The increase was 11.71 mg/kg. In the roots of the plants treated with the cytokinin m-topolin, the concentration of Mg^{2+} increased from the initial 4.02 mg/kg to the final 15.31 mg/kg, i.e. 11.29 mg/kg.

In the plants treated with auxin NAA, the concentration of Mg^{2+} in the leaves and stems of the plants increased from 4.91 mg/kg at the beginning of the experiment to 27.3 mg/kg at the end of the experiment, i.e. by 22.39 mg/kg. In the roots of

theplants treated with auxin NAA, the concentration of Mg^{2+} increased from the initial 4.77 mg/kg to the final 19.20 mg/kg, i.e. by 14.43 mg/kg.

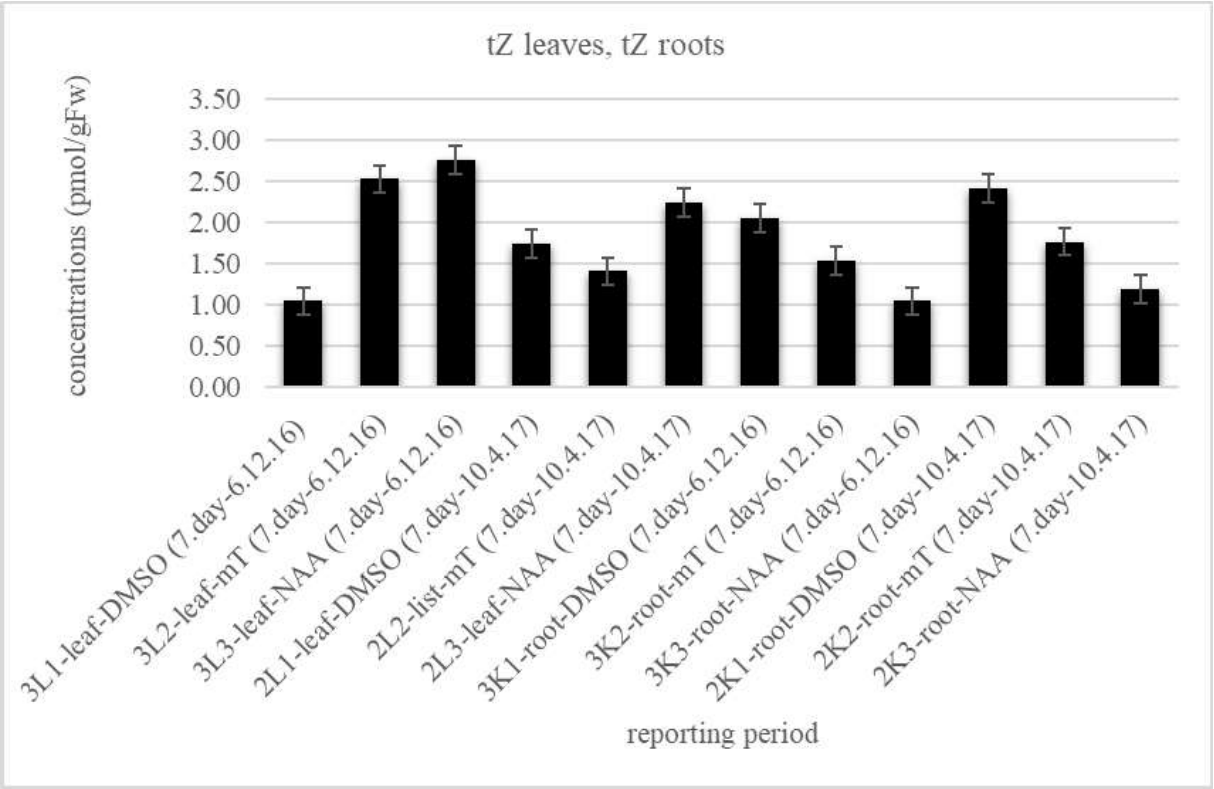

Figure 3. Cytokinin *trans*-zeatin levels in reed leaves and roots, taken for analysis at day 7 after treatment with exogenous mT and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

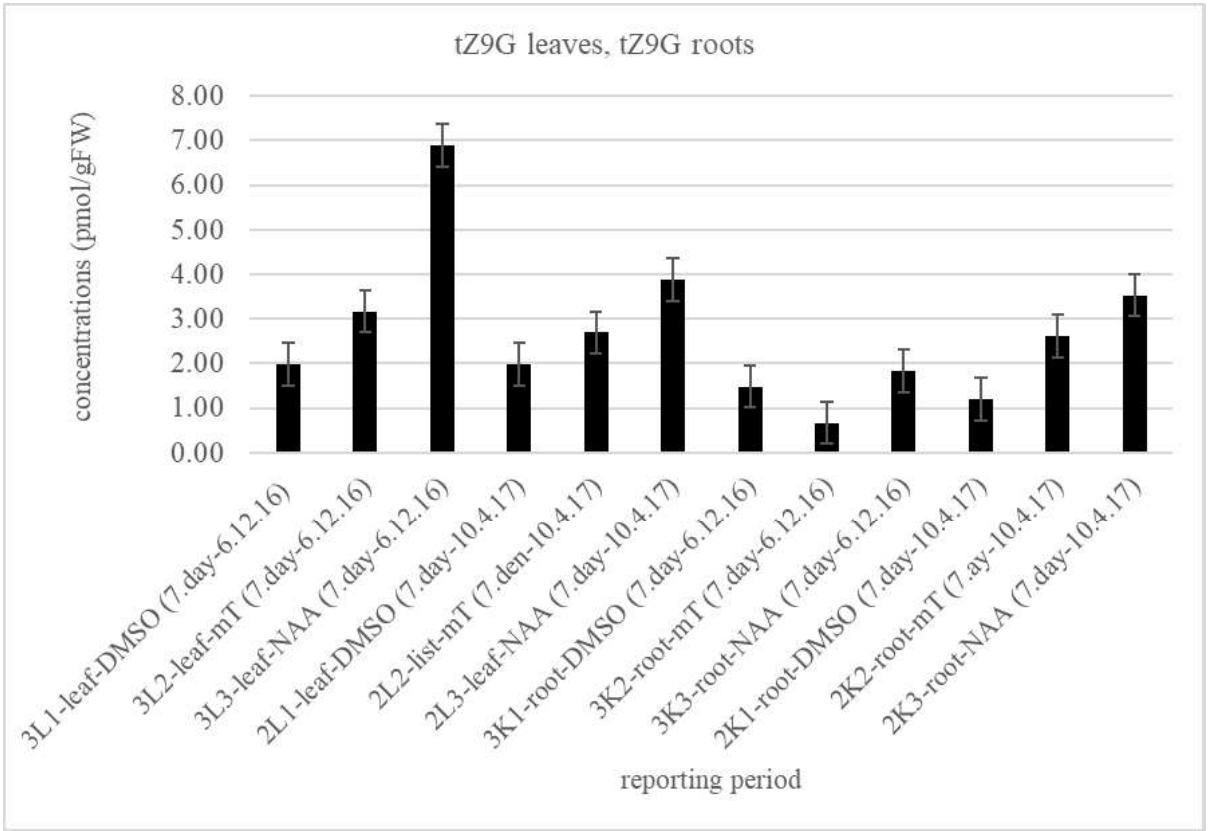

Figure 4. Cytokinin *trans*-zeatin-9-glucoside levels in reed leaves and roots taken for analysis at day 7 after treatment with exogenous mT and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

The concentration of Na^+ in the leaves and stems of the plants treated with cytokinin m-topolin at the beginning of the experiment was 16.0 mg/kg, at the end of the experiment 229.3 mg/kg. Na^+ concentration increased by 213.3 mg/kg. In the roots of the plants treated with the cytokinin m-topolin, the concentration of Na^+ increased from the initial 11.0 mg/kg to the final 108.3 mg/kg, i.e. 97.3 mg/kg.

In the plants treated with auxin NAA, the concentration of Na^+ in the leaves and stems of the plants increased from 18.1 mg/kg at the beginning of the experiment to 264.6 mg/kg at the end of the experiment, i.e. by 246.5 mg/kg. In the roots of the plants treated with auxin NAA, the concentration of Na^+ increased from the initial 11.2 mg/kg to the final 175.1 mg/kg, i.e. by 163.9 mg/kg.

The concentration of K^+ in the leaves and stems of plants treated with cytokinin m-topoline at the beginning of the experiment was 19.9 mg/kg, while at the end of the experiment was 345.5 mg/kg. K^+ concentration increased by 325.6 mg/kg. In the roots of the plants treated with the cytokinin m-topolin, the concentration of K^+ increased from the initial 15.80 mg/kg to the final 275.9 mg/kg, i.e. 260.1 mg/kg.

In the plants treated with auxin NAA, the concentration of K^+ in the leaves and stems of the plants increased from 23.8 mg/kg at the beginning of the experiment to 355.5 mg/kg at the end of the experiment, i.e. by 331.75 mg/kg. In the roots of the plants treated with auxin NAA, the concentration of K^+ increased from the initial 16.2 mg/kg to the final 317.8 mg/kg, i.e. by 301.6 mg/kg.

3.4. Evaluation of results of selected phytohormones

The determined levels of selected CKs are shown in the form of graphs and tables.

Table 2. Cytokinin *cis*-zeatin levels in reed leaves and roots, taken for analysis at day 7 after treatment with exogenous mT and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

Samples	*cis*-zeatin		
3L1-leaf-**DMSO** (7.day-6.12.16)	**4.71**	±	0.01
3L2-leaf-**mT** (7.day-6.12.16)	**9.11**	±	0.26
3L3-leaf-**NAA** (7.day-6.12.16)	**6.51**	±	0.65
2L1-leaf-**DMSO** (7.day-10.4.17)	**12.38**	±	0.54
2L2-leaf-**mT** (7.den-10.4.17)	**11.36**	±	0.32
2L3-leaf-**NAA** (7.day-10.4.17)	**13.16**	±	0.76
3K1-root-**DMSO** (7.day-6.12.16)	**1.37**	±	0.10
3K2-root-**mT** (7.day-6.12.16)		< LOD	
3K3-root-**NAA** (7.day-6.12.16)	**1.58**	±	0.10
2K1-root-**DMSO** (7.day-10.4.17)		< LOD	
2K2-root-**mT** (7.day-10.4.17)		< LOD	
2K3-root-**NAA** (7.day-10.4.17)		< LOD	

Table 3. Cytokinin *cis*-zeatin-9-glucoside levels in reed leaves and roots, taken for analysis at day 7 after treatment with exogenous *m*T and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

Samples	*cis*-zeatin-9-glucoside		
3L1-leaf-**DMSO** (7.day-6.12.16)	**1.14**	±	0.106
3L2-leaf-**mT** (7.day-6.12.16)	**0.82**	±	0.020
3L3-leaf-**NAA** (7.day-6.12.16)	**1.28**	±	0.096
2L1-leaf-**DMSO** (7.day-10.4.17)	**0.88**	±	0.050
2L2-leaf-**mT** (7.day-10.4.17)	**1.48**	±	0.027
2L3-leaf-**NAA** (7.day-10.4.17)	**1,09**	±	0.082
3K1-root-**DMSO** (7.day-6.12.16)	**0.89**	±	0.149
3K2-root -**mT** (7.day-6.12.16)		< LOD	
3K3-root-**NAA** (7.day-6.12.16)	**0.44**	±	0.03
2K1-root-**DMSO** (7.day-10.4.17)	**0.21**	±	0.012
2K2-root-**mT** (7.day-10.4.17)		< LOD	
2K3-root-**NAA** (7.day-10.4.17)	**0.36**	±	0.008

The following CK types were evaluated: *tZ* - *trans*-Zeatin, *cZ* - *cis*-Zeatin, *i*P - N6- (2-isopentenyl) adenine, *mT* - meta-topolin and their metabolites.

The level of *trans*-Zeatin, the most active naturally occurring cytokinin (Davies, 2010), was higher (6/12) in the reed leaf samples treated by exogenous *m*T and / or NAA at the beginning of the seventh day of the experiment, in comparison with control, treated by DMSO (Figure 3), followed by increased levels of its deactivating form, *trans*-zeatin-9-glucoside (*t*Z9G). The levels of *trans*-zeatin-o glucoside, *trans*-zeatin riboside concentrations determined at reed root are set below the limit of detection with the exception of *trans*-zeatin-9-glucoside (Figure 4).

Surprisingly, the levels of *trans*-zeatin were significantly decreased in roots after both treatments.

Also, the levels of *cis*-zeatin (*c*Z), a cytokinin with emerging significance and an interesting (though still largely unknown) biological function, were higher (6/12) in the reed leaf samples treated with exogenous mT and/or NAA on the seventh day of the experiment in comparison to DMSO – treated control (Table 2). The levels of *cis*-zeatin were significantly lower and almost identical in the roots treated with exogenous NAA and the control, treated only with DMSO on the seventh day of the experiment. The concentrations of *cis* -zeatin in the reed root of the plants treated with exogenous *m*T are determined below the detection limit. Very recently (Silva-Navas et al., 2019), *c*Z has been found to specifically stimulate root and hair elongation to increase the root-absorbing surface. Such an effect on plant morphology may be beneficial in the long run for the ability of reeds to absorb more salts from mine waters. In the case of this compound, the level of its deactivation product *cis*-zeatin-9-glucoside (*c*Z9G) (Table 3) was reduced after treatment, which also indicates the importance of *c*Z free base in the studied process and conditions.

The leaves of the plants contain much more *cis*-zeatin than *trans*-zeatin. In contrast, there are higher levels of *trans*-zeatin in the roots (Figure 3).

Levels of N6-(Δ2-isopentyl) adenine, which have been shown to slow leaf aging, inhibit root growth, and promote shoot regeneration (Davies, 2010) are higher in plant roots (Figure 5). In contrast, N6- (Δ2-isopentyl) adenine riboside levels are higher in plant leaves (Figure 6). The level of N6- (Δ2-isopentyl) adenine-9-glucoside was determined below the limit of detection.

The levels of *meta*-topolin, the only aromatic cytokinin found in the plant, were significantly increased in samples of *meta*-topolin-treated reed leaves and roots (Figure 7).

From the evaluation of cytokinin results, it is clear that the basal level of most measured cytokinins from auxin-free control leaf samples did not change or was even lower for some cytokinins and their metabolites compared to auxin samples. Opposite was true for root samples, where the levels of cytokinin, compounds known to inhibit root development, were lower in the plants treated by

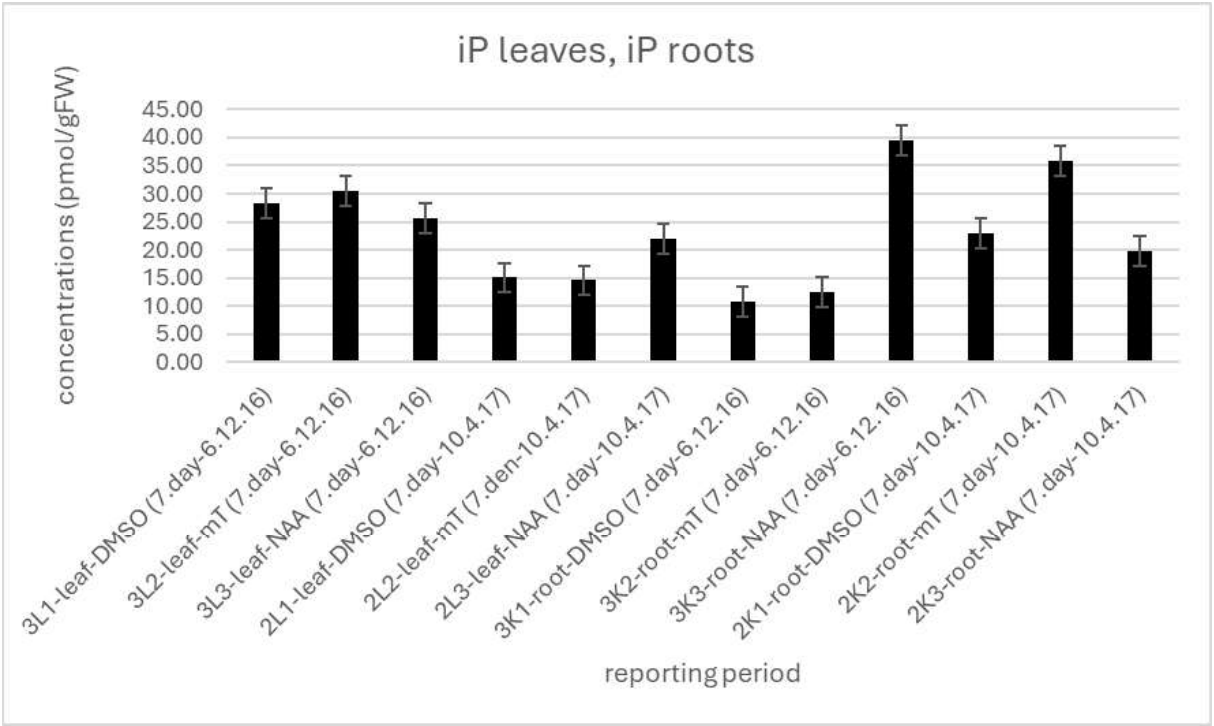

Figure 5. Cytokinin N^6-(Δ2-isopentyl) adenine levels in reed leaves and roots taken for analysis at day 7 after treatment with exogenous *m*T and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

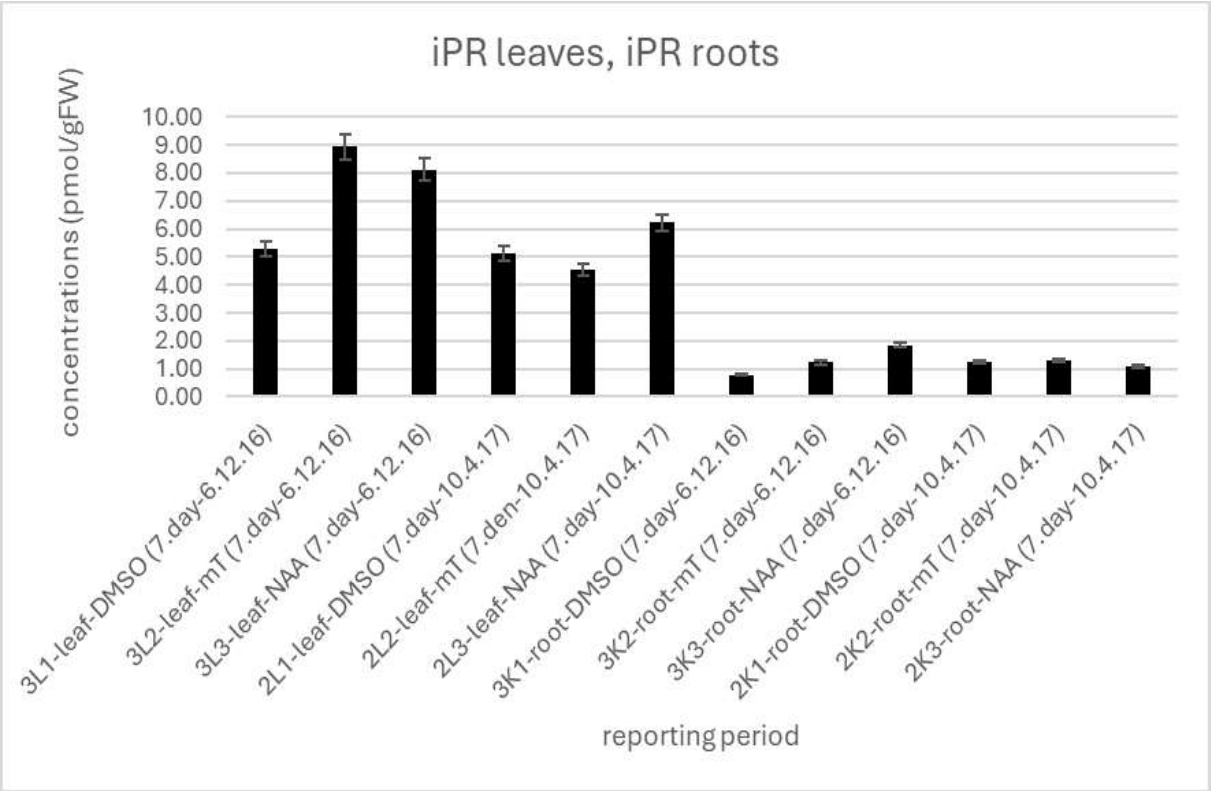

Figure 6. Cytokinin N^6-(Δ2-isopentyl) adenine ribosid levels in reed leaves and roots taken for analysis at day 7 after treatment with exogenous *m*T and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

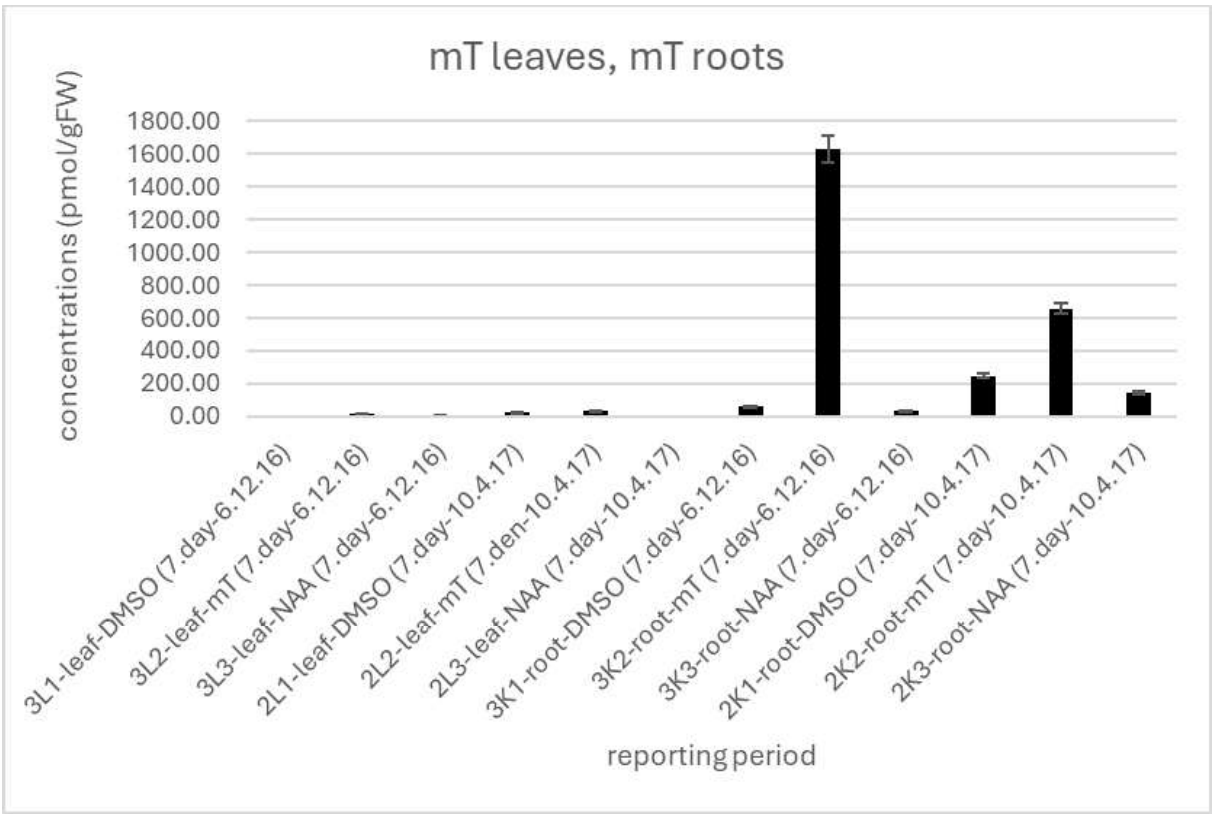

Figure 7. Cytokinin *meta*-topoline levels in reed leaves and roots, taken for analysis at day 7 after treatment with exogenous *m*T and/or NAA, with DMSO-treated control, determined by UHPLC-ESI-MS/MS.

plant hormones, in comparison with control. In contrast, for some cytokinin transport, storage or inactivation forms, the cytokinin levels have increased.

3.5. Statistical evaluation

To compare the type of treatment of plant parts, we used the Welch ANOVA test. The assumption of normal data distribution for classical ANOVA was verified using the Shapiro-Wilk test. And it was met for all selections. The second assumption, homogeneity of variances, was verified using Levene's test. This was fulfilled in all of them, so we used the classic analysis of variance ANOVA to verify the differences. Tests were performed at the 0.05 significance level (Tables 4-7).

4. DISCUSSION

Phytohormones play a very important role in controlling plant salinity stress adaptation response, which enable plants to adjust well to adverse soil conditions. Plant hormones and osmolytes have an important role in minimizing salinity stress-related detrimental effects on plants (Singh et al., 2022).

Only one article aimed at phytohormones applied to salt-affected plants was previously published. In Ma et al. (2016) exogenous cytokinin applications decreased salt-induced leaf senescence in perennial ryegrass. The positive impact of exogenous cytokinin were related to antioxidant enzyme activity enhancement, which is suppressed by Na^+ accumulation. Exogenous cytokinin increased K^+/Na^+ ratio associated with the up-regulation of high-affinity K^+ transporter.

The exogenous plant hormones in the presence of mine water were found to increase the levels of specific cytokinins in the shoots and decrease in the roots, with a proven stimulating effect on root elongation and root hair to increase the root-absorbing surface. Therefore, it can be assumed that in the long-range period, this could also have a positive effect on the ability of reeds to accumulate salts from mine waters (Ma et al., 2016).

Table 4. Statistical evaluation - differences in types of phytohormones in reed leaves (December 6, 2016).

Quantity	Tested criterion (ANOVA)	p-value	Significant differences
*t*Z	7.217	0.025	DMSO × NAA
*t*ZOG	6.350	0.033	DMSO ×NAA
*t*Z9G	3.138	0.117	Not significant
*c*Z	3.472	0.100	Not significant
*c*ZR	43.703	0.000	DMSO ×NAA, DMSO x *m*T
*c*ZOG	40.738	0.000	DMSO × mT, NAA × *m*T
*c*ZROG	6.564	0.031	NAA × *m*T
*c*Z9G	5.320	0.047	NAA × *m*T
*i*P	0.518	0.620	Not significant
*i*PR	1.052	0.406	Not significant
*m*T	67.990	0.000	DMSO × *m*T ×NAA

Table 5. Statistical evaluation - differences in the types of phytohormones in reed roots (December 6, 2016).

Quantity	Tested criterion (ANOVA)	p-value	Significant differences
*t*Z	13.013	0.007	DMSO × NAA
*t*Z9G	5.422	0.045	NAA × *m*T
*c*ZR	1.895	0.230	Not significant
*i*P	4.260	0.071	Not significant
*i*PR	4.307	0.069	Not significant
*m*T	110.051	0.000	DMSO × *m*T, NAA ×*m*T

Table 6. Statistical evaluation - differences in types of phytohormones in reed leaves (April 10, 2017).

Quantity	Tested criterion (ANOVA)	p-value	Significant differences
*t*Z	19.681	0.002	DMSO × NAA, *m*T × NAA
*t*ZOG	12.023	0.008	*m*T × NAA
*t*Z9G	16.365	0.004	DMSO × NAA, *m*T × NAA
*c*Z	5.062	0.052	Ntot significant
*c*ZR	52.718	0,000	DMSO × NAA, DMSO × *m*T
*c*ZOG	30.473	0,001	DMSO × NAA, DMSO × *m*T
*c*ZROG	5.938	0.038	DMSO × *m*T
*c*Z9G	54.798	0.000	DMSO × *m*T × NAA
*i*P	42.815	0.000	DMSO × NAA, *m*T × NAA
*i*PR	6.823	0.028	*m*T × NAA
*m*T	264.896	0.000	DMSO × *m*T × NAA
*m*TOG	177.983	0.000	DMSO × *m*T × NAA

Table 7. Statistical evaluation - differences in the types of phytohormones in reed roots (April 10, 2017).

Quantity	Tested criterion (ANOVA)	p-value	Significant differences
*t*Z	1.175	0.371	Not significant
*t*Z9G	7.513	0.023	DMSO × NAA
*c*ZR	0.404	0.570	Not significant
*c*ZROG	19.387	0.004	Not significant
*c*Z9G	9.027	0.033	DMSO × NAA
*i*P	14.274	0.005	DMSO × NAA, *m*T × NAA
*i*PR	0.396	0.692	Not significant
*m*T	54.828	0.000	DMSO × NAA, *m*T × NAA
*m*TOG	21.016	0.004	DMSO × NAA, *m*T × NAA

During our experiment, the highest concentrations of all monitored alkali metals were accumulated in leaves and stems of plant analysed (plant material parameters). This research is similar to that conducted by Shelef et al. (2012) with *Bassia indicia*. *Bassia indicia* plants tolerated a wide range of salinities and accumulated salts. The accumulation occurred mainly in leaves and branches, making *Bassia indicia* suitable for the phytoextraction of Na and other elements. These properties and the result are confirmed in a study by Pongrac et al. (2013) that focuses on the accumulation of Na^+, Mg^{2+}, and Cl^- in the halophytic plant. They found that the highest concentrations of all selected elements were accumulated mostly in the leaves. The test results of all monitored indicators in the plant show a ratio of root < stem < leaf. Furthermore, from the initial results of our experiment, it is clear that there is a higher accumulation of salts from mine waters in the plants treated with phytohormones, especially auxins. The result of our research confirms the results of the review Mishra and Tanna (2017), where halophytes are discussed as an excellent source of salt-responsive genes that can be further used to develop tolerance to salinity in crop plants through genetic engineering.

Mining water changes its composition due to mixing with other types of water, due to pollution, weathering products and biodegradation (Grmela, 1999). Furthermore, it also depends on the hydrogeological structure in the place where the mining works take place.

In the Ostrava - Karviná district, specifically in the Lazy mine, there is a salt mine water of the deposit type. The mineralization at Lazy Mine is in the range of 1000 - 10.000 mg/L (Grmela, 1999).

Deep mining waters have neutral or slightly alkaline pH. These waters mainly contain sulphates, chlorides but also iron and other solutes. This finding is confirmed by the results in this study (Younger et al., 2002).

The electrical conductivity of the water flowing out of the mine Lazy is many times higher, and over 30.000 µS/cm. In the tailings ponds, it is diluted and the water flows into the stream they no longer show such high values. In both cases, the conductivity decreases gradually by dilution with other waters from tributaries (Younger et al., 2002).

4.1. Plant material parameters

The ability of common reed *(Phragmites australis)* to absorb sodium into its tissues (leaves, stems and roots) was confirmed by the analysis of sodium in the biomass dry matter using the AAS method. A higher concentration of toxic sodium was measured in the leaves and stems of the plants, compared to a lower concentration of sodium measured in the roots. Highest bioaccumulation of sodium occurs in the plants treated with auxin.

A high concentration of Na^+ ions is toxic to unadopted plant species. In addition, Na^+ ions are capable of displacing other ions directly (e.g. Ca^{2+} from cell walls and membranes, K^+) (Silva-Navas et al., 2019).

However, sodium often prevents the very absorption of important ions from the soil solution into the plant's body, and this applies both to Ca^{2+}, K^+ and Mg^{2+}, as well to several micronutrients that plants cannot do without, due to the increase in soil pH: Fe, Mn, Zn and Cu (Silva-Navas et al., 2019).

Calcium is taken up by plants passively through the root tips as the divalent cation Ca^{2+} from the soil solution, where it is mostly the predominant cation (White & Broadley, 2003). It is transported to the above-ground part of the plant by xylem. Its mobility through the plant is low. The distribution of calcium in the plant after treatment with phytohormones is almost the same.

Magnesium is taken up by plants as the Mg^{2+} cation in smaller amounts than calcium (Tang & Luan, 2017). Its supply to the root hairs is mainly contributed by the flow of the soil solution and, to a lesser extent, by the growth of the roots. Magnesium is passively absorbed by plants. In Mg^{2+} uptake, there is an antagonistic relationship with K^+, NH_4^+, Ca^{2+}, Mn^{2+}, H^+. In the plant, magnesium is transported in the form of chelates and in some cases, it is dependent on calcium. Its movement is three times faster than that of

calcium. When treated with m-topolin, magnesium concentrations in the leaves and stems at the end of the experiment are almost the same as in the roots.

Potassium is a monovalent cation that the plant receives actively at lower concentrations (up to 0.5 mM) or passively at higher concentrations. The intake of potassium is significantly influenced by interactions of an antagonistic nature. Increasing concentration of K reduces the intake of Mg^{2+}, Ca^{2+}, NH_4^+, Zn^{2+}, Mn^{2+} and stimulates the intake of NO_3^-, $H_2PO_4^-$, Cl^-, SO_4^{2-}. When the plants are treated with both auxin and cytokinin, the concentration of potassium in the leaves and stems is higher than in the plant roots. When treated with auxin, there is a slightly lower concentration in the roots of plants than in the plants treated with cytokinin.

5. CONCLUSIONS

The article aimed to verify whether common reed *Phragmites australis* was able to improve the accumulation of selected indicators (especially sodium, potassium, calcium, and magnesium from the mine waters of Karviná mine and Lazy mine) by appropriate stimulation with phytohormones. This study was the first research in the Czech Republic, where endogenous auxins, cytokinins, and their metabolites were studied in reed plants. From the initial results, it is evident that during the period of our experiment, there was a higher accumulation of salts in the mine waters in favor of the samples with the addition of auxins at a concentration of 10^{-5} mol/L. The exogenous phytohormones in the presence of mine water were found to increase the levels of specific cytokinins in the shoots and decrease in the roots, with a proven stimulating effect on root elongation and root hair to increase the root-absorbing surface. Therefore, it can be assumed that in the long-range period, this could also have a positive effect on the ability of reeds to accumulate salts from mine waters.

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Received: 05.11.2024
Revised: 22.01.2025
Accepted: 24.01.20125
Published: 27.01.2025