Original Research Paper

Expression of Metallothionein-Like Protein 2B Gene Induced by Different Concentrations of Copper in White Spruce (*Picea glauca*)

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Abstract: The alterations to the expressions of metal transporter genes can induce metal ion uptake to maintain cell homeostasis when metal concentrations are low. Metallothioneins (MTs) are a class of low molecular mass (4-8 kDa), cysteine (Cys)-rich proteins that can bind metals via the thiol groups of their Cys residues. Studies on MT gene expressions in higher plants, such as conifers, are limited. White spruce (*Picea glauca*) is a North American native conifer species that are ecologically and economically important. The objective of the study is to determine the effects of different concentrations of copper on *MT2b* expression in white spruce (*P. glauca*). Seedlings were treated with three concentrations of copper sulfate including 1,312, 656, and 130 mg/kg. Potassium sulfate and water were used as controls. Total RNA was extracted from roots and needles. The levels of *MT2b* gene expression were measured using RT-qPCR. The present study reports for the first time, novel information on the effect of copper ions on the expression of *MT2b* in *P. glauca*. The *MT2b* gene was upregulated in roots when seedlings were exposed to 1,312 mg/kg of copper sulfate but were downregulated in genotypes treated with 656 mg/kg of copper sulfate in both tissues when compared to the water control. The expression of *the MT2b* gene was significantly downregulated in needles of genotypes treated with 656 mg/kg of copper sulfate when compared to the 1,312 mg/kg concentration. The expression of *MT2b* was higher in roots compared to needles regardless of the concentrations of copper sulfate or potassium sulfate used. *MT2b* was highly expressed in the roots of copper-resistant genotypes but was inhibited in the roots of susceptible seedlings when compared to the water control.

Keywords: Copper Toxicity, Copper Sulfate Resistance, Gene Expression, Metallothioneins, Picea Glauca

Introduction

Copper ions at high concentrations cause major stress due to the disruption of plant homeostasis. Copper toxicity plays a pivotal role in inhibiting plant growth by reducing the synthesis of nucleic acids, proteins, cell wall polysaccharides, and more (Li *et al.*, 2019). Fundamental biomolecules which are needed for healthy plant growth and seed germination will be altered and this may delay, or completely inhibit the germination process (Yruela, 2009). As well, root elongation, which is a necessary plant growth process, is inhibited by the interference of cell division, chromosomal aberrations, and abnormal mitosis during copper exposure (Jiang *et al.*, 2001) and other

metal ion exposure (L'Huillier *et al.*, 1996; Liu *et al.*, 2003; Jain *et al.*, 2010). Other enzyme activities such as α-amylases, β-amylases, invertase isoenzymes, and enolase, are repressed by copper metal toxicity (Chugh and Sawhney, 1996; Ahsan *et al.*, 2007; Pena *et al.*, 2011). These enzymes are involved in the breakdown of necessary biomolecules, such as starch (Sethy and Ghosh, 2013). The decreased enzyme activity will limit the ability of the breakdown products, such as starch, to reach the proper plant tissues and thereby affecting the overall seedling growth (Kabir *et al.*, 2008).

To mitigate the effects of copper toxicity, plants have developed mechanisms to cope with copper toxicity. This includes increased metal-binding ligands, such as

metallotheoneins and phytochelatins, or antioxidants (Mukherji and Gupta, 1972; Lidon and Henriques, 1994). Ligands are used to bind copper in the cytosol since they have a high affinity for metal ions (Chandrangsu *et al.*, 2017). As well, other copper-binding molecules can be found in plant vacuoles which sequester copper ions (Lidon and Henriques, 1994; Keinänen *et al.*, 2007). The increased synthesis of copper-binding compounds released from roots has been reported in *Anabaena cylindrica*l and *Plectonema boryanum* (Jardim and Pearson, 1984). Copper-bound complexes released from roots are efficient in reducing the effects of copper toxicity, but there may be a concentration limit that renders this mechanism ineffective, causing plants to rely on other copper resistance mechanisms (Jardim and Pearson, 1984).

Moreover, plants have developed mechanisms to adapt to various metals through transcriptional modifications (Zhigang *et al.*, 2006; Keinänen *et al.*, 2007; Singh *et al.*, 2016; Nishida *et al.*, 2020). Specific metal transporter genes can be upregulated, or repressed, under metal toxicity, as seen in *Brassica napus* or *Arabidopsis* (Wintz *et al.*, 2003; Meng *et al.*, 2017). The expressions of the genes involved in metal transport and detoxification are clear indications of the transcriptional caused by metal exposure (Keinänen *et al.*, 2007; Guo *et al.*, 2008; Kobayashi *et al.*, 2008; Hossain *et al.*, 2012). The alterations to the expressions of metal transporter genes can induce metal ion uptake to maintain cell homeostasis when metal concentrations are low (Grotz *et al.*, 1998). Excess metal ions can also repress the expression of metal transporter genes to prevent further uptake of ions, as documented for the copper transporters *COPT1* and *COPT2* in *Arabidopsis* (Sancenón *et al.*, 2003). Metallotheoneins (MTs) have low molecular mass (4-8 kDa) and are rich in cysteine (Cys) (Hamer, 1986). MTs can bind metals via the thiol groups of their Cys residues (Hamer, 1986). *MT2b* has previously been associated with copper resistance in *Arabidopsis thaliana* (Guo *et al.*, 2008).

White spruce (*Picea glauca*) is a North American native conifer species that are ecologically and economically important. There are currently no studies on the *MT2b* gene expression in higher plants, such as conifers. Hence, the objective of the study is to determine the effects of different concentrations of copper on *MT2b* expression in white spruce (*P. glauca*).

Materials and Methods

Genetic Materials and Seedling Treatments

Picea glauca seeds were collected from the City of Greater Sudbury (CGS) and grown at the College Boreal plant Center for six months. The seedlings were planted into pots containing a 50:50 sand/soil mixture composed of 75-81% peat moss, 13-17% perlite, 5-9% composted peat moss, along with ethoxylated alkylphenol (wetting agent), and dolomitic and calcitic limestone (Berger BM8 Soil Mix). They were then placed into a growth chamber (PGR15 with a CMP 4030 controller by Conviron) for an additional month to grow. The growing conditions included a 6 h dark and 18 h light cycle with the temperature ranging from 20 to 25°C. Plants were watered every other day to keep the soil mixture moist and they were fertilized once with equal amounts of nitrogen, phosphorus, and potassium (20-20-20) (Plant Prod Ultimate All Purpose Fertilizer).

The seedlings were treated with an aqueous solution of copper sulfate salt ($CuSO_4$) at the following concentrations: 130, 656, and 1,312 mg of copper per 1 kg of dry soil. These concentrations represent the bioavailable, half-total, and total levels of copper found in metal-contaminated soils in the City of Greater Sudbury, respectively (Nkongolo *et al.*, 2013). These levels correspond to 773.2, 3902, and 7,804 µmoL, of copper, respectively.

To control for any possible toxic effect due to sulfate ions (SO_4^{2-}), an aqueous solution of potassium sulfate (K_2SO_4) was used in equal molar amounts to each concentration of the copper sulfate treatments. The potassium sulfate control concentrations were 130, 656, and 1,312 mg/kg, which correspond to 773.2, 3902, and 7804 µmoL of sulfate respectively. Salt-free water was used as a negative control (0 mg of copper per 1 kg of dry soil). The experimental design was a completely randomized block design with six replications for the total copper sulfate treatment (1,312 mg/kg) and 10 replications for the other treatments and control groups, corresponding to 66 samples (Fig. 1). The damage caused by these different salts to seedlings were recorded seven days after each treatment using a 1 to 9 scale with genotypes with 1 to 3 scores considered copper resistant, 4 to 6 were moderately resistant and 7 to 9 were susceptible. Resistant and susceptible genotypes treated with 1,312 mg/kg of copper sulfate were also selected to determine the expression of the *MT2b* gene.

RNA Extraction

Total RNA was extracted from root and needle samples using the Plant/Fungi Total RNA Purification kit from Norgen Biotek. The quality check for the 132 (66 roots and 66 needles) extracted total RNA samples was performed using a 1% agarose gel. Quantification of RNA was performed using the Qubit® RNA BR assay kit from Life Technologies (Carlsbad, United States). One microgram of RNA from samples of the same treatment was pooled together for future analysis, for a total of seven different RNA pools.

RNA was then treated with DNase 1 (#EN0521) from Life Technologies. Overall, 2 µg of the RNA from the treatment pool, 1 µL of DNase, 1 µL of buffer, and water were added for a final volume of 10 µL for each of the pooled RNA samples. Each sample of the DNAse-treated

RNA was incubated at 37°C for 1 h and amplified using a 3-step PCR. The amplified RNA samples were then run on a 2% agarose gel to identify any DNA contamination. RNA samples with no DNA contamination were used for gene expression analysis and the DNase enzyme was inactivated using EDTA.

RT-qPCR

Primers were designed based on the *Picea glauca* genome. The *MT2b* gene associated with copper resistance was targeted in this study (Guo *et al.*, 2008). The sequence of this gene was retrieved from the NCBI database and analyzed by BLAST in the *P. glauca* transcriptome described in "The Hardwood Genomics Project" (http://hardwoodgenomics.org). When possible, primers were designed to span all the exons encoded by the genes. Primers were checked for hairpins and self and hetero-dimers using the OligoAnalyzer 3.1 by IDT (https://www.idtdna.com/calc/analyzer). *MT2B*, a gene associated with copper resistance was selected for gene expression analysis (Table 1). Elongation factor-1 alpha (*EF1-α*) was chosen as the reference gene (Table 1). The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies.

PCR was performed on both *P. glauca* DNA and cDNA. The cDNA amplicon size was verified by running the reactions on a 2% agarose gel as described in (Czajka *et al.*, 2019; Boyd and Nkongolo, 2021; Moarefi and Nkongolo,

2022). The primer pairs amplified a strong reproducible single band of the expected cDNA transcript size for the gene target and were used for RT-qPCR (Table 1). RT-qPCR was completed using the Dynamo HS SYBR Green Kit according to the manufacturer's protocol (Life Technologies). Each sample underwent amplification using the MJ Research PTC200 Thermal Cycler. The set program consisted of, (1) initial denaturation at 95°C for 10 min, (2) denaturation at 95°C for 15 sec, (3) optimized annealing temperature which depended on the primer (between 55-60°C) for 60 sec, (5) repeat step 2-4 for 41 cycles, (6) final elongation at 72°C for 7 min (7) melting curve 72-95°C, every 1°C, hold for 10 sec and (8) final elongation at 72°C for 3 min. RT-qPCR was performed two separate times for the *MT2b* and reference gene and samples were loaded in triplicates. This resulted in six data points per pooled sample. Outliers among the triplicates were excluded from further analysis.

Data Analysis

CFX Connect was used to analyze the data for gene expression. The data were exported to Excel and the C(t) values were quantified using the equation for the standard curve. They were normalized to the housekeeping gene (*EF1-α*) and water. SPSS 20 for Windows was used to determine statistical significance among means (P≤0.05). The Shapiro-Wilk test (P>0.05) was performed to verify the normal distribution of data for the C(t) values.

Table 1: Sequences and amplification product sizes for white spruce (*Picea glauca*) primers used for RT-qPCR

Target	Melting temp (°C)	Primer (5' TO 3')	Expected amplification	PCR product in cDNA (bp)
MT2b	F:66 R:67	F: GTGGATGCGGAAGTGGATGC		
		R: GCAGAATGGGCGAACCAACC	84	84
EF1-α	F: 67R: 69	F: TCTCCACACTCAGCTCGGCG		
		R: CCAGTGGTTGTTGACTTGCCGG	119	119

Fig. 1: White spruce (*Picea glauca*) seedlings treated with different concentrations of copper and potassium sulfate (130, 656, and 1312 mg/kg) in a growth chamber. Water was used as a negative control. The experimental design was a completely randomized block design. Resistant seedlings are completely green while susceptible genotypes are partially or completely yellow or dead

To determine the significant difference between the means, an Analysis of Variance (ANOVA) was performed and then Levene's 2-tailed test was conducted to analyze if the treatment variances were equal (P>0.05) or unequal (P≤0.05). Dunnett's T3 Post Hoc Test (P≤0.05) was performed if variances were unequal and Tukey's Post Hoc test (P≤0.05) was performed if variances were equal. An independent t-test was also performed to show the significance (P≤0.05) of the differences between the means of needles and root data for each treatment.

Results

Gene Expression Induced by Different Concentrations of Copper and Potassium Ions

MT2b Expression in Roots

MT2b expression was increased 1.4-fold in samples treated with 1,312 mg/kg of copper sulfate, but were repressed in those treated with 656 mg/kg of copper sulfate, when compared to water (Fig. 2a). Plants treated with 1,312 mg/kg of copper sulfate induced a higher gene expression compared to plants treated with the two lower copper sulfate concentrations (656 and 130 mg/kg) (Fig. 2a). *MT2b* expression was downregulated in samples treated with 656 mg/kg of copper sulfate coampared to those treated with 130 mg/kg of copper sulfate (Fig. 2a). There were no significant changes in *MT2b* expression for samples treated with 130 mg/kg of copper sulfate, when compared to the water control (Fig. 2a). *MT2b* expression showed a concentration-dependent decrease in samples treated with potassium sulfate (Fig. 2b). For samples treated with 656 mg/kg and 1,312 mg/kg of potassium sulfate, *MT2b* expression was significantly decreased when compared to samples treated with water and 130 mg/kg of potassium sulfate (Fig. 2b). *MT2b* expression was further downregulated in samples treated with 1,312 mg/kg of potassium sulfate compared to those treated with 656 mg/kg of potassium sulfate (Fig. 2b). There were no significant changes to *MT2b* expression between the water control and samples treated with 130 mg/kg of potassium sulfate (Fig. 2b). The *MT2b* gene expression was significantly elevated for samples treated with 1,312 mg/kg of copper sulfate when compared to the equivalent potassium sulfate treatment (Fig. 2c). For samples treated with 130 mg/kg potassium sulfate and copper sulfate, the potassium sulfate induced a significant increase of *MT2b* expression compared to the

equivalent copper sulfate concentration (Fig. 2c). There were no significant differences in *MT2b* expression when samples treated with 656 mg/kg of copper sulfate and potassium sulfate were compared (Fig. 2c).

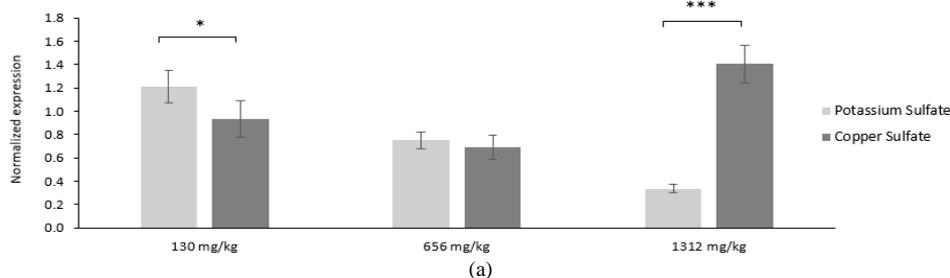### MT2b Expression in Needles

The *MT2b* gene expression was significantly downregulated in genotypes treated with 656 mg/kg of copper sulfate, compared to samples treated with 1,312 mg/kg of copper sulfate and the water control (Fig. 3a). The expression of *MT2b* was lower but statistically similar in genotypes treated with 130 mg/kg of copper sulfate, compared to the water control (Fig. 3a). *MT2b* expression was significantly downregulated in samples treated with 130, 656 and 1,312 mg/kg of potassium sulfate, compared to the water control (Fig. 3b). Overall, there were no significant differences in *MT2b* gene expression when the equivalent concentrations of potassium sulfate and copper sulfate treatments were compared (Fig. 3c).

MT2b Expression in Needle Vs. Root

The *MT2b* expression was significantly elevated in roots compared to needles for all three concentrations of copper sulfate (1,312, 656, and 130 mg/kg), (Fig. 4a). *MT2b* gene expression was significantly elevated in the roots for samples treated with 130 mg/kg and 656 mg/kg of potassium sulfate, compared to needles (Fig. 4b). No significant differences in *MT2b* expression was observed between root and needle tissues of samples treated with 1,312 mg/kg of potassium sulfate (Fig. 4b). Interestingly, the expression of *MT2b* was significantly elevated in needles of susceptible genotype compared to roots (Fig. 4c). The *MT2b* gene expression was not significantly different between the roots and needles of the resistant genotype (Fig. 4c).

MT2b Expression in Copper Susceptible and Resistant Genotypes

The expression of *MT2b* was higher in the roots of the copper-resistant genotypes compared to the water control (Fig. 5a). However, the *MT2b* gene was downregulated in the roots of susceptible genotypes compared to the water control and the resistant genotypes (Fig. 5a). There were no significant changes in *MT2b* expression in needle tissues when the water control and the resistant and susceptible genotypes were compared (Fig. 5b).
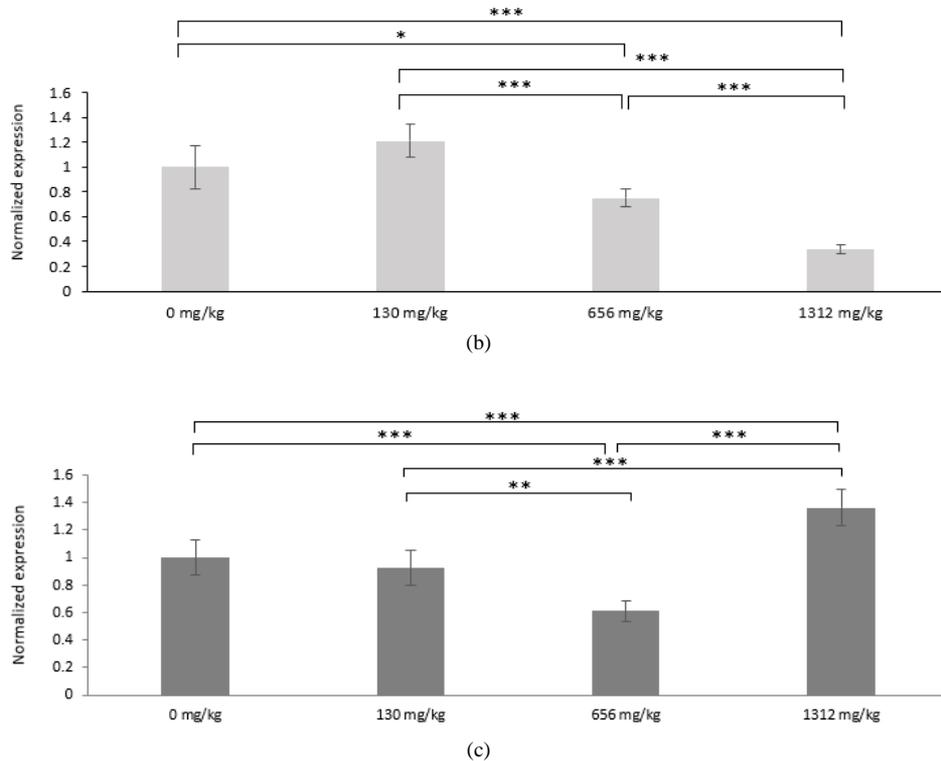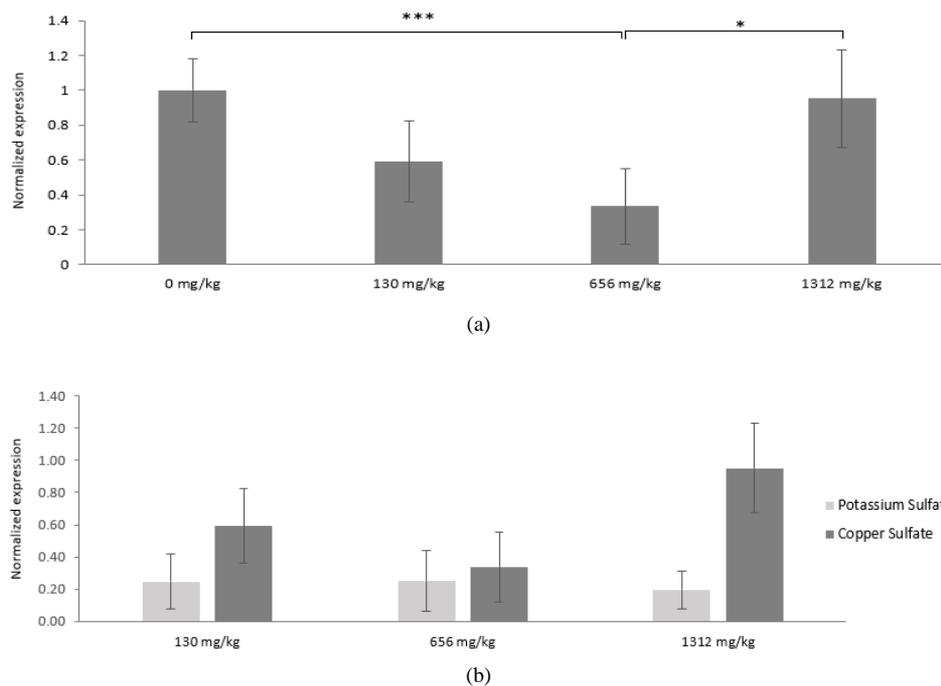

(a)

(b)

(c)

Fig. 2: *MT2b* gene expression in white spruce (*Picea glauca*) roots treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1-α*) and water was used as the negative control. Gene expression of (a) copper sulfate-treated roots, (b) potassium sulfate-treated roots, and (c) copper sulfate and potassium sulfate combined are presented. Significant differences among the means of the treatments are marked using * (p≤0.05), ** (P≤0.01), and *** (P≤0.001). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water
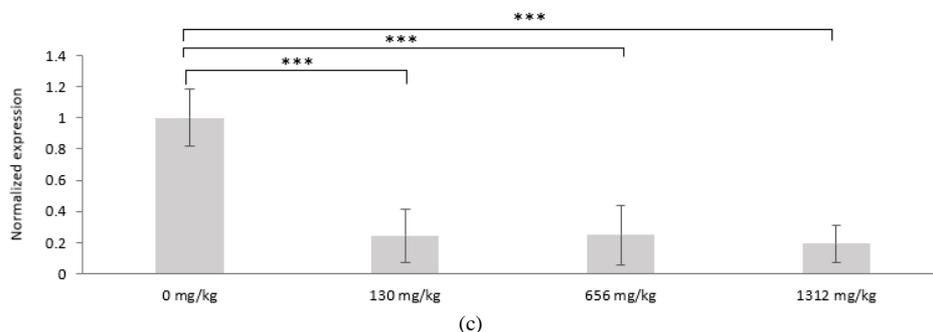

(a)

(b)

(c)

Fig. 3: *MT2b* gene expression in white spruce (*Picea glauca*) needles treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1-α*) and water was used as the negative control. Gene expression of (a) copper sulfate-treated needles, (b) potassium sulfate-treated needles, and (c) copper sulfate and potassium sulfate combined are presented. Significant differences among the means of the treatments are marked using * (P≤0.05), ** (p≤0.01), and *** (P≤0.001). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water
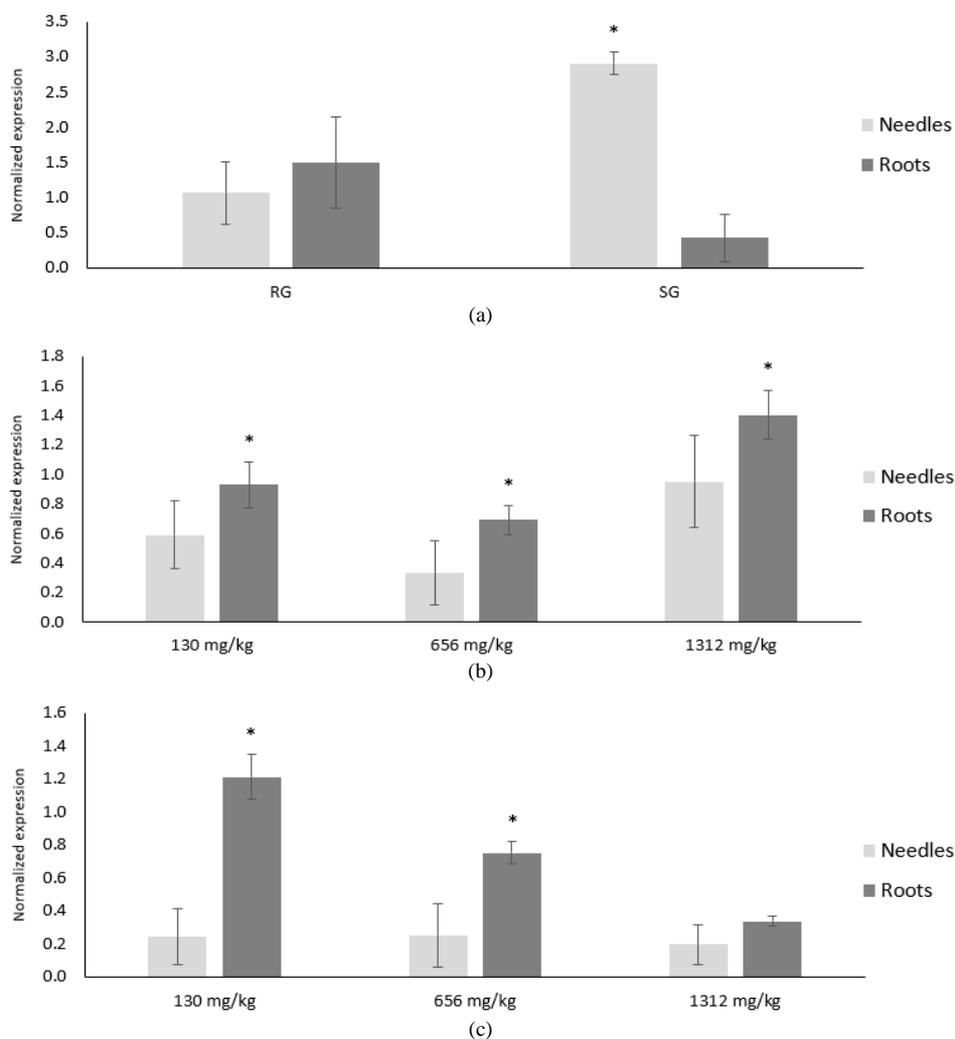

(a)

(b)

(c)

Fig. 4: *MT2b* gene expression in white spruce (*Picea glauca*) tissues. The gene expression was normalized to the housekeeping gene (*EF1-α*) and water was used as the negative control. Comparison of the gene expression between needles and roots; (a) copper sulfate treatments (b) potassium sulfate treatments and (c) Resistant (RG) and Susceptible (SG) genotypes treated with 1,312 mg/kg of copper sulfate. Significant differences (p≤0.05) between tissues are represented with an asterisk (*)
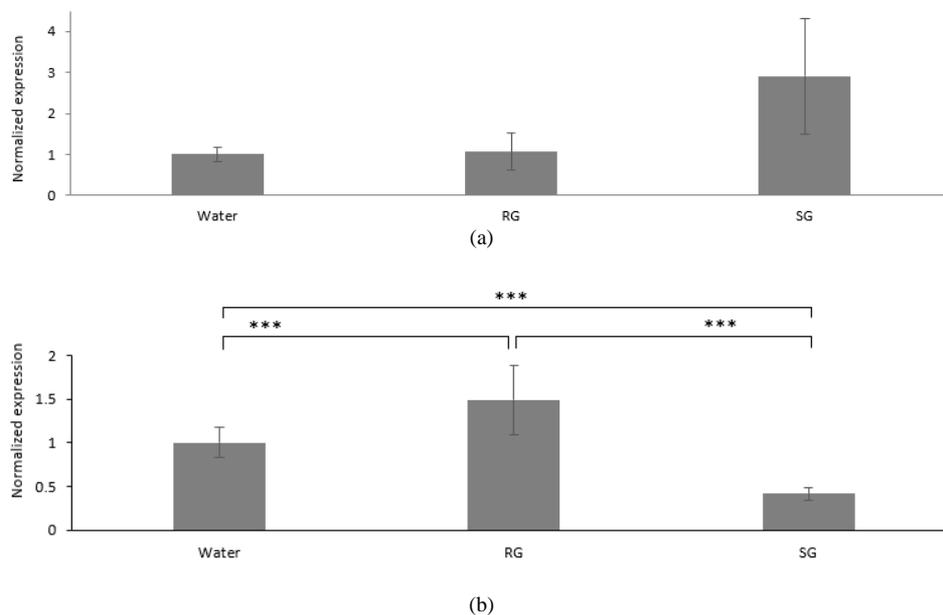
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Fig. 5: *MT2b* gene expression in white spruce (*Picea glauca*) (a) root and (b) needle tissues treated with 1,312 mg/kg of copper sulfate. The gene expression was normalized to the housekeeping gene (*EF1-α*) and water was used as the negative control. The phenotypic damage rating of the Resistant Genotype (RG) and Susceptible Genotype (SG) individuals was 2 and 7 respectively. Significant differences among the means of the treatments are marked using * (P≤0.05), ** (P≤0.01), and *** (P≤0.001). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water

Discussion

An excess of copper or free copper ions is toxic to plants and induces the production of multiple metal protein carriers, metal chelation mechanisms, or metal effluxes, to transport and remove the excess copper (Srivastava *et al.*, 2006). Metallothioneins are a gene family which is regulated by metals (Murphy *et al.*, 1997). Genes within this family contain a large distribution of cysteine residues within the amino acid terminals (Cobbett and Goldsbrough, 2002). They are high-affinity ligands which are one of the detoxification methods to prevent metal toxicity (Hall, 2002). Within this gene family, the *MT2a* and *MT2b* genes are induced under high copper concentrations and are expressed during oxidative stress (Zhou and Goldsbrough, 1995; Mir *et al.*, 2004). Many plant species contain two different isoforms of metallothioneins; *MT1* and *MT2* (Murphy and Taiz, 1995). The isoforms may be differently expressed. For example, the *MT2* gene was expressed during metal treatments and the *MT1* gene remained unaffected in *Arabidopsis* ecotypes (Murphy and Taiz, 1995). Interestingly, the expression of *MT2* genes was not affected in the leaves of *Avicennia germinans* when plants were exposed to different concentrations of copper (Gonzalez-Mendoza *et al.*, 2007). However, *Escherichia coli* and *Arabidopsis thaliana* both had an increased tolerance to copper ions due to the overexpression of the *MT2* gene (Zhigang *et al.*, 2006). The expression level of

MT2b in the copper-tolerant *Iris lactea* var. *chinensis* was found to be significantly increased in roots and leaves after a copper sulfate treatment (Gu *et al.*, 2015). When the *Iris lactea MT2b* gene was placed into *Arabidopsis* by a floral dip, the increased transgene expression showed greater root length growth compared to the non-transgenic plants after the copper treatment (Gu *et al.*, 2015). Interestingly, the expression of *MT2* expression in *Brassica campestris* did not significantly change when treated with copper but was induced with a cadmium treatment (Lv *et al.*, 2013). However, the *MT1* gene in *B. campestris* had an elevated expression after copper and cadmium treatments, suggesting that the MT gene family may work independently from one another, or have separate roles in plants dealing with metal toxicity (Lv *et al.*, 2013). The overexpression of the *MT2* gene in plants exposed to copper ions is an indication of their tolerance to copper (Guo *et al.*, 2008; Zhao *et al.*, 2012).

This is consistent with the current study showing that the highest concentration of copper sulfate had a significantly elevated *MT2b* gene expression in *P. glauca* roots when compared to the water control. The expression of MT2b was suppressed in the roots and needles of plants treated with 656 mg/kg of copper sulfate compared to the water control. It was observed that the potassium sulfate control was inhibiting the *MT2b* gene in a concentration-dependent manner in roots. The suppression of this gene in plants exposed to potassium sulfate was also observed in needles but this downregulation was not concentration-

dependent. This indicates that the repressed *MT2b* gene expression in plants treated with 656 mg/kg of copper sulfate may be caused by the sulfate ions, rather than the copper ions. However, it is also possible that the potassium ion is causing *MT2b* inhibition. Previous analyses demonstrated a significantly elevated *MT2b* expression in leaves of *Quercus rubra* treated with 1,312 mg/kg of potassium sulfate when compared to the control (Proulx *et al.*, 2017). Although the current study observed contrasting results, both studies indicate that the *MT2b* gene expression could be affected by the potassium or sulfate ions. Since samples treated with 1,312 mg/kg of copper sulfate showed an increased *MT2b* gene expression in *P. glauca* roots, it is suspected that the sulfate ions are inhibiting the *MT2b* gene at lower concentrations of copper sulfate (656 mg/kg). Once the copper ion concentration reaches the metal toxicity threshold, the expression of the *MT2b* gene is increased in roots rather than inhibited. The current study showed contrasting effects of sulfate and copper ions on the expression of *MT2b* in needle tissues. Samples treated with 1,312 mg/kg of copper sulfate have a similar level of *MT2b* expression in needles compared to the water control, but they have a significantly higher *MT2b* expression compared to samples treated with 656 mg/kg of copper sulfate. Potentially, the increased copper ion concentration is the cause of the differential *MT2b* expression between the two copper sulfate treatments (1,312 and 656 mg/kg) in the needles.

When comparing the expression of *MT2* in different tissues, it was found that *Brassica campestris* has a naturally high *MT2* expression in leaves (Lv *et al.*, 2013) and *B. napus* had a high *MT2b* expression in the cotyledons compared to hypocotyls (Pan *et al.*, 2018). Expression of *MT2b* within the *Arabidopsis* MT family was increased in roots after exposure to copper, whereas the young or maturing leaves showed no changes in *MT2b* expression (Guo *et al.*, 2003). On the other hand, the expression of *MT2* in *Thlaspi caerulescen's* shoot was neither induced nor repressed when treated with copper sulfate (Roosenstf *et al.*, 2005). Interestingly, the *MT2* RNA expression in *Thlaspi caerulescens* did not show significant differences between roots and shoots before the copper treatment (Roosens *et al.*, 2005). In the current study, the *MT2b* gene was highly expressed in the roots of *P. glauca* treated with all three copper sulfate concentrations and the two low concentrations of potassium sulfate. This is consistent with data reported in *Arabidopsis* (Guo *et al.*, 2003). This could indicate that the *MT2b* expression is generally higher in the roots of *P. glauca* to chelate the copper and sulfate ions before they translocate to the aerial tissues (Guo *et al.*, 2008). Previous data suggested that *IREG2,* which sequesters nickel, had a high expression in *N. japonica* roots, which led to nickel resistance and limited nickel translocation from roots to shoots (Nishida *et al.*, 2020). *MT2b* expression was elevated in *P. glauca* needles of the susceptible genotype but was similar in the needles and roots of the resistant genotype. It would be beneficial to measure the copper ion concentrations in *P. glauca* tissues to determine if there was a higher copper translocation in the susceptible genotype when compared to the resistant genotype, as this could affect the expression of the *MT2b* gene in different plant tissues.

When the *MT2* sequence from *Brassica campestris* was transfected to *Arabidopsis*, there was an increased tolerance to both the cadmium and copper metals, resulting in increased root and shoot lengths when compared to the control plants (Lv *et al.*, 2013). In this study, *MT2b* was highly expressed in roots of the copper-resistant genotypes but was inhibited in the susceptible genotype roots, when compared to the control. It should be pointed out that apoptotic signaling could disrupt the *MT2b* expression in susceptible plants by DNA or mRNA degradation, which limits the transcriptional and translational abilities of the *MT2b* gene. Further studies will need to analyze resistant and susceptible plants before the cell death process starts (Thomas *et al.*, 2015). However, apoptosis might not affect *MT2b* expression in *P. glauca*, since no significant changes to the *MT2b* expression within the needle tissues were observed when the water control, the resistant genotype, and the susceptible genotype (with dying needle tissues) were compared.

Conclusion

This study aimed to determine the effects of different concentrations of copper ions on MT2b expression in white spruce (*P. glauca*). The present study reports for the first time, novel information on the effect of copper on the expression of MT2b in P. glauca. The MT2b gene was upregulated in roots when plants were exposed to 1,312 mg/kg of copper sulfate but were downregulated in genotypes treated with 656 mg/kg of copper sulfate in both tissues, compared to the water control. The *MT2b* gene expression was significantly downregulated in needles of genotypes treated with 656 mg/kg of copper sulfate when compared to samples treated with 1,312 mg/kg of copper sulfate and the water control. The expression of *MT2b* was higher in roots compared to needles regardless of the concentrations of copper sulfate or potassium sulfate used. Differential expression of *MT2b* was observed in copper-resistant and susceptible genotypes. A transcriptome analysis is warranted to determine which genes of the *P. glauca* genome are affected by copper toxicity.

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Author's Contributions

Megan McKergow: Conducted experiments, analyzed data, and wrote the first draft.

Kabwe Nkongolo: Designed and monitored the experiments and data analysis; wrote the final manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved**.**

References

Ahsan, N., Lee, D. G., Lee, S. H., Kang, K. Y., Lee, J. J., Kim, P. J., ... & Lee, B. H. (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. *Chemosphere*, *67*(6), 1182-1193. https://doi.org/10.1016/J.CHEMOSPHERE.2006.10.075

Boyd, M., & Nkongolo, K. K. (2021). Nickel induces changes in the expression of genes encoding 1-aminocyclopropane-1-carboxylic acid deaminase and glutathione reductase in Picea glauca. *Chemistry and Ecology*, *37*(7), 589-602. https://doi.org/10.1080/02757540.2021.1937140

Chandrangsu, P., Rensing, C., & Helmann, J. D. (2017). Metal homeostasis and resistance in bacteria. *Nature Reviews Microbiology*, *15*(6), 338-350. https://doi.org/10.1038/nrmicro.2017.15

Chugh, L. K., & Sawhney, S. K. (1996). Effect of cadmium on germination, amylases and rate of respiration of germinating pea seeds. *Environmental Pollution*, *92*(1), 1-5. https://doi.org/10.1016/0269-7491(95)00093-3

Cobbett, C., & Goldsbrough, P. (2002). Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, *53*(1), 159-182. https://doi.org/10.1146/annurev.arplant.53.100301.135154

Czajka, K. M., Michael, P., & Nkongolo, K. (2019). Differential effects of nickel dosages on in vitro and in vivo seed germination and expression of a high affinity nickel-transport family protein (AT2G16800) in trembling aspen (Populus tremuloides). *Ecotoxicology*, *28*(1), 92-102. https://doi.org/10.1007/S10646-018-2003-8

Gonzalez-Mendoza, D., Moreno, A. Q., & Zapata-Perez, O. (2007). Coordinated responses of phytochelatin synthase and metallothionein genes in black mangrove, Avicennia germinans, exposed to cadmium and copper. *Aquatic Toxicology*, *83*(4), 306-314. https://doi.org/10.1016/J.AQUATOX.2007.05.005

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., & Eide, D. (1998). Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. *Proceedings of the National Academy of Sciences*, *95*(12), 7220-7224. https://doi.org/10.1073/PNAS.95.12.7220

Gu, C. S., Liu, L. Q., Deng, Y. M., Zhu, X. D., Huang, S. Z., & Lu, X. Q. (2015). The heterologous expression of the Iris lactea var. chinensis type 2 metallothionein IlMT2b gene enhances copper tolerance in Arabidopsis thaliana. *Bulletin of Environmental Contamination and Toxicology*, *94*(2), 247-253. https://doi.org/10.1007/s00128-014-1444-x

Guo, W. J., Bundithya, W., & Goldsbrough, P. B. (2003). Characterization of the Arabidopsis metallothionein gene family: Tissue-specific expression and induction during senescence and in response to copper. *New Phytologist*, 369-381. https://doi.org/10.1046/j.1469-8137.2003.00813.x

Guo, W. J., Meetam, M., & Goldsbrough, P. B. (2008). Examining the specific contributions of individual Arabidopsis metallothioneins to copper distribution and metal tolerance. *Plant physiology*, *146*(4), 1697-1706. https://doi.org/10.1104/PP.108.115782

Hall, J. Á. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, *53*(366), 1-11. https://doi.org/10.1093/JEXBOT/53.366.1

Hamer, D. H. (1986). Metallothionein. *Annual review of Biochemistry*, *55*(1), 913-951. https://doi.org/10.1146/ANNUREV.BI.55.070186.004405

Hossain, M. A., Piyatida, P., da Silva, J. A. T., & Fujita, M. (2012). Molecular mechanism of heavy metal toxicity and tolerance in plants: Central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *Journal of Botany*, *2012*. https://doi.org/10.1155/2012/872875

Jain, R., Srivastava, S., Solomon, S., Shrivastava, A. K., & Chandra, A. (2010). Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (*Saccharum spp.*). *Acta Physiologiae Plantarum*, *32*(5), 979-986. https://doi.org/10.1007/S11738-010-0487-9

Jardim, W. F., & Pearson, H. W. (1984). A study of the copper-complexing compounds released by some species of cyanobacteria. *Water Research*, *18*(8), 985-989. https://doi.org/10.1016/0043-1354(84)90249-5

Jiang, W., Liu, D., & Liu, X. (2001). Effects of copper on root growth, cell division and nucleolus of Zea mays. *Biologia Plantarum*, *44*(1), 105-109. https://doi.org/10.1023/A:1017982607493

Kabir, M., Iqbal, M. Z., Shafiq, M., & Farooqi, Z. R. (2008). Reduction in germination and seedling growth of Thespesia populnea L., caused by lead and cadmium treatments. *Pak. J. Bot*, *40*(6), 2419-2426.

Keinänen, S. I., Hassinen, V. H., Kärenlampi, S. O., & Tervahauta, A. I. (2007). Isolation of genes up-regulated by copper in a copper-tolerant birch (Betula pendula) clone. *Tree Physiology*, *27*(9), 1243-1252. https://doi.org/10.1093/TREEPHYS/27.9.1243

Kobayashi, Y., Kuroda, K., Kimura, K., Southron-Francis, J. L., Furuzawa, A., Kimura, K., ... & Koyama, H. (2008). Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in Arabidopsis. *Plant physiology*, *148*(2), 969-980. https://doi.org/10.1104/PP.108.119933

L'Huillier, L., d'Auzac, J., Durand, M., & Michaud-Ferrière, N. (1996). Nickel effects on two maize (Zea mays) cultivars: Growth, structure, Ni concentration and localization. *Canadian Journal of Botany*, *74*(10), 1547-1554. https://doi.org/10.1139/B96-187

Li, L., Long, M., Islam, F., Farooq, M. A., Wang, J., Mwamba, T. M., ... & Zhou, W. (2019). Synergistic effects of chromium and copper on photosynthetic inhibition, subcellular distribution and related gene expression in Brassica napus cultivars. *Environmental Science and Pollution Research*, *26*(12), 11827-11845. https://doi.org/10.1007/s11356-019-04450-5

Lidon, F. C., & Henriques, F. S. (1994). Subcellular localisation of copper and partial isolation of copper proteins in roots from rice plants exposed to excess copper. *Functional Plant Biology*, *21*(4), 427-436. https://doi.org/10.1071/PP9940427

Liu, D., Jiang, W., & Gao, X. (2003). Effects of cadmium on root growth, cell division and nucleoli in root tip cells of garlic. *Biologia Plantarum*, *47*(1), 79-83. https://doi.org/10.1023/A:1027384932338

Lv, Y., Deng, X., Quan, L., Xia, Y., & Shen, Z. (2013). Metallothioneins BcMT1 and BcMT2 from Brassica campestris enhance tolerance to cadmium and copper and decrease production of reactive oxygen species in Arabidopsis thaliana. *Plant and Soil*, *367*(1), 507-519. https://doi.org/10.1007/s11104-012-1486-y

Meng, J. G., Zhang, X. D., Tan, S. K., Zhao, K. X., & Yang, Z. M. (2017). Genome-wide identification of Cd-responsive NRAMP transporter genes and analyzing expression of NRAMP 1 mediated by miR167 in Brassica napus. *Biometals*, *30*(6), 917-931 https://doi.org/10.1007/s10534-017-0057-3

Mir, G., Domenech, J., Huguet, G., Guo, W. J., Goldsbrough, P., Atrian, S., & Molinas, M. (2004). A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *Journal of Experimental Botany*, *55*(408), 2483-2493. https://doi.org/10.1093/JXB/ERH254

Moarefi, N., & Nkongolo, K. K. (2022). Contrasting tolerance and gene expression between white pine (Pinus strobus) and jack pine (P. banksiana) exposed to an increasing nickel concentration. *Ecological Genetics and Genomics*, 100124. https://doi.org/10.1016/J.EGG.2022.100124

Mukherji, S., & GUPTA, B. D. (1972). Characterization of copper texicity in lettuce seedlings. *Physiologia Plantarum*, *27*(2), 126-129. https://doi.org/10.1111/J.1399-3054.1972.TB03588.X

Murphy, A., & Taiz, L. (1995). Comparison of metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes (correlation with copper tolerance). *Plant Physiology*, *109*(3), 945-954. https://doi.org/10.1104/PP.109.3.945

Murphy, A., Zhou, J., Goldsbrough, P. B., & Taiz, L. (1997). Purification and immunological identification of metallothioneins 1 and 2 from Arabidopsis thaliana. *Plant Physiology*, *113*(4), 1293-1301. https://doi.org/10.1104/pp.113.4.1293

Nishida, S., Tanikawa, R., Ishida, S., Yoshida, J., Mizuno, T., Nakanishi, H., & Furuta, N. (2020). Elevated expression of vacuolar nickel transporter gene IREG2 is associated with reduced root-to-shoot nickel translocation in Noccaea japonica. *Frontiers in Plant Science*, *11*, 610. https://doi.org/10.3389/FPLS.2020.00610

Nkongolo, K. K., Spiers, G., Beckett, P., Narendrula, R., Theriault, G., Tran, A., & Kalubi, K. N. (2013). Long-term effects of liming on soil chemistry in stable and eroded upland areas in a mining region. *Water, Air, & Soil Pollution*, *224*(7), 1-14. https://doi.org/10.1007/s11270-013-1618-x

Pan, Y., Zhu, M., Wang, S., Ma, G., Huang, X., Qiao, C., ... & Qu, C. (2018). Genome-Wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in Brassica napus L. *International journal of molecular sciences*, *19*(8), 2181. https://doi.org/10.3390/ijms19082181

Pena, L. B., Azpilicueta, C. E., & Gallego, S. M. (2011). Sunflower cotyledons cope with copper stress by inducing catalase subunits less sensitive to oxidation. *Journal of Trace Elements in Medicine and Biology*, *25*(3), 125-129. https://doi.org/10.1016/J.JTEMB.2011.05.001

Proulx, M., Michael, P., Djeukam, C., & Nkongolo, K. (2017). Differential gene transcription in red oak (*Quercus rubra*) genotypes resistant to copper toxicity. *American Journal of Biochemistry and Biotechnology*, 13, 215–255. https://doi.org/10.3844/AJBBSP.2017.215.225

Roosens, N. H., Leplae, R., Bernard, C., & Verbruggen, N. (2005). Variations in plant metallothioneins: The heavy metal hyperaccumulator Thlaspi caerulescens as a study case. *Planta*, *222*(4), 716-729. https://doi.org/10.1007/s00425-005-0006-1

Sancenón, V., Puig, S., Mira, H., Thiele, D. J., & Peñarrubia, L. (2003). Identification of a copper transporter family in Arabidopsis thaliana. *Plant Molecular Biology*, *51*(4), 577-587. https://doi.org/10.1023/A:1022345507112

Sethy, S. K., & Ghosh, S. (2013). Effect of heavy metals on germination of seeds. *Journal of Natural Science, Biology and Medicine*, *4*(2), 272. https://doi.org/10.4103/0976-9668.116964

Singh, S., Parihar, P., Singh, R., Singh, V. P., & Prasad, S. M. (2016). Heavy metal tolerance in plants: Role of transcriptomics, proteomics, metabolomics and ionomics. *Frontiers in Plant Science*, *6*, 1143. https://doi.org/10.3389/fpls.2015.01143

Srivastava, S., Mishra, S., Tripathi, R. D., Dwivedi, S., & Gupta, D. K. (2006). Copper-induced oxidative stress and responses of antioxidants and phytochelatins in Hydrilla verticillata (Lf) Royle. *Aquatic Toxicology*, *80*(4), 405-415. https://doi.org/10.1016/J.AQUATOX.2006.10.006

Thomas, M. P., Liu, X., Whangbo, J., McCrossan, G., Sanborn, K. B., Basar, E., ... & Lieberman, J. (2015). Apoptosis triggers specific, rapid and global mRNA decay with 3′ uridylated intermediates degraded by DIS3L2. *Cell Reports*, *11*(7), 1079-1089. https://doi.org/10.1016/J.CELREP.2015.04.026

Wintz, H., Fox, T., Wu, Y. Y., Feng, V., Chen, W., Chang, H. S., ... & Vulpe, C. (2003). Expression profiles of Arabidopsis thaliana in mineral deficiencies reveal novel transporters involved in metal homeostasis. *Journal of Biological Chemistry*, *278*(48), 47644-47653. https://doi.org/10.1074/JBC.M309338200

Yruela, I. (2009). Copper in plants: Acquisition, transport and interactions. *Functional Plant Biology*, *36*(5), 409-430. https://doi.org/10.1071/FP08288

Zhao, H., Wu, L., Chai, T., Zhang, Y., Tan, J., & Ma, S. (2012). The effects of copper, manganese and zinc on plant growth and elemental accumulation in the manganese-hyperaccumulator Phytolacca americana. *Journal of Plant Physiology*, *169*(13), 1243-1252. https://doi.org/10.1016/J.JPLPH.2012.04.016

Zhigang, A., Cuijie, L., Yuangang, Z., Yejie, D., Wachter, A., Gromes, R., & Rausch, T. (2006). Expression of BjMT2, a metallothionein 2 from Brassica juncea, increases copper and cadmium tolerance in Escherichia coli and Arabidopsis thaliana, but inhibits root elongation in Arabidopsis thaliana seedlings. *Journal of experimental botany*, *57*(14), 3575-3582. https://doi.org/10.1093/JXB/ERL102

Zhou, J., & Goldsbrough, P. B. (1995). Structure, organization and expression of the metallothionein gene family inArabidopsis. *Molecular and General Genetics MGG*, *248*(3), 318-328. https://doi.org/10.1007/BF02191599