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Differential Gene Transcription in Red Oak (*Quercus rubra*) Genotypes Resistant to Copper Toxicity

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Abstract: Toxicity of metals is a major abiotic stressor of plants. Copper (Cu) is one of the most abundant metals in soils from the Greater Sudbury Region (GSR) due to its mining history. Recent studies have described several transporters and chelating proteins involved in copper resistance. *Quercus rubra* (Red Oak) is a common species that grows in metal contaminated soils in Northern Ontario (Canada). The main objectives of this study were1) to evaluate the toxicity of copper to *Q. rubrum* plants and 2) assess the level of transcriptionof genes associated with copper resistance (RAN1, MT2b and MRP4). *Q. rubra* seedlings were grown in growth chambers and treated with copper at different doses. Total RNA was extracted from leaves and amplified by RT-qPCR. All the plants tested were resistant to Cu even at high concentrations of 1312 mg of Cu per kg of dry soil since no damage associated with Cu toxicity was observed after 14 days of treatment. RAN1 transporter and the chelating protein MT2b were significantly downregulated ($p \leq 0.05$) at high doses of 656 mg and 1312 mg of copper per kg of dry soil compared to water control. The transcription of MRP4 was significantly increased in the presence of copper at a concentration of 1312 mg/kg. No gene demonstrated differential transcription in samples treated with a low dose of 130 mg of copper/kg of dry soil that is equivalent to the bioavailable amount of copper under natural conditions in the Greater Sudbury Region.

Keywords: Copper Toxicity, Red Oak (*Quercus rubra*), Gene Transcription, RT – qPCR, Northern Ontario

Introduction

Soil metal contamination is a serious problem for mining regions around the world. The Greater Sudbury Region (GSR), home to one of the world's largest copper and nickel mining operations (Winterhalder, 1995; Adamo *et al*., 2002), has been subject to mining since the end of 19th century (Belzile *et al*., 2004). These ongoing operations have resulted in higher levels of heavy metals such as copper and nickel in soils in this region (Adamo *et al*., 2002). Several studies have reported high concentrations of copper and nickel near smelters in the GSR compared to more remote areas (Narendrula *et al*., 2012; 2013). Although the amount of total metals in soil is high, recent studies have shown that it is essential to consider bioavailable metals to predict their toxicity and impact on vegetation (Violante *et al*., 2010; Abedin *et al*., 2012; Mehes-Smith *et al*., 2013). The bioavailable metals represent the potion of total elements that can be absorbed by the root system (Theriault and Nkongolo, 2016). Copper plays a role in the development and growth of several plant species (Yruela, 2005). It is a microelement that acts as a cofactor for several metalloproteins involved in various physiological processes such as electron transport in photosynthesis (Raven *et al*., 1999), mitochondrial respiration and cell membrane metabolism (Yruela, 2005).

At concentrations similar to the level found in soil from the GSR, copper has adverse effects on plants. It can impair plant growth and several cellular processes (Yruela, 2005). In the 1970s, the GSR had about 17,000 hectares of arid land and 72,000 semi-arid land near smelters (Winterhalder, 1995). Despite a significant reduction in metals and sulfur dioxide emissions, copper and nickel concentrations remain high. Yet the populations of several tree species in northern Ontario proliferate despite the high amount of metals in the ecosystem (Nkongolo *et al*., 2013; Tran *et al*., 2014;

Theriault *et al*., 2014). Species such as red oak (*Quercus rubra*), some birch and poplar, recolonized metal contaminated areas within the GSR (Winterhalder, 1995; Makela *et al*., 2016). *Q. rub*ra is a native species of GSR and has played an important role in the restoration process of this region (Beckett and Negusanti, 1990).

It is known that plants avoid the toxic effect of metals by maintaining a safe internal level (Manara, 2012). These protective physiological mechanisms vary greatly among species. Some species have developed a strategy to reduce the amount of bioavailable heavy metals by forming non-toxic metal complexes in the rhizosphere (Ryan *et al*., 2009; Maron *et al*., 2013). Other species try to avoid excessive absorption of heavy metals. This exclusion is based on a decrease in the transcription of genes coding for metal transporters in the root system resulting in a decrease in metal influx (Lin and Aarts, 2012). On the other hand, accumulators translocate metals in their aerial tissues (Baker, 1981). For example, copper accumulates in *Q. rubra* leaves at certain concentrations (Tran *et al*., 2014).

Genetic mechanisms involved in plant resistance to toxicity of metals are not well understood, especially in woody species such as *Q. rubra*. Several studies on model plant species and other non-model species have demonstrated that genes encode transporters (Sancenón *et al*., 2003; Keinänen *et al*., 2007; Kobayashi *et al*., 2008) and chelating proteins (Guo *et al*., 2008) which are associated with copper resistance. Some of the targeted genes, RAN1 and MRP4 code for ATPase transport protein families of the P-type (Yruela, 2005) and the ABC transporter (ATP Binding Cassette) (Rea, 1999; Keinänen *et al*., 2007), respectively for copper transport. Moreover, the selected MT2b gene codes for a protein of the metallothionein family which plays an important role in the chelation of metals (Guo *et al*., 2008). Some recent studies have demonstrated the link between these genes and copper resistance in some species and this coupled with the high survival of *Q. rubra* in metal contaminated sites in the GSR (Beckett and Negusanti, 1990; Adamo *et al*., 2002), leads us to hypothesize that resistance to the copper toxicity in *Q. rubra* might be mediated by RAN1, MRP4 and MT2b genes.

Hence, the main objectives of this study were to (1) evaluate the toxicity of copper to *Q. rubrum* plants and (2) assess the level of transcriptionof genes associated with copper resistance (RAN1, MT2b and MRP4).

Materials and Methods

Samplings

Quercus rubra seeds (seedlot#007228) were collected from zone 34 in Southern Ontario, Canada (https://files.ontario.ca/seed_zones_of_ontario.jpg. The seedlings were germinated and grown in jiffy pots for five months. They were then transplanted in 50:50 mix of quartz sand and potting soil and acclimatized for a week before treatment.

To assess Cu toxicity on *Q. rubra*, six – month old seedlings were treated with different doses of copper. The copper treatments consisted of an aqueous solution of copper sulfate salt ($CuSO_4$) at final copper concentrations of 130 mg, 656 mg and 1312 mg of copper per kg of dry soil. These levels corresponded to the bioavailable (fraction of the total copper available to biota), half total and total copper amounts in metal-contaminated soils within the GSR, respectively. To determine any potentially toxic effect of excess sulfate ion (SO_4) on plants, three potassium sulfate (K_2SO_4) treatments corresponding to each Cu dose were included as controls. Salt-free water was used as the main control. The experimental design was a completely randomized block with 15 replications.

Copper toxicity was measured using a damage rating scale ranging from 1 to 9, 1 being no visible toxicity symptoms and 9 dead plants. Individual plants with scores of 1 to 3 were considered copper resistant, 4 to 6, moderately resistant and 7 to 9 susceptible. Plant heights were measured every two days throughout the experiment to measure the effects of each treatment on plant growth.

RNA Extraction

Total RNA was extracted from samples using the procedure described by Theriault and Nkongolo (2016; Djeukam *et al*., 2016). RNA was quantified using the Qubit RNA BR Assay kit from Life Technologies (Carlsbad, United States). RNA quality was verified on a 1% agarose gel. One microgram of RNA from samples of the same treatment was pooled together for further processing.

RT-qPCR

RNA was treated with DNase1 (#EN0521) from Life Technologies. Sequences for each gene were retrieved from the NCBI database and analyzed by BLAST in the *Q rubra* transcriptome described in "The Hardwood Genomics Project" (http://hardwoodgenomics.org).When possible, primers were designed to span all the exons encode by the genes. Primers were checked for hairpins, self and hetero-dimers using the OligoAnalyzer 3.1 by IDT (https://www.idtdna.com/calc/analyzer). The list of candidate genes with their associated primer pairs can be found in Table 1 and 2. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies.

PCR was performed on both *Q. rubra* DNA and cDNA. Size of the amplicons derived from primer pair amplification were verified on agarose gels. Only primers that showed a reproducible single band of the appropriate size were used for RT-qPCR. RT-qPCR was performed using the Dynamo HS SYBR Green Kit by Life Technologies according to the manufacturer's protocol.

Table 1: Candidate genes involved in copper resistance in model and non-model plant species

Targeted genes	Species	References
RAN 1	*Arabidopsis thaliana*	Kobayashi *et al*. (2008)
MRP4	*Betula pendula*	Keinänen *et al*. (2007)
MT2b	*Arabidopsis thaliana*	Guo *et al*. (2008)

Table 2: Sequences of red oak (*Quercus rubra*) primers used for RT-qPCR and expected amplification product size

Targeted and housekeeping gene	Primer pair sequences	Expected amplicon (pb)	Hybrization tempearture (°C)
Targeted gene			
RAN 1	F: TGCTTGCTCCAATTCTGTTG	210	55
	R: AGAGCTCCATGTGGCTTTGT		
MT2b	F: GGAAACTGTGGCTGTGGAAC		
	R: CTGAGGAGCAACACCAACAA	123	55
MRP4	F: TTGTTGGGACTGTGGGATCT		
	R: TGGTGCCATTTTGTAATCCA	139	55
House keeping gene			
α-tubuline	F: GACGTGTCTGTGCTCTTGGA	147	55
	R: AGCCCCATCAAATCTCAATG		

Each sample was amplified with the MJ Research PTC-200 Thermal Cycler in triplicates. The process included (1) initial denaturing at 95°C for 15min; (2) denaturing at 94°C for 30sec; (3) 30sec at 55°C annealing; (4) elongation at 72°C for 30sec; (5) read (6) repeat step 2–6 for 41 cycles; (7) final elongation at 72°C for 7min; (8) melting curve 72–95°C, every 1°C, hold for 10sec; and (9) final elongation at 72°C for 3min. This qPCR, was run three separate times with each sample in triplicate. This resulted in a total of nine data point for each bulked sample.

The data was analyzed using the MJ Opticon Monitor 3.1 by BioRad and delta C(t) values were exported to excel. Delta C(t) values of samples from growth chamber assays were normalized to a separate housekeeping gene and the relative transcription was calculated using the water control.

Statistical Analysis

Data were analyzed using SPSS 20 for Windows, with all data being transformed to achieve a normal distribution. ANOVA, followed by Dunnett T3 were performed to determine significant differences among means. Student T test was performed to determine significant differences between gene transcription levels for different $CuS0_4$ treatments and the controls. All the differences were determined at $p \leq 0.05$.

Results

Copper Toxicity

Analysis of damage ratings and plant growth revealed no significant difference among the treatments during the 14 days of the assays. All the seedlings treated showed no symptoms of copper toxicity even at the highest concentration of 1,312 mg/kg for 14 days (Table 3).

Gene Transcription

All the primer pairs developed for the targeted genes were functional for qRT-PCR analysis since they generated expected size amplifications. Analysis of qRT-PCR data showed that transcription of two genes (RAN1 and MT2b) were significantly decreased demonstrated a compared to controls for 656 and 1312 mg Cu/kg treatment while MRP4 transcription was increased only at the dosage of 1,312 mg/kg.

Sulfates at the three concentrations tested had no effect on gene transcription. Detailed data on plant reaction to sulfate are summarized in Fig. 1S and 2S. RNA1 gene was significantly suppressed by copper treatments at 1,312 mg/kg and 656 mg/kg compared to their respective water and potassium sulfate control treatments (Fig. 1 and 1S) but not between treatment of 1,312 mg/kg and 656 mg/kg (Fig. 1). The same result was observed when the 130 mg/kg treatment was compared to water control (Fig. 1).

No significant difference in transcription was observed between sulfate and water controls (Fig. 2S). Significant down regulation (suppression) of the MT2b gene was observed for high copper treatments (656 mg/kg and 1,312 mg/kg) compared to the respective water and sulfate control treatments ($p \leq 0.05$) (Fig. 2 and 2S). No significant difference was observed between 1,312 mg/kg and 656 mg /kg treatments (Fig. 2). Similarly, the level of transcription of MT2b gene was similar when 130 mg/kg treatment was compared with water control (Fig. 2).

On the other hand, MRP4 was significantly upregulated at the high dose of 1312 mg of copper/kg of dry soil compared to water and sulfate controls (Fig. 3 and 3S). Similarly, at this dose, Copper treatments at 130 mg/kg and 656 mg/kg did not result in significant differences in transcription with their respective water and sulfate control (Fig. 3 and Fig. 3S).

Table 3: Damage ratings of *Quercus rubra* seedlings after 14 days of treatments with different doses of copper sulfate ($CuSO_4$) and potassium sulfate (K_2SO_4)

Treatments	Copper Concentrations (mg/kg)	SO_4^{2-} Concentration (mg/kg)	Damage rating
$CuSO_4$	130	0.20	1.1± 0.1
	656	0.99	1.0 ± 0.0
	1312		
	1.98	2.0 ± 0.4	
K_2SO_4	0	0.20	1.0 ± 0.0
	0	0.99	1.0 ± 0.0
	0		
	1.98	1.4 ± 0.4	
Water	0	0	1.0 ± 0.0

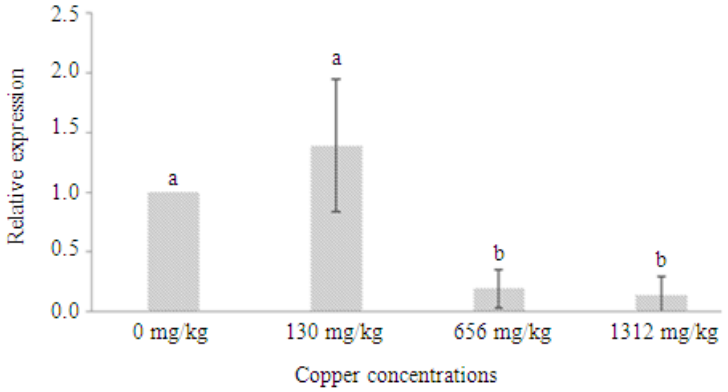

Fig. 1: Transcription of RAN1 in red oak (*Quercus rubra*) treated with different doses of copper. Transcription of RAN1 was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p<0.05$)

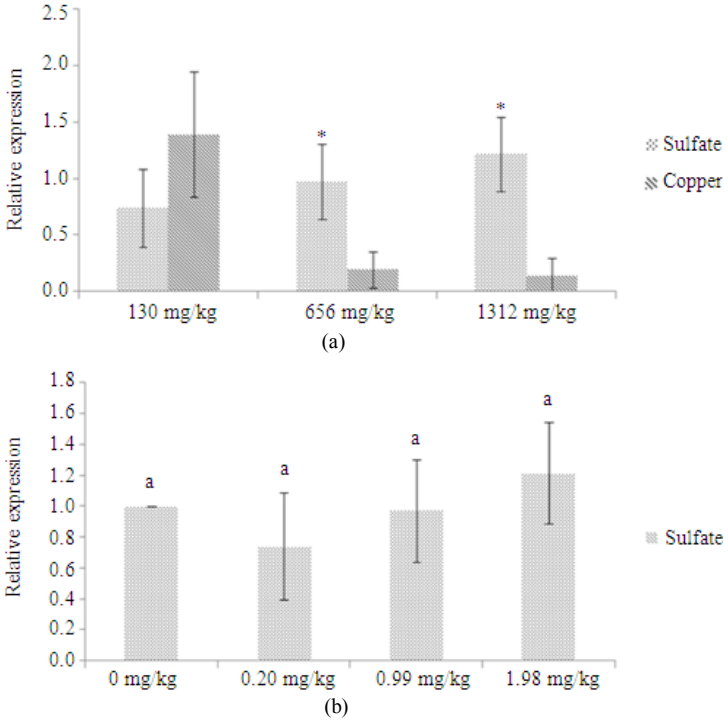

Fig. 1S: Transcription of RAN1 in red oak (*Quercus rubra*) treated with different doses of copper: (a) treatments with 130 mg/kg, 656 mg/kg and 1312 mg/kg with respective sulfate controls (0.20, 0.99 and 1.98 mg/kg); b) treatments with sulfate and water controls. Transcription of RAN1 was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p<0.05$)

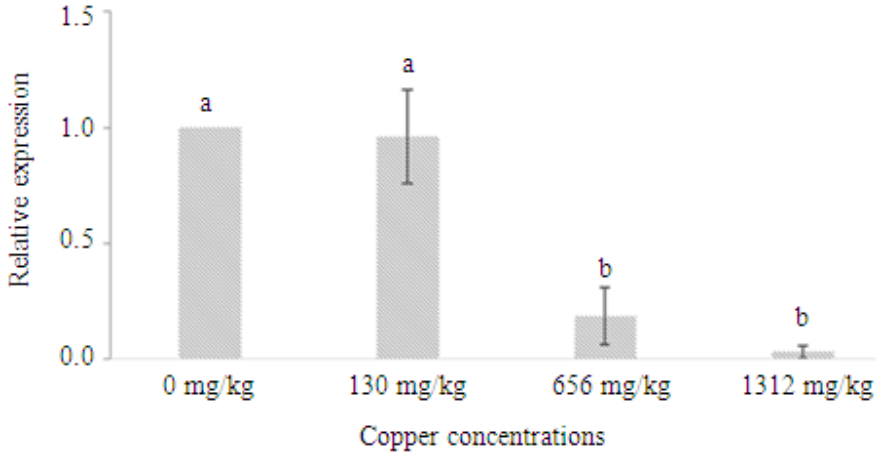

Fig. 2: Transcription of MT2b in red oak (*Quercus rubra*) treated with different doses of copper. Transcription of MT2b was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p<0.05$)

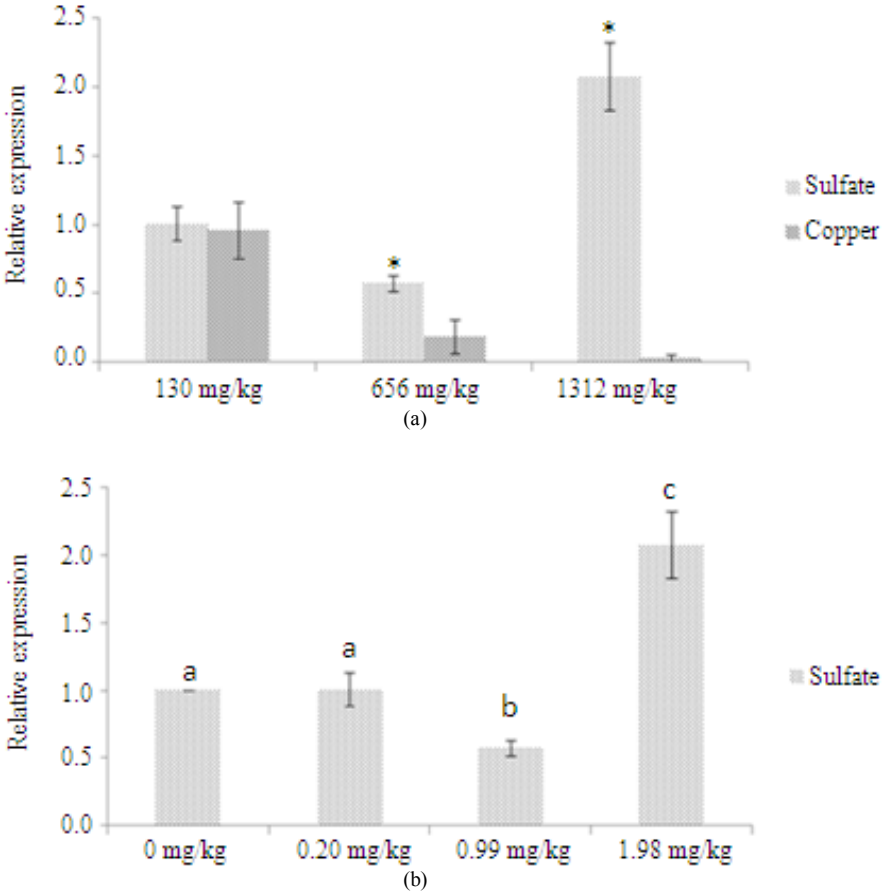

Fig. 2S: Transcription of MT2b in red oak (*Quercus rubra*) treated with different doses of copper: (a) treatments with 130 mg/kg, 656 mg/kg and 1312 mg/kg with respective sulfate controls (0.20, 0.99 and 1.98 mg/kg); (b) treatments with sulfate and water controls. Transcription of MT2b was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p<0.05$)

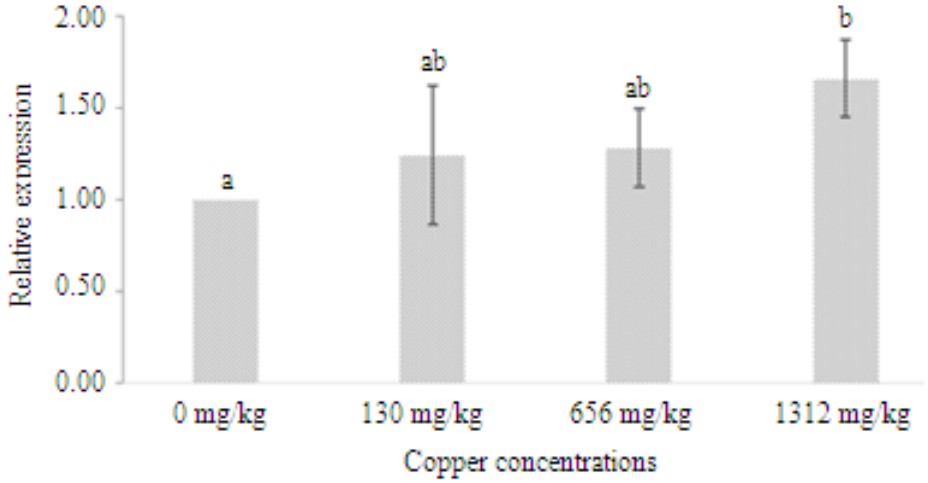

Fig. 3: Transcription of MRP4 in red oak (*Quercus rubra*) treated with different doses of copper. Transcription of MRP4 was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p<0.05$)

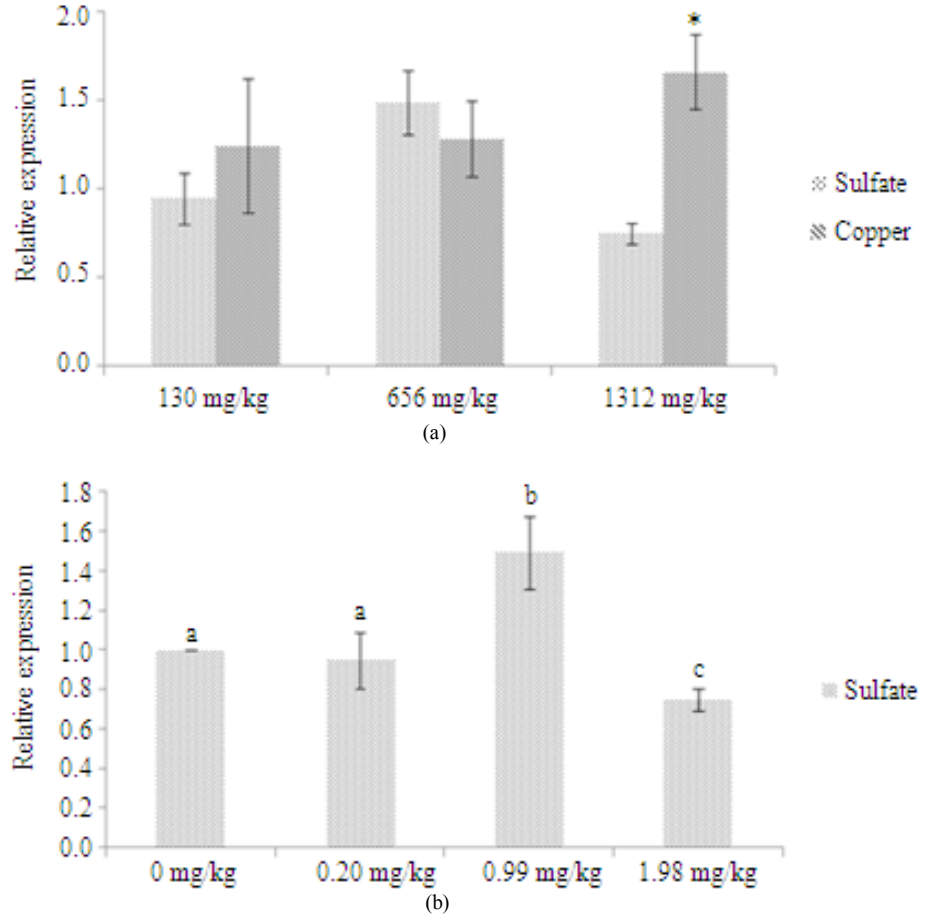

Fig. 3S: Transcription of MRP4 in red oak (*Quercus rubra*) treated with different doses of copper (a) treatments with 130 mg/kg, 656 mg/kg and 1312 mg/kg with respective sulfate controls (0.20, 0.99 and 1.98 mg/kg) (b) treatments with sulfate and water controls. Transcription of MRP4 was standardized based on the housekeeping gene α-tubulin 1. Significant differences were found among treatments based on ANOVA and Dunnett T3 tests. Means with different letters are significantly different ($p\leq0.05$)

Discussion

Importance of Multiple Controls

Plant root systems absorb only solubilized and bioavailable forms of metals in soil. In the present study, copper sulfate ($CuSO_4$) was the salt used in copper treatments because of its high solubility that enables it to ionize to Cu^{+2} and SO_4^{-2} when mixed in water. Thus, solubilized copper is available for assimilation by *Q. rubra* roots. However, the second component of the solubilized salt, the sulfate ion, is also absorbed by the roots and can cause adverse effects to plants at high concentrations as demonstrated by Djeukam *et al*. (2016). Indeed they showed the adverse effects of potassium sulfateon another woody species (*Betula papyrifera*) that was associated with an increase in concentrations (Djeukam *et al*., 2016). Therefore, it is important to consider the potential effects of this ion on phenotypic damage and gene transcription change, even at low concentrations. Surprisingly, in this study, no significant differences were found between the control treatments for the sulfate ion (K_2SO_4) and the water control based on the level of damage to plants and their growth.

Copper Toxicity

Copper is an essential microelement that is toxic toplants in large amounts (Wuana and Okieimen, 2011). A wide range of studies have shown that an excess of copper has the potential to be toxic and result in several physical symptoms in several plant species (Adrees *et al*., 2015). Specifically, it can induce several symptoms such as chlorosis (Ali *et al*., 2002; Shahbaz *et al*., 2010; Feigl *et al*., 2013), necrosis (Drazkiewicz *et al*., 2004; Yruela, 2009; Foy *et al*., 1978; Barbosa *et al*., 2013; Aly and Mohamed, 2012) and inhibition of root and aerial plant tissue growth (Ali *et al*., 2002; Benimeli *et al*., 2010; Cook *et al*., 1997). In addition, recent studies have demonstrated the presence of such symptoms in woody plant species (Theriault and Nkongolo, 2016; Djeukam *et al*., 2016). It is therefore essential for plants to develop a mechanism to regulate copper homeostasis.

A recent study has shown that copper is found in *Q. rubra* leaves in GSR (Tran *et al*., 2014) where it can cause oxidative stress (Drazkiewicz *et al*., 2004) and a decreased chlorophyll concentration (chlorosis) (Pätsikkä *et al*., 2002; Feigl *et al*., 2013). In this study, a damage score was assigned to plants based on the percentage of leaf area covered by chlorosis and/or necrosis (Table 1). But, no significant differences among the different treatments for plant damage were observed. Thus, copper at the concentrations used in this study (130-1312 mg/kg) does not cause symptoms of oxidative stress such as chlorosis and necrosis in *Q. rubra* leaves. Many other studies have shown a reduction in plant growth in the presence of high copper concentrations. For example, Benimeli *et al*. (2010), as well as Barbosa *et al*. (2013), observed a decrease of *Zea mays* plants in the presence of high concentrations of copper. The same effects were observed in other species such as *Brassica juncea, Brassica napus* (Feigl *et al*., 2013) and *Triticum aestivum* (Cook *et al*., 1997). Djeukam *et al*. (2016) observed a reduction in the growth of *B. papyrifera* seedlings treated with copper at a concentration dose of 1312 mg/kg or more compared to a water control. In this study, growth of *Q. rubra* plants was not significantly affected by Cu at all the doses used (130, 656 and 1312 mg/kg) and their respective control treatments.

Our results also contrast with data from other studies that focused on *B. papyrifera* (a hard wood species) showing a decrease in growth and the presence of leaf chlorosis and necrosis after exposure to copper (Djeukam *et al*., 2016) and nickel (Theriault and Nkongolo, 2016). However, Keller *et al*. (2003) suggests that the root system surface may have an impact in the absorption of metals such as copper and potentially lead to a difference in assimilation of metals between these species (Keller *et al*., 2003). This difference in species copper assimilation needs further study.

Effect of Copper on Gene Regulation

A toxic amount of copper and other metals associate with oxidative stress, the production of Reactive Oxygen Species (ROS) (Drazkiewicz *et al*., 2004). Damage to plants can be induced by copper directly or by the ROS that it has formed. ROS can denature proteins, create mutations in DNA and cause membrane instability by lipid peroxidation (Salin, 1988; Bowler *et al*., 1992; Scandalios, 1993; Yadav, 2010). Some plant cells have therefore developed defense systems that protect them against ROS damage (Drazkiewicz *et al*., 2004). Other plants have instead developed mechanisms to regulate copper absorption and intracellular distribution by using metal transporters. Some transporters such as the COPT and ZIP families transport copper intracellularly (Burkhead *et al*., 2009) and then through internal membranes to organelles using transporters known as ATPases for Heavy Metals (HMA) (Baloun *et al*., 2014). At present, eight transporters of the HMA family (HMA1-8) have been identified in *A. thaliana*. HMA1-4 has been identified as playing a role in the transport of zinc, cadmium and lead, while HMA5-8 transports silver and copper. A specific transporter of this family, HMA7 (also called RAN1 (responsive to antagonist 1) plays a role in the transmembrane transport of copper at the cytosol level to the Golgi apparatus (Woeste and Kieber, 2000).

More specifically, Baloun *et al*. (2014) identified the presence of the RAN1 gene in two ecotypes of the species *Silene vulgaris*, one being tolerant to copper and the second susceptible. A significant increase in RAN1

expression was observed in copper-tolerant plants (Baloun *et al*., 2014) and an increase in expression was observed in the presence of excess copper in all plants. A homologous gene (HMA5) of RAN1 has a very similar DNA sequence (Yruela, 2005) is involved in copper resistance in *A. thaliana* (Kobayashi *et al*., 2008). On the other hand, Del Pozo *et al*. (2010) observed a suppression of RAN1 in aerial parts of *A. thaliana* exposed to copper. In this study, significant suppression of the RAN1 gene was observed in *Q. rubra* exposed to high copper doses (656 and 1312 mg/kg) compared to controls (Fig. 2a). The different expression of the RAN1 gene in different species suggest that the mechanisms of resistance to copper developed by plants vary from one species to another.

Chelation by a metal ligand is another mechanism of detoxification of an excessive amount of essential metals (copper, for example). Metallothioneins (MTs) are cysteine-rich proteins found in the cytoplasm of cells of a wide variety of organisms such as animals, microorganisms and plants (van Hoof *et al*., 2001). There is evidence that MTs play a role in protecting plants against the harmful effects of copper by chelation making the excess copper unavailable to activate (van Hoof *et al*., 2001; Guo *et al*., 2008). The implication of MTs in metal resistance is attributed to the presence its cysteine residues. Some studies have demonstrated their implications in the detoxification of copper in *Saccharomyces cerevisiae* (Ecker *et al*., 1989). A copper-sensitive yeast colony was transformed with the *Silene vulgaris* MT2b gene, which conferred copper tolerance Other studies have shown that plants tolerant to copper have a greater expression of the MT2b gene in *Silene vulgaris* (van Hoof *et al*., 2001) and *Silene paradoxa* (Mengoni *et al*., 2003), whereas we observed a significant suppression of this gene in *Q. rubra* at high concentrations of copper compared with sulfate and water controls. However, the two studies involve different tissues (roots or leaves) in different species and it is not clear whether the transcription of MT2b is increased in *Q. rubra* roots exposed to copper. For example, copper treatment with *A. thaliana* increases the expression of MT2b in roots (Guo *et al*., 2003). Other studies have shown that *Silene vulgaris* (van Hoof *et al*., 2001) and *Silene paradoxa* (Mengoni *et al*., 2003) populations that are tolerant to copper have a greater expression of the MT2b gene. But, in the present study a significant suppression of this gene was observed in *Q. rubra* in the presence of high concentrations of copper (656 and 1312 mg/kg) compared with their respective control treatments of sulfate and water control. However, the two studies are not similar since they involve different tissues (roots and leaves) in different species. Thus, it would be interesting to observe the expression of MT2b in *Q. rubra* roots exposed to Cu.

Proteins associated with Multidrug Resistance-Associated Proteins (MRPs) belong to the ABC transporter family (Rea, 1999). Through energy (ATP), this family of proteins transports several substances such as amino acids, lipids, sugars and chelated metals (Rea, 1999). These transporters play an integral role in the detoxification of plants. It has been suggested that one of its potential functions is the vacuolar sequestration of toxic elements (e.g., heavy metals). But the precise role of these transporters in woody species such as *Q. rubra* remains unknown. Bovet *et al*. (2003) reported an increase in expression of several genes encoding MRPs in response to cadmium exposure in *A. thaliana*. In addition, Keinänen *et al*. (2007) compared copper-resistant and susceptible birch (*Betula pendula*) and revealed differential transcription of the MRP4 gene.

Indeed, a high increase in the transcription of the MRP4 gene has been observed in copper-resistant *B. pendula* in the presence of a high level of this metal (Keinänen *et al*., 2007). In the present study, no significant difference was found between MRP4 gene transcriptionin plants treated with 656 mg and 130 mg of Cu per kg of soil compared to their respective control treatments. However, treatment of copper at a dose of 1,312 mg/kg resulted in a significant increase in transcription of this gene compared with control. Thus, copper tolerance in *Q. rubra* appears to be partially mediated by this gene when copper concentration in soil is greater than 1,312 mg/kg. Keinänen *et al*. (2007) demonstrated that the increase in MRP4 gene transcription was at the level of the root system and the stem. Similarly, the increased transcription of MRP4 in the presence of cadmium was evident at the root level (Bovet *et al*., 2003). Since the RNA in this study was extracted from *Q. rubra* leaves and not from roots or stems, it is difficult to compare the results with those of other authors. Therefore, future studies should focus on evaluating the transcription of the MRP4 gene in these different tissues.

Conclusion

In this study, an analysis of plant damage and growth changes in response to copper contamination was performed. No significant differences were found among treatments based on damage rating and plant growth. An analysis of the genes playing a role in copper resistance in other species was carried out to determine whether they associate with this phenomenon in *Q. rubra*. The study revealed that the transcription of each targeted gene is affected by the highest copper dose and supports the hypothesis that RAN1, MT2b and MRP4 are involved in the copper resistance in *Q. rubra*. On the other hand, this reinforces the fact that the bioavailable copper in metal-contaminated soils from the GSR are therefore insufficient to induce a genetic response in *Q. rubra*

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Author's Contributions

Proulx Migueal: Corrducted research, analyzed the data and revised the mamuscript.

Paul Michael: Monitored research experiments and data analysis.

Charnelle Djeukam: Assisted with molecular analysis and data analysis.

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

Conflict of Interest

The authors declare no conflict of interest.

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