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High Level of *Nicotianamine Synthase* (*NAS3*) and *Natural Resistance Associated Macrophage Protein (NRAMP4)* Gene Transcription Induced by Potassium Nitrate in Trembling Aspen (*Populus tremuloides*)

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Abstract: Changes in gene transcription in response to excess metal concentrations have been reported in many organisms, including yeast, microorganisms and plants. Most investigations on the effects of nickel toxicity in plants use commercial salts whose effects have not been analyzed in detail. The main objective of the present study was to determine the effects of different doses of nickel nitrate and potassium nitrates on gene transcription in *Populus tremuloides*. Four month-old *P. tremuloides* seedlings were treated with different doses of nitrate salts including 150 mg/kg, 500 mg/kg, 800 mg/kg and 1, 600 mg/kg. A significant increase of *Nicotianamine Synthase* (*NAS*3) gene transcription was induced by the 400 mg/kg and 800 mg/kg of nickel nitrate doses compared to water. This upregulation was driven by nitrate rather than nickel. Likewise, the 800 mg/kg and 1,600 mg/kg doses of potassium nitrate resulted in significant increase in the transcription of *Natural Resistance Associated Macrophage Protein* (*NRAMP*4) gene compared to water control and the 150 mg/kg dose. This differential transcription of this gene was caused by potassium. Our results also confirmed that the low level of bioavailable nickel in metal–contaminated soils (<150 mg/kg) cannot induce differential transcription of *NAS3* and *NRAMP4*. The use of nitrate without nickel should be required as additional controls in any study assessing effects of Ni using nickel nitrate salts.

Keywords: Trembling Aspen (*Populus temuloides*), Gene Transcription, Nickel Toxicity, Nickel Nitrate, Potassium Nitrate, RT-qPCR

Introduction

Nickel is a micronutrient required for plant growth and physiological functions. An excess amount will cause Ni toxicity that is detrimental to plant development. With the increasing levels of Ni contamination in the environment, it is essential to understand the functional roles and toxic effects of Ni in plants. Changes in gene transcription in response to excess metal concentrations are commonly seen in metal tolerant plants like hyperaccumulators. Genes that have been identified to possibly play a role in metal tolerance in plants can be species-specific and metal-specific. Recent reports showed that *Populus tremuloides* is a metal accumulator species. It grows readily in sites contaminated with a high content of Ni in Northern Ontario (Canada) (Mehes-Smith *et al*., 2013). This species does translocate excess Ni and zinc (Zn) from roots to leaves. Metal accumulator plants such as *P. tremuloides* use genes that have a similar function and are commonly involved in metal transport (Kalubi *et al*., 2016).

A recent review of literature revealed 11 genes associated with nickel resistance in model and non-model plants. These genes include *1-Aminocyclopropane-1-*

Carboxylic acid deaminase (ACC), high affinity nickel transporter family protein (AT2G16800), Iron-Regulated protein (IREG), Glutathione Reductase (GR), glutathione-s-transferase, Metal Transporter (NRAMP 1,2,3,4), Nicotianamine Synthase (NAS3), Putative Transmembrane Protein (TMP), Serine acetyltransferase (SAT), Thioredoxin family protein, Zn finger protein of Arabidopsis thaliana (ZAT11) and MRP4 (Freeman *et al*., 2004; Lemaire *et al*., 2004; Stearns *et al*., 2005; Mizuno *et al*., 2005; Schaaf *et al*., 2006; Mari *et al*., 2006; Visioli *et al*., 2012; Liu *et al*., 2014; Theriault *et al*., 2016a). *NAS3* and *NRAMP 4* are associated with Ni transport and are well studied in many microorganisms and plant species. Information on their transcription under metal stress in tree species is very limited.

The *Natural Resistance Associated Macrophage Protein (NRAMP)* transporter is a family of genes whose main function is to bind and transport divalent metal ions. This is a highly conserved gene family during evolution and homologues have been identified in a large range of organisms including bacteria, yeast, mammals and higher plants (Williams *et al*., 2000).

The *Nicotianamine Synthases* genes (*NAS1, 3* and *4*) on the other hand, are a group of enzymes that synthesize the metal chelator Nicotianamine (NA). This nonproteinogenic amino acid can bind to some transition metals like Fe, Cu, Zn, Mn and Ni and immobilize them for transport. NA has already been implicated in transporting Fe, Cu and Zn for long distances within plants (Wintz *et al*., 2003).

It should be pointed out that most studies using nickel nitrate as salts to study the effect of Ni on gene transcription ignore the potential effects caused by these nitrate anions (Freeman *et al.*, 2004; Douchkov *et al*., 2005; Merlot *et al*., 2014). However, there is evidence that high nitrate concentrations can be phytotoxic when they exceed the thresholds that plants can tolerate (Parker *et al*., 1983; Goyal and Huffaker, 1984). We hypothesize that both nickel and nitrate at high concentrations can trigger an increase of *NAS (1, 3 and 4)* and NRAMP transcription in plants and that potassium will have no effects on these genes.

The main objectives of the present research were to determine the effects of different doses of nickel nitrate and potassium nitrates on gene transcription in *P. tremuloides*.

Materials and Methods

Nickel Toxicity Assessment

Populus tremuloides seeds were provided by the Canadian Forest Services seed bank (Fredericton). These seeds were collected in Woodstock, NB (seedlot# 20061003.0) and stored at 4°C. Seeds were germinated in "Petawawa" germination boxes and seedlings were grown in a deep tray with soil. Four month-old seedlings were transplanted into pots containing a 50:50 sand/soil mixture and left to grow for an additional month and a half in a growth chamber. Plants were watered as needed and fertilized twice a week with equal amounts of nitrogen, phosphorus and potassium (20-20-20**)**.

Ni toxicity was assessed by treating seedlings with an aqueous solution of nickel nitrate salt [$Ni(NO_3)_2$] at the following concentrations: 150 mg, 400 mg, 800 mg and 1,600 mg of nickel per 1 kg of dry soil. To control for any possible toxic effect due to an increase in nitrate ions (NO_3) in the plants, an aqueous solution of commercial potassium nitrate (KNO_3) salts was used for controls in equal molar amounts to each dose of the nickel salts. The nitrate controls for 1,600 mg/kg, 800 mg/kg, 400 mg/kg and 150 mg/kg corresponds to 603.38 µmol, 301.69 µmol, 150.85 µmol and 113.08 µmol of nitrate respectively. Salt-free water was used as a negative control (0 mg Ni per 1 kg of dry soil). The experimental layout was a completely randomized block design with 12 replications per each nickel treatment, 11 for the water control and 5 per nitrate control. Roots were harvested from seedlings after 7 days of treatments, frozen in liquid nitrogen and stored at -20°C for RNA extraction.

RNA Extraction and RT-qPCR

Total RNA was extracted from the root samples using the protocol described previously by Theriault *et al*. (2016b) with some modifications. Only 0.3 g of root material were used to extract RNA. The chloroform phase separation steps were carried out with 1 ml of CTAB solution: 1 mL phenol chloroform. RNA was precipitated in 100 µL of SDS extraction buffer and chloroform steps were scaled down accordingly. Extracted RNA from 49 samples was run on a 1% agarose gel to verify quality. It was quantified with the Qubit® RNA BR assay kit by Life Technologies (Carlsbad, United States). Samples from the same treatment were pooled together in equal amounts resulting in a total amount of 10 micrograms of root RNA per treatment.

Pooled RNAs were treated with DNase 1 (#EN0521) (Life Technologies). A PCR reaction was performed for each pool and the samples were run on a 1% agarose gel to verify the absence of DNA contamination before the DNase reaction was inactivated. Pools that had no bands of PCR products from DNA amplification were used for gene transcription analysis.

RT-qPCR was performed for target genes (Table 1) associated with nickel resistance in other plant species. BLAST search for target genes was performed based on the *Populus trichocarpa* genome in the NCBI database.

Table 1: Sequences of trembling aspen (*Populus tremuloides*) primers used for RT-qPCR

Target	Melting temp (°C)	Primer	Expected amplicon (bp)	PCR product in cDNA (bp)
NAS3	F: 60.02	F:AAAGTTGCGTTTGTGGGTTC	232	232
	R: 59.98	R:CTGCCAAGAAGACGACATCA		
NRAMP4	F: 59.96	F: CCTTGTAAATGCAGGGCAAT	292	292
	R: 60.02	R: TGACTGCAGCACATTTAGCC		
Housekeeping α-tubulin	F: 60.21	F: GGCAAGCAGGGATTCAAGTA	150	150
	R: 60.36	R: GGCACATGTTTTCCAGAACC		

*Primers were designed by matching gene sequences to the Populus trichocarpa genome. When possible primers were designed to span the exon-exon border of the gene

Primers flanking the sequences of these genes were designed to span the exon-exon border of the gene, when possible. The OligoAnalyzer 3.1 program by IDT (https://www.idtdna.com/calc/analyzer) was used to check primer sequences for potential hairpins, self and hetero-dimers**.** Then, the cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit by Life Technologies. PCR was performed on cDNA and DNA. Reactions were run on a 1% agarose gel to verify the amplicon size. Primer pairs were used for RT-qPCR only if they showed a strong reproducible single band of the expected cDNA transcript size for the gene target. The RT-qPCR was performed according to the manufacturer's protocol using the Dynamo HS SYBR Green Kit (Life Technologies). Each reaction was run in triplicates using the MJ Research PTC-200 Thermal Cycler. The set program includes (1) initial denaturing at 95°C for 15 min; (2) denaturing at 94°C for 30 sec; (3) 30 sec at 55°C annealing; (4) elongation at 72°C for 30 sec; (5) read (6) repeat step 2-6 for 41 cycles; (7) final elongation at 72°C for 7 min; (8) melting curve 72-95°C, every 1°C, hold for 10 sec; and (9) final elongation at 72°C for 3 min as described in Kalubi *et al*. (2018). The RT-qPCR was performed two separate times per gene target and samples were loaded in triplicates. The result was six quantitation data points per bulked sample. Outliers among the triplicates were excluded in further analysis.

Data Analysis

The MJ Opticon Monitor 3.1 program by BioRad was used to analyze the data. The run data was exported to Excel. The C(t) Values were quantified using the equation for the standard curve and then normalized to the housekeeping gene α-tubulin. SPSS 20 for Windows was used to determine statistical significance among means ($p<0.05$). The Shapiro Wilk test was performed to verify normal distribution of data. Data sets that did not meet requirements were log transformed to achieve normal distribution. Analysis of Variance (ANOVA) and Games-Howell Post-hoc Test were used to determine any significant differences among means for different treatments and controls.

Results

All the genotypes treated with different doses of nickel nitrate showed a high level of resistance to nickel toxicity. The transcription of *NAS3* and *NRAM4* genes in roots of *P. tremuloides* subject to these treatments was assessed using RT-qPCR. The primer pairs used to amplify the housekeeping and target genes are listed in Table 1.

A significant increase of *NAS3* transcription was induced by the 400 mg/kg and 800 mg/kg of nickel nitrate doses compared to water. But no significant difference was observed between the 1, 600 mg/kg dose and the water control (Fig. 1a). There was no significant difference in gene transcription when the 150 mg/kg dose of potassium nitrate was compared to water (Fig. 1b). Surprisingly, *NAS3* transcription was significantly upregulated at the 400 mg/kg, 800 mg/kg and 1,600 mg/kg dose of potassium nitrate with a 1.5X, 3X and 3X fold increase compared to water, respectively. Significant differences between nickel nitrate and potassium nitrate were observed at 150 mg/kg, 800 mg/kg and 1,600 mg/kg (Fig. 1c).

No significant difference in *NRAMP4* transcription was observed for any of the four nickel nitrate doses compared to water (Fig. 2a). On the other hand, there was an unexpected trend of increased *NRAMP4* transcription when samples were treated with increasing concentrations of potassium nitrate (Fig. 2b). In fact, the 800 mg/kg dose and the 1,600 mg/kg dose of potassium nitrate resulted in a significant difference in this transcription compared to water control and the 150 mg/kg dose. When nickel nitrate and potassium nitrate were compared, there was a significant upregulation of *NRAMP4* induced by 150 mg/kg of nickel nitrate compared to potassium nitrate used as control; and a significant downregulation for the 800 mg/kg and the 1,600 mg/kg (Fig. 2c) doses.

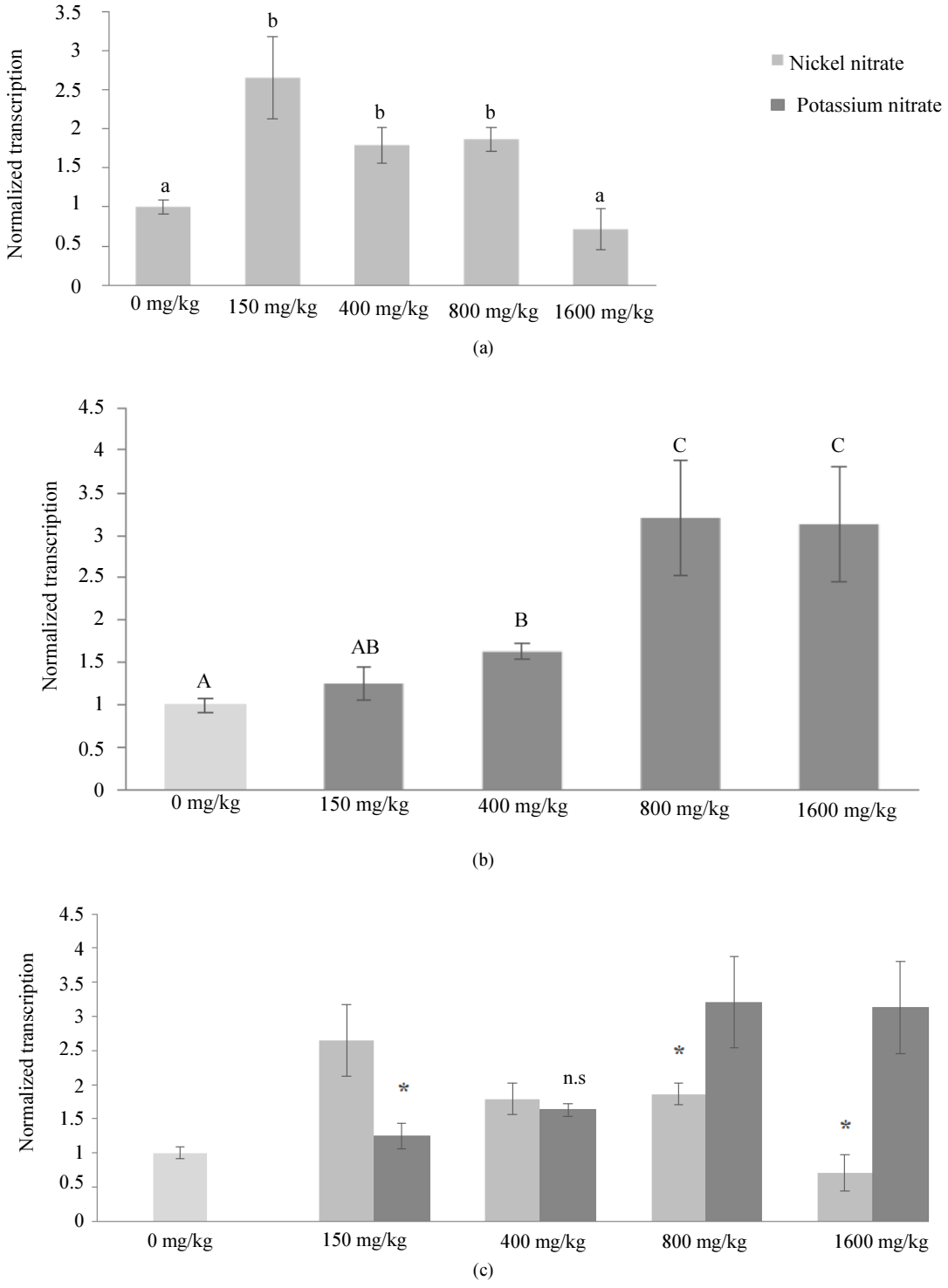

Fig. 1: *NAS3* gene transcription in trembling aspen (*Populus tremuloides*) treated with different doses of (a) nickel nitrate and (b) potassium nitrate. The gene transcription was normalized to housekeeping gene α-tubulin and water was used as the negative control. (c) Gene transcription of all the treatments combined. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water. Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk (*). In Fig. 1c, n.s. means no significant difference was found between means

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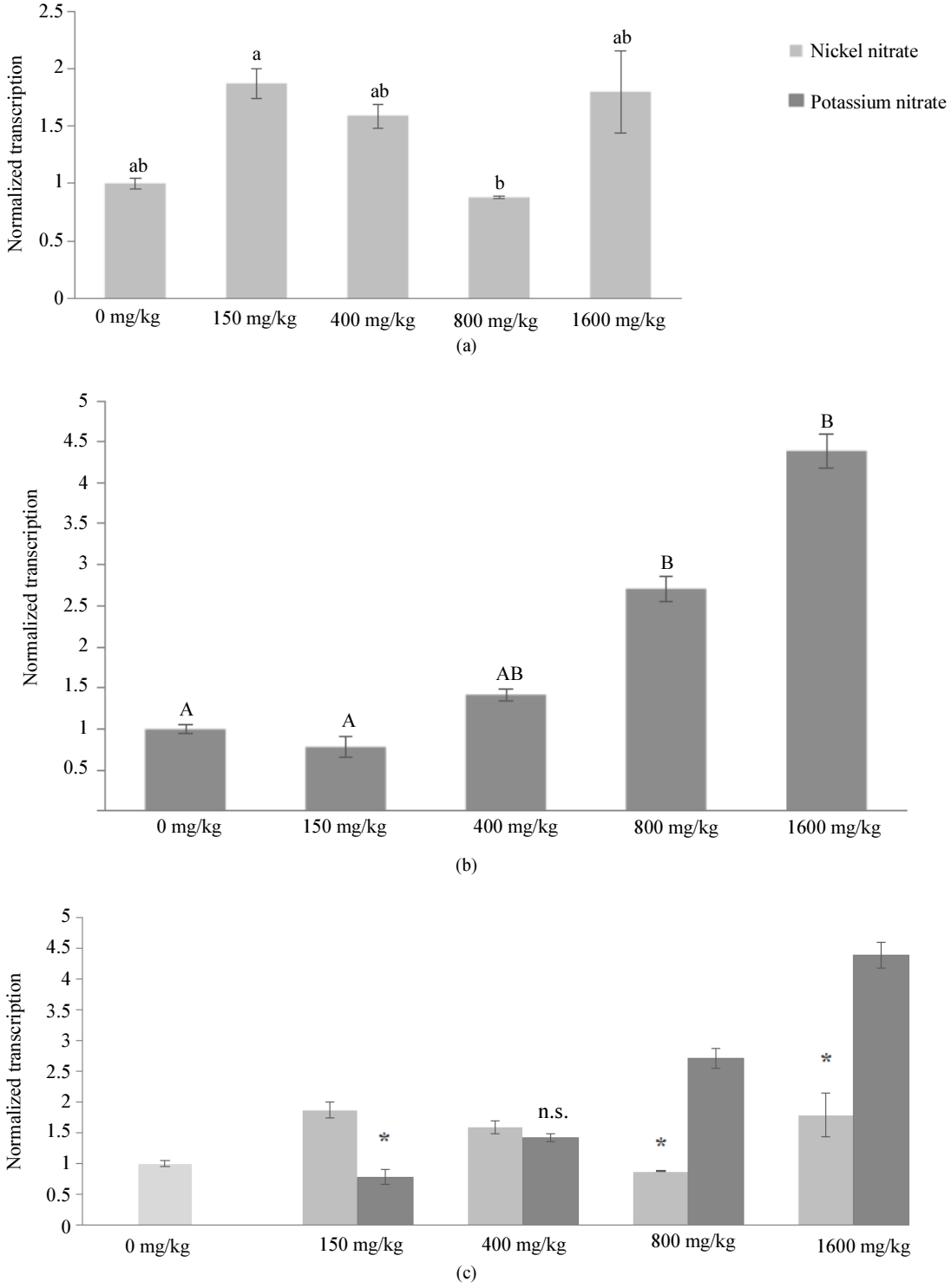

Fig. 2: *NRAMP4* gene transcription in trembling aspen (*Populus tremuloides*) treated with different doses of (a) nickel nitrate and (b) potassium nitrate. The gene transcription was normalized to housekeeping gene α-tubulin and water was used as the negative control. (c) Gene transcription of all the treatments combined. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water. Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk (*****). In Fig. 1c, n.s. means no significant difference was found between means

Discussion

This study aimed to determine if genes associated with nickel tolerance in other model and non-model species are involved in the *Populus tremuloides* response to nickel and to assess if there is any nickel dosage effect. The two genes studied are involved in metal transport, which appears to be the main mechanism involved in metal resistance.

All the *P. tremuloides* genotypes treated with potassium nitrates showed no significant toxicity symptoms or damage. Unexpectedly, both *NAS3* and *NRAMP4* transcription had a trend of upregulation with increasing concentration of potassium nitrate used as controls. This difference was most significant at the 800 mg/kg dose for both genes. This highlights the importance of including control treatments when using metal salts to assess the toxicity of a specific metal.

The *Nicotianamine Synthase (NAS)* genes are important for the synthesis of the cross-metal chelator protein Nicotianamine (NA) which can bind numerous transition metals. Recently, Ni has been identified to have high binding affinity to NA, particularly in the Ni and Zn hyperaccumulator species *T. caerulescens* (Vacchina *et al*., 2003). The *NAS3* gene was found to be overexpressed and high levels of NA were produced in *T. caerulescens*, *Arabidopsis halleri*, *Noccaea caerulescens* exposed to Ni (Vacchina *et al*., 2003; Deinlein *et al*., 2012; Visioli *et al*., 2014). It appears that an increase in nicotianamine contributes to metal tolerance in hyperaccumulator plants via metal chelation and facilitates metal translocation (Weber *et al*., 2004; Deinlein *et al*., 2012). In contrast, *NAS* genes seem to play a role in metal homeostasis (i.e., Fe, Cu, Zn and Mn) only in non-accumulating plants such as *Arabidopsis thaliana* (Curie *et al*., 2009).

It was expected that *NAS3* gene transcription would also be affected by Ni toxicity in *P. tremuloides*. But, our results show a trend of higher *NAS3* transcription in samples treated with both nickel nitrate and potassium nitrate. This suggests that nitrate rather than nickel affects the transcription of *NAS3* in our assays. This gene doesn't appear to play any role in *P. tremuloides* tolerance to nickel. The lower effect of the 1,600 mg/kg dose of nickel nitrate could be caused by an interaction of nickel with nitrate at high dose where nickel interferes with nitrate.

The *NRAMP* transporters gene family is conserved in many organisms including plants. This gene codes for *NRAMP* proteins that can bind and complex heavy metal ions for transport. The metal ions binding are dependent on the species and the protein. Some divalent cations that can be bound by *NRAMP* metal ion transporters include Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} (Supek *et al*., 1997; Liu *et al*., 1997; Chen *et al*., 1999; Nevo and Nelson, 2004). Mizuno *et al*. (2005) were the first to identify the association *NRAMP4* and Ni in a yeast study. They found an increase of *NRAMP4* transcription in yeast cells exposed to nickel. In plants, Oomen *et al.* (2009) found that *NRAMP3* and *NRAMP4* are highly expressed in *Thlaspi caerulescens* which is a metal hyperaccumulator. The two genes can bind Fe, Mn and Cd while Zn can be only bound by *NRAMP4*. Theriault *et al*. (2016a) further investigated *NRAMP* genes involved in Ni transport in white birch (*Betula papyrifera*). They found that plants that showed resistance to excess nickel may do so partially via the downregulation of genes associated with binding and transport activity like *NRAMP1-2*. In fact, the transcriptome analysis that they conducted associated the gene transcription of two *NRAMP* transporters with nickel resistance and accumulation in *B. papyrifera*. They found also a downregulation of five genes involved in metal transport including *NRAMP1* and *NRAMP2* in Ni resistant genotypes. We initially presumed that the transcription of *NRAMP* transporters may have a similar role in nickel resistance in *P. tremuloides*. The *NRAMP4* primers used in RT- qPCR confirmed the presence of this gene in *P. tremuloides*. But the results showed no significant differences in *NRAMP4* transcription in any of the nickel treatments compared to water. Unexpectedly, high concentrations of potassium nitrates at the 800 mg/kg and 1,600 mg/kg dose did significantly increase *NRAMP4* transcription in comparison with the water treatment. The direct comparison of nickel nitrate and potassium nitrate suggests that *NRAMP4* transcription seems to be more affected by potassium than the nickel and these effects increase as the concentration increases. Similar studies that we have conducted demonstrated that potassium nitrate can induce a high level of transcription of *1-aminocyclopropane-1-carboxylic acid (ACC) deaminase* and *NAS3* genes in leaves of *Quercus rubra* seedlings exposed to at low dose (150 mg/kg) of Ni. Our results also confirmed findings pf a field study on *P. tremuloides* and *Acer rubrum* indicating that the low level of bioavailable nickel in metal–contaminated soils (< 150 mg / kg) cannot induce differential transcription of *NAS3* and *NRAMP4* (Kalubi *et al*., 2018).

Conclusion

The present study shows that nickel nitrate increases *NAS3* transcription. This upregulation decreased as the salt concentration increased. Potassium nitrate also triggers an increase of the *NAS3* transcription. But this gene upregulation increases as the salt concentration increased. On the other hand, *NRAMP4* transcription was affected only by potassium. In fact, repeated assays revealed that potassium induced an upregulation of *NRAMP4* that was dose – dependent. The use of nitrate without

nickel should be required as additional controls in any study assessing effects of Ni using nickel nitrate salts.

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Authors Contributions

Karolina M. Czajka: Conduct all the experiments, analyzed the data and contributed to the writing of the manuscript.

Kabwe Nkongolo: Designed the project activities, coordinated the experiments and wrote the manuscript.

Conflict of Interest

Karolina M. Czajka declares that she has no conflict of interest. Kabwe Nkongolo declares that he has no conflict of interest.

Compliance with Ethical Standards

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

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