Gene regulation and global DNA methylation mechanisms involved in white spruce (*Picea glauca*) response to copper toxicity

By

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Abstract

Plants require metals to survive, but an excess of metals can have phytotoxic effects. The objectives of this study were to 1) analyze the transcription of genes associated with copper resistance (*RAN1, MT2b,* and *MRP4*) and 2) assess the effects of copper and potassium on global DNA methylation in white spruce (*P. glauca*). Seedlings were treated with three concentrations of copper sulfate including 1,312 mg/kg, 656 mg/kg, and 130 mg/kg. Potassium sulfate and water used as controls. DNA and RNA were extracted from roots and needles. The levels of gene expression were measured using RT-qPCR and the global 5 methyl cytosine was assessed using the Abcam ELISA kit procedure. Overall, the highest concentration of copper sulfate (1,312 mg/kg) induced the most severe damages to plants compared to the 656 mg/kg and 130 mg/kg concentrations. Copper ions at the 1312 mg/kg concentration induced an upregulation of the *MRP4* gene in roots and needles. The 656 mg/kg concentration of copper sulfate and potassium sulfate induced an upregulation of *MRP4* only in needles. The *MT2b* gene was upregulated in roots when plants were exposed to 1,312 mg/kg of copper sulfate, but was downregulated in genotypes treated with 656 mg/kg of copper sulfate in both tissues, compared to the water control. The *RAN1* gene was upregulated in roots treated with 1,312 mg/kg copper sulfate. Differential expression of *RAN1, MT2b,* and *MRP4* genes were observed in copper-resistant and susceptible genotypes. The present study revealed that potassium ions induced a hypomethylation of DNA at the 1,312 mg/kg concentration while copper did not induce changes in the level of global cytosine methylation.

Key words: *Picea glauca*; copper toxicity; gene expression; DNA methylation.

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Chapter 1: Literature Review

1.1. Metal Toxicity in Plants

Soils found in many parts of the world can begin as, or transform into, soils that are contaminated with metals. Soil contamination can be from anthropogenic activities such as mining, use of fertilizers, industrial wastes (Sharma and Dietz 2009; Yadav 2010), or natural occurrences such as metal movement through the food chains (Foy et al. 1978). Plants require metals for physiological processes, but high concentrations of metals can cause harm (Dekock 1956; Simpson and Batley 2009). Phytotoxicity is prominent in locations where there is an elevated concentration of metals that can be absorbed by roots, and, eventually, translocated to other parts of plants (Foy et al. 1978; Chatterjee and Chatterjee 2000). Phytotoxicity can be caused by many different metals including nickel (Ni), cobalt (Co), copper (Cu), lead (Pb), iron (Fe), chromium (Cr), zinc (Zn), manganese (Mn), molybdenum (Mo), and cadmium (Cd) (Godbold and Kettner 1991; Chatterjee and Chatterjee 2000; Sun et al. 2007; Anjum et al. 2015).

When plants are under stress, they can evolve and adapt to environmental changes over time. Metal-adapted ecospecies, such as metal hyperaccumulators, have been established due to the stress of metal-contaminated soil (Foy et al. 1978; Colpaert et al. 2011). The new ecospecies are adaptive to the soil conditions, but metal contamination can decrease species diversity (Antonovics et al. 1971). Symptoms of metal toxicity can be species-specific. Some metals may interfere with other nutrient binding needed for growth (Sharma and Dietz 2009). Metals can hinder the ability of root growth and absorption, or induce an increase in reactive oxygen species (ROS) accumulation (Foy et al. 1978; Ebbs and Kochian 1997; Sharma and Dietz 2009). In addition to metal toxicity, the effects of stress caused by temperature and soil pH can be leading factors of physical plant abnormalities and limited growth (Gentili et al. 2018). The physiological

homeostasis within plants allows a stable equilibrium between different molecules and ions required for survival. When a stress develops, such as a high concentration of heavy metals in the soil, the homeostasis can be disrupted, which has the potential to lead to inhibited growth or morphological abnormalities (Das and Roychoudhury 2014). One equilibrium, which is highly disturbed during heavy metal contamination, involves the production and scavenging of ROS (Das and Roychoudhury 2014).

The oxidative stress induced by metals is associated with the metal ions inducing and catalyzing reactions in the cell, or causing stomata closure (Bazzaz et al. 1974; Cheignon et al. 2016). Stomatal closure may favor the formation of more ROS production through abscisic acid inhibition of stomatal opening (Yan et al. 2007). In a cell without stress, there is a minimum amount of ROS production, as well as antioxidant mechanisms to help scavenge and remove the ROS (Foyer and Noctor 2005). Most of the antioxidant mechanisms contain enzymatic components such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) (Miller et al. 2010; Gill and Tuteja 2010). Commonly, there is an elevated enzyme activity from the antioxidant enzymes that help prevent oxidative stress, as seen in the species *Vaccinium myrtillus* (Kandziora-Ciupa et al. 2013), tobacco (Eltayeb et al. 2007), and *Arabidopsis* (Ushimaru et al. 2006).

Iron

Iron is an essential metal for plants as it is a micronutrient needed to survive (Rout and Sahoo 2015). Difficulties arise when there is too much iron in the soil, and the concentration exceeds the toxicity threshold (Sharma et al. 2016). The two main reasons plants will be affected by a high iron concentration is due to a direct iron toxicity or an indirect iron toxicity (Saaltink et al. 2017). A direct iron toxicity happens when a superabundant amount of iron is absorbed by the plant roots,

compared to an indirect iron toxicity where an iron plaque forms around the roots (Wheeler et al. 1985; Laan et al. 1991). An iron plaque would limit iron and nutrient uptake into the plant (Saaltink et al. 2017). Iron deficiency in plants causes many stresses since it has key roles in the physiological plant processes, such as chlorophyll synthesis (Hu et al. 2017). It was found that a decrease in the amount of iron in *Vigna sinensis* causes a deficiency in beta-aminolevulinic acid (Marsh et al. 1963). The decreased amount of amino acid decreased the rate of chlorophyll synthesis, and in toll, caused leaf chlorosis (Marsh et al. 1963). Vine plants were sequestered to soil with sufficient and deficient iron concentrations (Covarrubias and Rombola 2013). It was found that concentrations of organic acids within the plants, such as citrate, tartrate, and ascorbate, were changed due to deficient iron concentrations, demonstrating the importance of this metal for normal plant functions (Covarrubias and Rombola 2013).

To date, there are high concentrations of iron in the soil of wetlands, and iron toxicity is more abundant in these locations (Becker and Asch 2005). Reduced iron (Fe 2+) is known to be absorbed by plants, such as rice (*Oryza sativa*), in water filled lowlands (Becker and Asch 2005). The increase in the reduced iron causes chlorosis and will prevent plant physiological processes from commencing (Becker and Asch 2005). However, the survival of the lowland species comes from the ability of soluble ferrous iron to be oxidized into insoluble ferrous iron in the soil (Batty and Younger 2003). The insoluble ferrous iron will linger around the roots of the species and remain within the soil, which limits the uptake of iron and prevents iron toxicity (Batty and Younger 2003).

Zinc

Zinc is another main micronutrient found in plants which has key functions in the ribosomes, carbohydrate production, and aids in the oxidation processes (Nagajyoti et al. 2010). As being a

cofactor to RNA polymerase, zinc is a well-known metal with different roles related to various needs within plants (Nagajyoti et al. 2010). As seen in *Phaseolus vulgaris* (Cakmak and Marschner 1993), and *Brassica juncea* (Prasad et al. 1999), zinc deficient and toxic conditions were found to induce oxidative stress, which led to symptoms such as growth inhibition. Zinc deficiency decreased antioxidant enzyme functionality (Cakmak and Marschner 1993) while zinc excess caused increased superoxide dismutase, catalase, and guaiacol peroxidase activity, among other antioxidant enzymes (Prasad et al. 1999). Growth inhibition was seen in the roots and shoots of other plant species grown with zinc exposure, and generally, there were chlorosis symptoms in the younger leaves (Ebbs and Kochian 1997; Fontes and Cox 1998). However, different species will have variable effects from zinc contamination. Agarwala et al. (1977) observed no decrease in chlorophyll content in barley grown in soils with a high zinc concentration. However, Schlegel et al. (1987) reported a decreased chlorophyll content in spruce plants after treatment with excess zinc ions.

Zinc can replace iron in certain reactions because of the similarities between the radius of both ions (Marschner 1986). One example of this occurrence is an uptake of zinc through the iron-regulated transporters in the plant roots during increased levels of zinc in the soil (Vert et al. 2002). This is illustrated through the iron-regulated transporter 1 (*IRT1*), where the transporter will be attracted to the zinc metal ions, as well as the iron metal ions (Lin and Aarts 2012).

Nickel

As a trace element, nickel is very important for physiological reactions in plant cells (Yusuf et al. 2011). Nickel can be found naturally in the environment through the high concentration in igneous rock and surrounding soil, or introduced through anthropogenic activities (Mitchell 1945; Yadav 2010). Toxicity caused by nickel is due to an excess of nickel ions being absorbed and exceeding

the concentration threshold in plant species (Yusuf et al. 2011). Nickel affects metabolic processes, photosynthesis, water absorption and transpiration, and the overall plant growth (Sreekanth et al. 2013). Seed germination and seed growth has been found to decrease in species of pigeonpea (Rao and Sresty 2000), cabbage (Pandey and Sharma 2002), and corn (Bhardwaj et al. 2007), when placed in a soil with a surplus of nickel. The excess nickel will compete with other metals within the cell, or cause oxidative stress through an increased formation of free oxygen radicals (di Toppi and Gabbrielli 1999; Schützendübel and Polle 2002; Sreekanth et al. 2013). Previously, oxidative stress tolerance was measured in *A. bertolonii* and *N. tabacum* (Boominathan and Doran 2002). It was found that both species had high levels of hydrogen peroxide after nickel treatment, but only the growth of *N. tabacum* was stunted (Boominathan and Doran 2002). Further investigations found that there was a large increase in catalase activity in *A. bertolonii*, which is an antioxidant mechanism, and could be considered one of the mechanisms that caused nickel tolerance (Boominathan and Doran 2002).

Cadmium

Unlike other heavy metals, cadmium (Cd) and lead (Pb) are not essential elements for plant processes, and are well known to be toxic when present (Benavides et al. 2005). Cadmium concentrations in the soil are directly related to the anthropogenic activities which occur near a soil habitat (Benavides et al. 2005). Cadmium is able to inhibit many plant functions, such as the iron reductase and the nitrate reductase activity, which was seen in *Cucumis sativus* L. and *Silene cucubalus* plants respectively (Mathys 1975; Alcántara et al. 1994). Other harmful factors observed after cadmium treatments include a reduced function of ATPase activity, and the beginning of lipid peroxidation (Fodor et al. 1995). As well, cadmium can play a large role in oxidative stress by producing oxygen-free radicals, which damage cells, proteins, and DNA (Fodor

et al. 1995). Cadmium inhibits the detox ability of antioxidants, and thus, increases the amount of free oxygen radicals in plants (Unsal et al. 2020). Overtime, the effects of the oxidative stress will cause degradation of lipids, or even cell death (Stohs and Bagchi 1995; Gallego et al. 1996; Cho and Seo 2005). However, some species, such as *Sedum alfredii*, are cadmium accumulators, and are tolerant to its damaging effects (Sun et al. 2007). Cadmium hyperaccumulators pose risks to humans and animals which may digest a large concentration of cadmium without warning (Zhi et al. 2020).

Lead

As one of the most abundant metals in terrestrial ecosystems, lead toxicity in plants and animals has many repercussions. High levels of lead in soils can lead to plant morphological deformities and inhibition of plant growth (Nagajyoti et al. 2010). Morphological abnormalities include reduced stem and leaf elongation, as seen in *Allium* species (Gruenhage and Jaeger 1985) and barley (Juwarkar and Shende 1986). More issues can arise when lead accumulation affects the accumulation of other metal ions (Godbold and Kettner 1991). It was reported that an accumulation of lead in *Picea abies* needles had a negative effect on the concentrations of calcium and manganese in cells (Godbold and Kettner 1991).

One of the plant growth regulators, indole-3-acetic acid (IAA), is affected by lead and other metal ions (Dimkpa et al. 2012). Interestingly, it is the reduced biosynthesis of auxins, or the upregulation of IAA peroxidases, which are metal catalyzed, that eventually lower the IAA plant levels (Potters et al. 2007). Other inhibitory effects of lead include the decreased activity of carboxylating enzymes, and of several other enzymes (Stiborova et al. 1987), since lead will react with sulfhydryl groups in many of the enzymes (Nagajyoti et al. 2010).

In conclusion, there can be a direct toxicity with metal ions interacting with molecules that contain thiols and carboxylates, but there is a high probability for the occurrence of an indirect metal toxicity as well (Sreekanth et al. 2013; Saaltink et al. 2017). When the concentration threshold for any of the metal ions is exceeded, it was seen that there was very limited and retarded growth; shorter roots which cannot go as deep into the soil, and limited elongation of stems and leaves (Foy et al. 1978; Gruenhage and Jaeger 1985; Ebbs and Kochian 1997; Fontes and Cox 1998). Indirectly, the limited root growth will prevent the plant from accessing nutrients and water deep within the soil, and in turn, can cause metal deficiencies. As well, if there is a metal deficiency in the plant, the cell's metal transporters may substitute one metal for another, which can cause issues with redox reactions, or induce the formation of ROS (van Assche and Clijsters 1990; Lin and Aarts 2012; Bloom 2019; Bosnić et al. 2019). The main concern for metal toxicity is the decrease in enzyme functionality, changes made to the redox reactions, and the accumulation of ROS in the plant cells (Stiborova et al. 1987; Yan et al. 2007; Bloom 2019). In each of those cases, the plant homeostasis is disrupted, and normal plant processes are delayed or stopped.

1.2. Copper Toxicity in Plants

Copper is one of the many important micronutrients for plant species, but it can also lead to toxic effects (Foy et al. 1978). Similar to contamination of other heavy metals, an overabundance of copper is added into the environment naturally, or by anthropogenic activities (Foy et al. 1978; Sharma and Dietz 2009; Yadav 2010). As for instance, fertilizer components in Florida (Reuther and Smith 1954), or chemical plants and waste disposal in places, such as India (Khatun et al. 2008), are known to produce high levels of copper, and elevated pollutions.

When copper is present in the cells, it goes through many biological processes, and is important to the survival of the plant (Letelier et al. 2005). With the addition of other ions and molecules, copper

is mainly known to be a part of cellular respiration, antioxidant defense, cellular iron metabolism, photosynthesis, phosphate uptake, and more (Nalewajko and Olaveson 1995; Letelier et al. 2005; Krupanidhi et al. 2008). However, copper levels above the threshold concentration can lead to copper toxicity (Khatun et al. 2008). The effects of copper toxicity on plants are dependent on the species itself. For example, maize (*Zea mays*) was reported to be less sensitive to copper exposure compared to tomato plants (*Lycopersicon esculentum*) (Adriano 1986; Mocquot et al. 1996).

Higher copper concentrations found in the leaf tissues is a general indication of copper toxicity, and the potential for cell damage (Buss et al. 2012). Species within the Cruciferae, Caryophyllaceae, Gramineae, Leguminosae, and Asteraceae families are known to be less sensitive to metal toxicity (Xiong and Wang 2005). However, *Prunus cerasifera* plants were reported to be tolerant to low and intermediate copper ion concentrations (Lombardi and Sebastiani 2005). It was also determined that once there was a high copper concentration, *P. cerasifera* showed symptoms of copper toxicity, such as leaf discoloration, and eventually, necrosis (Lombardi and Sebastiani 2005).

Copper ions in soils enter plant cells through root uptake, and in some species, there is a possibility of foliar ion uptake (Watmough et al. 1999; Barbosa et al. 2013). Copper transport protein 1 (*COPT1*) plays an important role in copper ion uptake (Sancenón et al. 2003). On the other hand, different *COPT* proteins are commonly used for intracellular transport of copper (Sancenón et al. 2003). P1B-type ATPase (*HMA*) transporters also have roles to move copper into different organelles and tissues (Zimmermann et al. 2009). When there is excess copper in soil, transporters for other metal ions may use copper as a substitution instead of absorbing the metal ion that the transporter generally attracts (Bosnić et al. 2019). Examples of transporters which can be used for copper translocation include members of the Zn/Fe permeases (*ZIP*s) family (Wintz et al. 2003),

or the iron transporter 1 (*IRT1*) (Korshunova et al. 1999). Copper transport using the *ZIP* family and *IRT1* has been observed in *Arabidopsis thaliana* and yeast (Korshunova et al. 1999; Wintz et al. 2003).

Once root uptake of metals has occurred, the copper will be translocated through the plant by the xylem and the phloem (Pätsikkä et al. 1998). Copper can only be transported in a complexed form, such as being paired with an amino acid in xylem (Graham 1979). Primarily, copper will be found in soil as copper-soil organic matter (SOM) complexes, as opposed to free ions (Strawn and Baker 2009). Copper can be bound to cells because of its high affinity for peptide, sulfhydryl, carboxylic, and phenolic groups (White et al. 1981; Pich and Scholz 1996; Liao et al. 2000). High copper concentrations are unstable, and provide damaging effects to plant cells (Bosnić et al. 2019). However, in low and intermediate copper concentrations, there may not be visible toxicity effects. This is possible when the process of xylem and root binding molecules, and other mechanisms, can bind the free copper ions, which prevent them from participating in deadly reactions (Welch and Shuman 1995).

The root apex is one of the first tissues targeted by many metal ions (Ambrosini et al. 2015). The root apical meristem is a location of the root system, which divides easily and quickly, and is the location of new cell growth (Verbelen et al. 2006). It is common to find a higher accumulation of copper in roots than in leaves and other higher parts of plants, as is seen in *Zea mays* roots (Cathala and Salsac 1976; Lexmond 1980). When an excess of copper uptake and translocation transpires, it can cause nutritional disorders, which may be linked to a shorter root apical meristem (Ambrosini et al. 2015). The shorter length would have detrimental consequences on plant health. With a shorter root system, it would be merely impossible for plants to gain access to the nutrients and water in the deeper soil (Pierret et al. 2016). Thus, copper toxicity effects may cause direct damage

to plant cells, but there may be a larger impact caused by indirect nutrient and water deficiency. An increased lateral root length growth has been observed in *Arabidopsis* (Lequeux et al. 2010) and *Pinus pinaster* (Arduini et al. 1995) exposed to copper. In other species, such as soybean (Kulikova et al. 2011), *Pinus pinea* (Arduini et al. 1995), and grapevines (*Vitis* spp.) (Ambrosini et al. 2015), lateral root length growths were suppressed by copper exposure. As well, there is evidence of plasmolysis of epidermis cells induced by copper in aquatic *Lemna minor*, *Elodea canadensis*, and *Leptodictyum riparium* (Basile et al. 2012; Brunetto et al. 2016).

Nutrient deficiency, especially iron, can be caused by an excess of copper ions in soil (Yau et al. 1991). Commonly, deficiencies due to higher copper concentrations cause physical symptoms, such as discoloration in leaves (Cambrollé et al. 2015). For instance, a well-known toxicity symptom is chlorosis due to iron deficiencies in plant cells (Yau et al. 1991). Chlorosis caused by copper will likely be seen as a yellow leaf with dark green veins. Evidence of these symptoms caused by copper toxicity can be found in many species such as clove (*Syzygium aromaticum*) (Yau et al. 1991), *Zea maize* (Mocquot et al. 1996), and Indian ginseng (*Withania somnifera* (Rout et al. 2013). Sunflower seedlings (*Helianthus annuus*) and peach rootstock (*Prunus cerasifera*) were identified as resistant to copper based on the lack of chlorosis symptoms after being treated with copper (Lin et al. 2003; Lombardi and Sebastiani 2005).

Chlorophyll and carotenoid pigments in plant leaves may be broken down during copper exposure (Malec et al. 2009). It has been shown that elevated copper levels inhibited leaf pigment concentrations within certain species, such as *Withania somnifera* (Khatun et al. 2008) and barley (Capsi et al. 1999). In that case, there will be a decrease in the photosynthetic ability of the plant, which would produce oxidative stress, and a lack of glucose, oxygen, and water (Cambrollé et al.

2015). However, the Chinese cabbage (*Brassica pekinensis Rupr*) chlorophyll pigments were increased when it was treated with high concentrations of copper (Xiong and Wang 2005).

Due to copper toxicity, plants have developed mechanisms which can diminish the effects that the high copper concentration has on the plant cells. Increased metal binding ligands, such as metallothioneins and phytochelatins, or antioxidants, has been observed when there is an excess of copper (Mukherji and Gupta 1972; Lidon and Henriques 1994). The 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 the copper ions (Lidon and Henriques 1994). The increased synthesis of copper-binding compounds released from roots was reported in *Anabaena cylindrica* and *Plectonema boryanum* (Jardim and Pearson 1984). Copper-bound complexes released from the roots are efficient in reducing the effects of copper toxicity, but there may be a concentration limit which renders this mechanism ineffective, causing the plant to rely on other copper resistance mechanisms (Jardim and Pearson 1984).

Another mechanism which can aid in plant survival when exposed to lower, and intermediate concentrations of copper, is preferential absorption of cations and anions (Jacobson and Ordin 1954). Plants, much like animals, have an electrochemical charge due to the nutrients and ions present within cells. Copper, being a cation, will cause an increase of organic acids to be produced by plants (Jacobson and Ordin 1954), as well as decreasing the pH of the soil (Brunetto et al. 2016). Interestingly, the addition of anions, such as inorganic nitrogen, could increase the copper contaminated soil pH (Neina 2019). An increased soil pH may favor copper-complexing processes, which remove a portion of the free copper ions in soil, as seen in *Daphnia magna* (Meador 1991). However, Meador (1991) reported increased toxicity from ionic copper concentrations as the pH increased.

In the presence of iron, and other trace metal ions, hydrogen peroxide can enter a multistep reaction as an oxidant to form byproducts of ROS (Halliwell and Gutteridge 1984; Zhao et al. 2005). The process is called the Fenton reaction, and in effect, the dangerous ROS produced can rapidly induce DNA damage (Zhao et al. 2005). Most commonly, this reaction takes place in the chloroplasts of the plant cells (Pätsikkä et al. 1998). Copper is one of the metals which is used to catalyze the Fenton reactions, and an increase of copper could cause oxidative stress by producing an increased amount of ROS (Halliwell and Gutteridge 1984). The Haber-Weiss reaction is similar to the Fenton reaction, but it uses superoxide and hydrogen peroxide to form ROS (Haber and Weiss 1934). It has been determined that the reaction is not favorable, and in turn, must need a catalyst (Kehrer 2000). Copper can be used as the metal catalyst which is needed for the superoxide and hydrogen peroxide to react (Kehrer 2000; Liochev and Fridovich 2002).

During copper toxicity, the plant cells are under major stress due to the disruption of the 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 stress (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 growth of the seeds (Kabir et al. 2008).

Overall, copper toxicity symptoms can easily lead to cell necrosis through nutrient deficiency, starvation, lack of water uptake, down-regulation of key metabolic enzymes, and the inhibition of seed germination (Yau et al. 1991; Lombardi and Sebastiani 2005; Kabir et al. 2008; Ambrosini et al. 2015).

1.3. Gene Expression Associated with Metal Contamination

Environmental and biological stresses will have adverse effects on plant survival. Conditions including, but not limited to, flooding, high salt concentrations, UV light exposure, or heat stress, have been determined to be factors inducing the synthesis of stress proteins (Sachs and Ho 1986). Each of the individual stress factors alter gene expressions and the synthesis of specific proteins (Panikulangara et al. 2004). As an example, the synthesis of heat-shock proteins will be targeted by temperature stress (Sachs and Ho 1986; Ahsan et al. 2007).

Several plant species will be affected when exposed to a high concentration of metal ions. However, some plant species are known to be metal hyperaccumulators (Pollard et al. 2002). It has been established that accumulator species have an increased metal concentration in roots and shoots compared to non-accumulator species (Clemens 2001; Rascio and Navari-Izzo 2011). The ability for hyperaccumulators to accumulate high metal concentrations in plant tissues is associated with an adapted gene expression of certain proteins and transporters (Pollard et al. 2002). Species such as *Arabidopsis halleri*, *T. caerulescens* and *Alyssum bertolonii*, are examples of plants that have a higher antioxidative response when compared to non-hyperaccumulator species (Sharma and Dietz 2009). Metal transporters which have been known to increase expression during metal

exposure are members of *ZIP*, *HMA*, *MATE*, *YSL*, *MTP*, *NRAMP*, and *YSL* families (Rascio and Navari-Izzo 2011; Ding et al. 2017).

The *COPT/Ctr* proteins are found in eukaryotic species (Hanikenne et al. 2005). This family of transporters is involved in cellular copper transport (Yuan et al. 2011). The three main features which are found within transporters of the gene family are three transmembrane domains, two methionine rich regions in the N terminus, and a methionine rich region in transmembrane domain II (Petris 2004). Generally, genes within this family are expressed in the absence of copper, and they are repressed when there are sufficient copper concentrations in the cells (Puig and Thiele 2002; Yuan et al. 2010; van den Berghe and Klomp 2010). In rice (*Oryza sativa*), each of the seven *COPT* genes were affected by other bivalent ions tested (Yuan et al. 2011). Yuan et al. (2011) and Carrió-Seguí et al. (2019) reported that the *COPT* genes were either induced, or suppressed under iron, manganese, zinc, and copper deficiencies. Genes within the *COPT* family may work independently, or may require working cooperatively with one another (Yuan et al. 2010, 2011).

ZIP (Zinc-regulated transporter Iron-regulated transporter Proteins) family members control the intake of zinc and iron ions and is commonly expressed during zinc or iron deficiencies (Assunção et al. 2001; Li et al. 2013). Within this family of transporters, *ZIP2* and *ZIP4* have been discovered to transport copper in *Arabidopsis*, and other species (Grotz et al. 1998; Puig et al. 2007). It was also discovered that the transport of divalent cations, using the *ZIP2* and *ZIP4* genes, was regulated by copper ion availability (Wintz et al. 2003; del Pozo et al. 2010). Wintz et al. (2003) reported that *Arabidopsis thaliana* had an overexpression of *ZIP2* and *ZIP4* under copper deficiency, but were repressed when the concentrations of copper were elevated. Other studies found that the expression of *Arabidopsis ZIP4* gene was unchanged by copper deprivation compared to normal copper concentrations (Yamasaki et al. 2009; Wu et al. 2015). *ZNT1* is another zinc transporter

gene within the *ZIP* family (Assunção et al. 2001). In *T. caerulescens*, the expression of this gene was highly expressed under zinc exposure (Assunção et al. 2001). However, in its non-hyperaccumulating relative, *Thlaspi arvense, ZNT1* was only expressed under zinc deficiency conditions (Assunção et al. 2001).

One of the first genes discovered in the *ZIP* transporter family was the *IRT1* transporter in *A. thaliana* (Zheng et al. 2018). It was identified as an iron transporter, and it is highly expressed during iron deficiency (Eide et al. 1996; Guerinot 2000). This transporter is also involved in zinc and manganese transport (Korshunova et al. 1999; Guerinot 2000). *IRT1* is one of the few cadmium transporters in plants, and cadmium ions have been found to lower iron and zinc ion uptake in yeast cells when the *IRT1* gene is expressed (Eide et al. 1996; Rogers et al. 2000). By transporting cadmium, plants with an overexpressed *IRT1* gene can be analyzed as potential cadmium accumulators and be used for phytoremediation (Yan et al. 2020). Furthermore, in *A. thaliana,* high concentrations of nickel ions can induce the *IRT1* gene, causing nickel uptake in plant species (Thomine et al. 2000; Nishida et al. 2012).

The heavy metal ATPase (*HMA*s) transporter family is important for selective nutrient and metal absorption, metal resistance, and transportation of many transition metals (Mills et al. 2003; Gravot et al. 2004; Hussain et al. 2004). The transported ions include essential metals such as copper, zinc, and cobalt, or non-essential metals such as cadmium and lead (Li et al. 2015). Transport of non-essential metals can be detrimental in some species. In *Arabidopsis thaliana*, for example, an overexpression of *AtHMA3* induces cadmium absorption (Morel et al. 2009). Other relevant *HMA* type genes, found in *Arabidopsis thaliana,* are the *AtHMA1* and *AtHMA4* genes involved in zinc, cobalt, and cadmium transport (Mills et al. 2003, 2005; Moreno et al. 2008). The ATPase activity induced by *AtHMA1* gene is increased under high cadmium, copper, cobalt, and zinc

concentrations (Moreno et al. 2008). Studies found that within the *HMA* gene family, there are similar gene motifs needed for heavy metal transport, and a mutation in the CPC motif of *AtHMA4* stopped cadmium and zinc tolerance in yeast (Mills et al. 2005).

NRAMP (natural resistance-associated macrophage protein) family members are found in eukaryotes (Cellier et al. 2001). This transporter protein is commonly known for manganese, copper, cadmium, and iron transport, but can also transport and distribute other divalent metal ions (Ullah et al. 2018). There are two model plant species, *Arabidopsis thaliana* and *Oryza sativa* (rice), which have been used for *NRAMP* observations (Vatansever et al. 2016). The *NRAMP*s assist in reducing intercellular metal ion availability (Nelson 1999; Bereczky et al. 2003). These transporters are controlled by iron ions at the post-transcriptional, and post-translational levels (Gunshin et al. 1997; Portnoy et al. 2002; Bereczky et al. 2003). Meng et al. (2017) discovered at least 10 genes within the *NRAMP* family involved in cadmium transportation in *Brassica napus.* *BnNRAMP1b* was one of the genes that have been studied in detail and was upregulated in response to cadmium (Meng et al. 2017). In *Arabidopsis thaliana*, *Atnramp1*, *Atnramp3*, and *Atnnramp4*, were highly expressed during iron deficiencies (Curie et al. 2000; Thomine et al. 2000). Interestingly, an increased tolerance to high iron concentrations was observed when the *Atnramp1* gene was overexpressed (Curie et al. 2000). However, an increased *Atnramp3* expression caused a hypersensitivity to cadmium (Thomine et al. 2000).

Adaptation of different species to each of the metals through transcriptional modifications has been reported (Singh et al. 2016). 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 changes a species has generated during metal exposure (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 (Krishna et al. 2020). They can also repress the expression of the transporter genes to prevent further uptake of ions (Hossain et al. 2012).

1.4. Epigenetics

Epigenetics involves the modification of the chromatin structure within a plant species through molecular tools such as DNA methylation, modifications of histones, or chromatin remodeling (Bird 2007). Gene expressions can become enhanced or repressed when the chromatin structure is modified (Johnson et al. 2004; Marfil et al. 2009; Chinnusamy and Zhu 2009). The alterations to the gene expressions can be heritable, without changing the genomic sequences (Trerotola et al. 2015). Regardless of the mode of chromatin modification, the adaptability of the plant species which undergo epigenetic modifications increases due to the rapid responses to environmental stimuli and stress factors (Klironomos et al. 2013).

1.4.1. DNA Methylation

DNA methylation can inhibit transcription factors from binding to the DNA, or can introduce proteins which repress gene expressions (Moore et al. 2013). *Solanum ruiz-lealii* is a flowering plant which had normal and abnormal flowering phenotypes, but it was found that there was no genetic variation between the two phenotypes (Marfil et al. 2009). This is possible through epigenetics and any abiotic stresses placed on the plant, which could increase or decrease the level of DNA methylation (Marfil et al. 2009; Richards 2011). These abiotic stresses, such as an elevated metal ion concentration, has decreased DNA methylation of *Trifolium repens* L. and *Cannabis sativa* L., which are metal sensitive and metal tolerant plants respectively (Aina et al. 2004).

Epigenetic changes occurring through DNA methylation induced by osmotic, temperature, and salinity stresses have been documented in *Pisum sativum* L., *Arabidopsis*, *Zea mays*, and *Nicotiana tobaccum* (Kovařik et al. 1997; Steward et al. 2002; Labra et al. 2002; Boyko et al. 2010). More specifically, osmotic stress increased DNA methylation in *Pisum sativum* roots (Labra et al. 2002), low NaCl concentrations caused hypermethylation in *Nicotiana tobaccum* cells (Kovařik et al. 1997), while cold stress decreased nucleosome DNA methylation in *Zea mays* (Steward et al. 2002).

1.4.2. Histone Modifications

Chromatin architecture is largely controlled by histone proteins. Histone modifications can lower the accessibility to the DNA strand (Feng and Jacobsen 2011). The acetylation and deacetylation of histones is reversable and catalyzed by histone acetyl transferases and histone deacetylases (Pandey et al. 2002). For example, toxins, such as *Helminthosporium carbonum* (HC-Toxin), which affects *Zea mays*, can inhibit histone deacetylase (Brosch et al. 1995). The inhibition can be reversed by reducing the toxin to an alcohol, and allowing the chromatin to revert to the original structure and gene expression levels (Brosch et al. 1995). Plant species can benefit from histone modifications that allow them to survive under environmental stresses. *Arabidopsis* can quickly adapt to cold temperatures by histone deacetylation and repressive histone methylation involving the flowering locus C (FLG) gene (Pfluger and Wagner 2007). A link between DNA methylation and the methylation of lysine 9 of histone 3 has been reported in *Neurospora crassa*, a commonly used a model fungal species in genetics (Tamaru et al. 2003).

1.5. Species of Interest: White Spruce

White spruce (*Picea glauca*) is a North American native species. There are thirty-four different species of trees within the genus *Picea* which are distributed around the world, mostly abundant in the northern areas (Ran et al. 2015). *Picea* species are one of the most commonly planted trees in Canada, and are economically and ecologically important (Beckett and Negusanti 1990; Hodgetts et al. 2001). These conifers are well adapted to mild temperatures and colder regions, which are found in Northern locations (Porth and Torre 2020). White spruce is one of the few transcontinental species, along with black spruce (*Picea mariana*) (Farrar 1995). White spruce belongs to the *Pinaceae* family, and they are dominant trees in the boreal forest (Hodgetts et al. 2001; Ran et al. 2015). The soils found in the boreal forests are commonly acidic in nature (Bilodeau-Gauthier et al. 2011) with pH values varying between 3.7 and 4.3 (Nosko et al. 1988). The podzolic soil is acidic due to the large composition of minerals and metal ions which are seeped into the different layers of soil from decomposed organic matter and rain (Brock et al. 2020).

Five coniferous species including *P. glauca* were tested to observe the effect of metals on the growth of plants (Patterson and Olson 1983). *P. glauca* was one of least susceptible species to copper, cobalt, and nickel exposure (Patterson and Olson 1983). It was observed that *P. glauca* radical elongation was inhibited during cobalt and nickel exposure at lower concentrations, but there was no effect from low concentrations of copper (Patterson and Olson 1983). However, for each of the metals tested, roots were significantly shorter after treatment with metal concentrations above 50 ppm, when compared to the untreated plants (Patterson and Olson 1983).

Picea glauca's symbiotic relation with an ectomycorrhizal fungus, *Suillus luteus*, was evaluated under cadmium, copper, nickel, lead, and zinc exposures (Dixon and Buschena 1988). Metals

hampered the fungi growth while *P. glauca* seedlings grown with these ectomycorrhizal fungi had larger root and shoot weights (Dixon and Buschena 1988). At high concentrations for each of the metals tested, the seedling growth was inhibited, with or without the fungi (Dixon and Buschena 1988). This means that the fungi may help with the survival through heavy metal contamination, but only at low metal concentrations.

The sensitivity of the *Picea* genus with different metal ions is expected to be variable among species, as seen with varying toxicity symptoms in *Picea abies* after being treated with cadmium, mercury, and zinc (Schlegel et al. 1987). It was found that 0.1 micromolar of mercury, compared to the 1 micromolar of cadmium and 30 micromolar of zinc, eventually decreased the chlorophyll content in *P. abies* needles (Schlegel et al. 1987). However, Boyd and Nkongolo (2021) reported no significant physical toxicity symptoms in *P. glauca* treated with 302 micromolecular concentrations of nickel.

The pathway of lead uptake in *P. glauca* was evaluated using an application of rainfall at certain locations on the needles (Watmough et al. 1999). The translocation of the lead ions from the needles to the shoot was not significant compared to the concentration of lead ions at the site that lead was applied to the needles (Watmough et al. 1999). Thus, suggesting that lead foliar uptake is not the major pathway of lead accumulation in this species (Watmough et al. 1999). Further investigations of foliar uptake for other metals are important in locations where there can be persistent acid rain and increased atmospheric pollution (Schreck et al. 2012). The height of tree species may allow further metal uptake in the foliage which can add to metal toxicity.

1.6. Rationale and Objectives

Much of the current research focusses on the plant survival and seed germination under metal stress. There is limited information on the effects of metal toxicity in the genus *Picea*. In areas where there are high metal concentrations, such as the City of Greater Sudbury, it would be useful to determine the effects of metals on conifer species since they are widely used in the Sudbury regreening program. This research will provide inside knowledge on the susceptibility or resistance of white spruce (*P. glauca*) to higher soil copper concentrations. The expressions of metal resistance genes in *P. glauca*, as well as epigenetic mechanisms involved in metal resistance, have yet to be investigated in detail, compared to model and other non-model plant species. An understanding of the regulation of genes associated with copper resistance, as well as the epigenetic mechanisms associated with *P. glauca* responses to copper toxicity, will help determine its potential in the regreening of copper contaminated areas.

The main objectives of this study are to 1) determine the effects of different concentrations of copper on the level of expression of genes associated with copper resistance (*RAN1, MT2b, MRP4*) and 2) evaluate the level of global DNA methylation in white spruce (*P. glauca*) exposed to copper contamination.

Chapter 2: Expression of genes associated with copper resistance induced by different concentrations of copper and potassium sulfates in white spruce (*Picea glauca*)

2.1. Introduction

Metals, such as copper, found within the environment, continue to create damaging effects at higher concentrations in many different plant species (Dekock 1956). Several metals are required nutrients for many plant and animal physiological processes. An increased metal concentration is detrimental to the species diversity within ecosystems, and may cause metabolic disruptions within the plant itself (Antonovics et al. 1971; Yruela 2009; Ambrosini et al. 2015; Yadav et al. 2016). At optimum levels, copper is one of the many micronutrients which plays a part in plant respiration, cell wall metabolism, photosynthesis, protein synthesis, among many more physiological and biochemical processes (Nalewajko and Olaveson 1995; Letelier et al. 2005; Krupanidhi et al. 2008; Nazir et al. 2019; Shabbir et al. 2020). When this element is present at non-optimal concentrations, many of these processes will be disturbed to allow plants to focus on antioxidant mechanisms to deal with an excess copper, or to get more copper when it is deficient is soils (Sancenón et al. 2003; Li et al. 2019; Shabbir et al. 2020). An excess of metal ions causes a decline in plant growth, an increased production of stress biomarkers, and a disruption in the translocation of nutrients (Arduini et al. 1995; Kulikova et al. 2011; Singh et al. 2015; Bosnić et al. 2019). The decline in the overall plant growth can be caused by a combination of factors including nutritional imbalances or increased oxidative stress (Halliwell and Gutteridge 1984; Kehrer 2000; Cambrollé et al. 2015; de Conti et al. 2020). If plants do not obtain the proper amount of nutrients, there may be nutrient deficiency symptoms, which can be just as damaging as the toxicity symptoms (Adrees et al. 2015). Environmental remediation techniques, such as tree planting or soil liming, can be useful for plants

with limited growth due to factors such as incompatible soil pH or high metal concentrations (Beckett and Negusanti 1990; Nkongolo et al. 2013).

Several plant species have developed tolerance and avoidance mechanisms to cope with copper toxicity as reported in *Quercus rubra* (Proulx et al. 2017), *Solidago chilensis* (Lillo et al. 2019), and in *A. rubrum* and *P. tremuloides* (Kalubi et al. 2016) studies. Copper ions can suppress or upregulate transporter genes for other ions, causing reduced intake of necessary molecules, such as phosphorous (Feil et al. 2020). The changes to the metal transporter proteins can occur at the transcript level due to the deficiency or abundance of copper ions, as seen with *MT2*, *MRP4*, *RAN1*, and *COPT* differential gene expressions (Andrés-Colás et al. 2006; Zhigang et al. 2006; Keinänen et al. 2007; Yuan et al. 2011; Aguirre and Pilon 2016). On the other hand, plants have the ability to reduce oxidative stress through enzymatic [such as superoxide dismutase (*SOD*), polyphenol oxidase (*POD*), and catalase (*CAT*)] and non-enzymatic (glutathione) antioxidant mechanisms (Adrees et al. 2015; Yadav et al. 2016; Hasanuzzaman et al. 2017). Many of the antioxidant mechanisms can be enhanced with the addition of molecules. For example, castasterone, a plant hormone, increased chlorophyll content in the leaves and improved *SOD, POD,* and *CAT* activities under copper exposure (Yadav et al. 2016). As well, the addition of molecules such as gibberellin, auxin, citric acid and calcium, have shown to limit copper accumulation in roots and shoots in *Pisum sativum* L., and may limit copper accumulation in other plant species as well (Massoud et al. 2019). The limitation and avoidance mechanisms will prevent possible copper toxicity symptoms since the copper does not enter metal uptake or assimilation processes (Lillo et al. 2019).

The objective of the study is to determine whether *Picea glauca* is resistant or susceptible to copper toxicity, and to assess the levels of gene expression induced by an increased concentration of copper. I hypothesize that *P. glauca* will be resistant to a low concentration of copper equivalent

to the bioavailable levels of this element in the City of Greater Sudbury (Nkongolo et al. 2013). I also predict that this concentration will have no effect on gene expression and that higher concentrations of copper will cause damage to *P. glauca* seedlings and induce changes to gene expression.

2.2. Materials and Methods

2.2.1. Assessment of *Picea glauca* Resistance to Copper

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 include a 6-hour dark and 18-hour light cycle with the temperature ranging from 20 ^{0}C and 25 ^{0}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 mg, 656 mg, and 1,312 mg of copper per 1 kg of dry soil (Appendix 2). These concentrations represent the bioavailable, half total, and total levels of copper, at metal-contaminated soils in the CGS, respectively (Nkongolo et al. 2013). These levels correspond to 773.2 µmol, 3902 µmol, 7,804 µmol, of copper, respectively.

To control for any possible toxic effects 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 controls for 130 mg/kg, 656 mg/kg, and 1,312 mg/kg correspond to 773.2 µmol, 3902 µmol, 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 6 replications for the total copper sulfate treatment (1312 mg/kg), and 10 replications per the other treatments and control groups, corresponding to 66 samples (Appendix 1). A resistant and susceptible genotype treated with 1312 mg/kg of copper sulfate was also chosen for individual analysis.

Damages were measured seven days after treatment using a 1 to 9 scale, 1 indicating no damage and 9 indicating dead plants. Needle and root samples were collected, individually wrapped, placed into liquid nitrogen, and then stored at -20 ℃ until RNA extraction. Prior to freezing, all the collected roots were rinsed under water to remove all soil, and then dried with a paper towel to remove any excess water.

2.2.2. 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 RNA samples was performed using a 1% agarose gel (Appendix 4). Quantification of RNA samples were performed using the Qubit® RNA BR assay kit from Life Technologies (Carlsbad, United States). One microgram of RNA from samples of the same treatment were 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℃ for 1 hour 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.

2.2.3. RT-qPCR

Primers were designed based on the *Picea glauca* genome. Sequences for each gene were 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 encode 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). The selected genes associated with copper resistance include *RAN1, MRP4, MT2b* (Table 2 and 3). Elongation factor-1 alpha (*EF1- α*) was chosen as the reference gene (Table 3). 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 (Appendix 5). Primer bands which were used for RT-qPCR contained a strong reproducible single band of the expected cDNA transcript size for the gene target (Table 3). 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℃ for 10 minutes, 2) denaturation at 95°C for 15 seconds, 3) optimized annealing temperature which depended on the primer (between 55-62°C) for 60 seconds, 4) read, 5) repeat step 2-4 for 41 cycles, 6) final elongation at 72°C for 7 minutes 7) melting curve 72 – 95°C, every 1°C, hold for 10 seconds, and 8) final elongation at 72°C for 3 minutes. RT-qPCR was performed two separate times for each targeted 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.

2.2.4. 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. Afterwards, they were normalized to the housekeeping gene (*EF1- α*) and water. SPSS 20 for Windows was used to determine statistical significance among means ($P \leq 0.05$). The Shapiro Wilk test ($P > 0.05$) was performed to verify normal distribution of data for the C(t) values. To determine the significant difference between means for the many treatments and controls, an analysis of variance (ANOVA) was performed and then the Levene's 2-tailed test was conducted to analyze if the treatment variances were equal ($P > 0.05$) or unequal ($P \leq 0.05$). Dunnett's T3 Post Hoc Test ($P \leq 0.05$) was performed if variances were unequal, and a Tukey's Post Hoc test ($P \leq 0.05$) was performed if variances were equal. An independent t-test was also performed to show the significance ($P \leq 0.05$) of the differences between needles and roots within the pooled groups for each gene target and treatment.

To test for significant differences between the damage ratings of the plants within the different treatments, the Shapiro Wilk test ($P > 0.05$) was performed to verify abnormal distribution of data. A Kruskal-Wallis non-parametric test was performed to determine any significant ($P \leq 0.05$) differences for the damage rating data. A Dunn's Post Hoc Test was then performed for a pairwise comparison and significantly ($P \leq 0.05$) different ranks were adjusted using the Bonferonni correction for multiple tests.

2.3. Results

2.3.1. *Picea glauca* Resistance to Copper Toxicity

Copper resistant and susceptible genotypes were identified. The damage rating values varied between 1 and 9 for samples treated with 1,312 mg/kg of copper sulfate. This indicates segregation in screened samples for resistance to the highest copper sulfate concentration (Appendix 3). Genotypes with damage scores of 1 to 3 were considered copper resistant, 4 to 6 were moderately resistant, and 7 to 9 were susceptible. Overall, the highest concentration of copper sulfate (1,312 mg/kg) induced the most severe damages to plants compared to the 656 mg/kg and 130 mg/kg concentrations (Table 1). The other treatments, including the water control, the 656 mg/kg and 130 mg/kg copper sulfate, and the potassium sulfate treatments, did not cause any physical toxicity symptoms with their respective mean damage ratings (≤ 1.5) (Table 1).

Table 1. Damage rating of *P. glauca* treated with different copper sulfate and potassium sulfate concentrations after seven days after treatment. Values are represented as means with the corresponding SE

Treatment	**Concentration (mg/kg)**	**Mean Damage Rating***
Water control	0	1 **a**
Potassium Sulfate	130	1.13 ± 0.13 **a**
	656	1.29 ± 0.18 **a**
	1,312	1.50 ± 0.34 **a**
Copper Sulfate	130	1.07 ± 0.07 **a**
	656	1.36 ± 0.17 **a**
	1,312	5.67 ± 0.92 **b**

*Mean damage ratings with different letters are significantly different ($P \leq 0.05$) based on Kruskal-Wallis and Dunn's post hoc test (N = 66).

Table 2. Selected genes involved in copper resistance in model and non-model plant species

Targeted genes	Species	Reference
RAN1	*Arabidopsis thaliana*	(Kobayashi et al. 2008)
MRP4	*Betula pendula*	(Keinänen et al. 2007)
MT2b	*Arabidopsis thaliana*	(Guo et al. 2008)

Table 3. 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)
RAN1	F:65	F: AGCTGCAGATGTTGGCATGG	195	195
	R:64	R: CCAGCTGCCAGTGGTATTGC		
MRP4	F:63	F: GGTGTAGTGGGAAGGACAGGC	74	74
	R:64	R: GCAGCAGGCTCAACTATTCGG		
MT2b	F:66	F: GTGGATGCGGAAGTGGATGC	84	84
	R:67	R: GCAGAATGGGCGAACCAACC		
EF1- α	F: 67	F: TCTCCACACTCAGCTCGGCG	119	119
	R: 69	R: CCAGTGGTTGTTGACTTGCCGG		

2.3.2. Gene Expression Analysis

2.3.2.1. MRP4 expression in roots

An increased expression of *MRP4* was observed for samples treated with 1312 mg/kg of copper sulfate compared to the water control (Figure 1a). In fact, the *MRP4* gene expression was 2.18 folds higher for samples treated with 1312 mg/kg of copper sulfate compared to the water control (Figure 1a). The expression of this gene was also higher at 1,312 mg/kg compared to 656 mg/kg and 130 mg/kg. Interestingly, there were no changes to *MRP4* expression in the half copper (656 mg/kg), or bioavailable copper (130 mg/kg) concentrations in roots compared to the water control (Figure 1a). There was no significant difference in the expression of *MRP4* induced by each of the potassium sulfate concentrations (Figure 1b). *MRP4* expression was significantly higher at the 1,312 mg/kg copper sulfate treatment when compared to the 1312 mg/kg potassium sulfate treatment (Figure 1c). No significant difference was observed between samples treated with copper sulfate and potassium sulfate at the low concentrations used (656 mg/kg and 130 mg/kg) (Figure 1c).

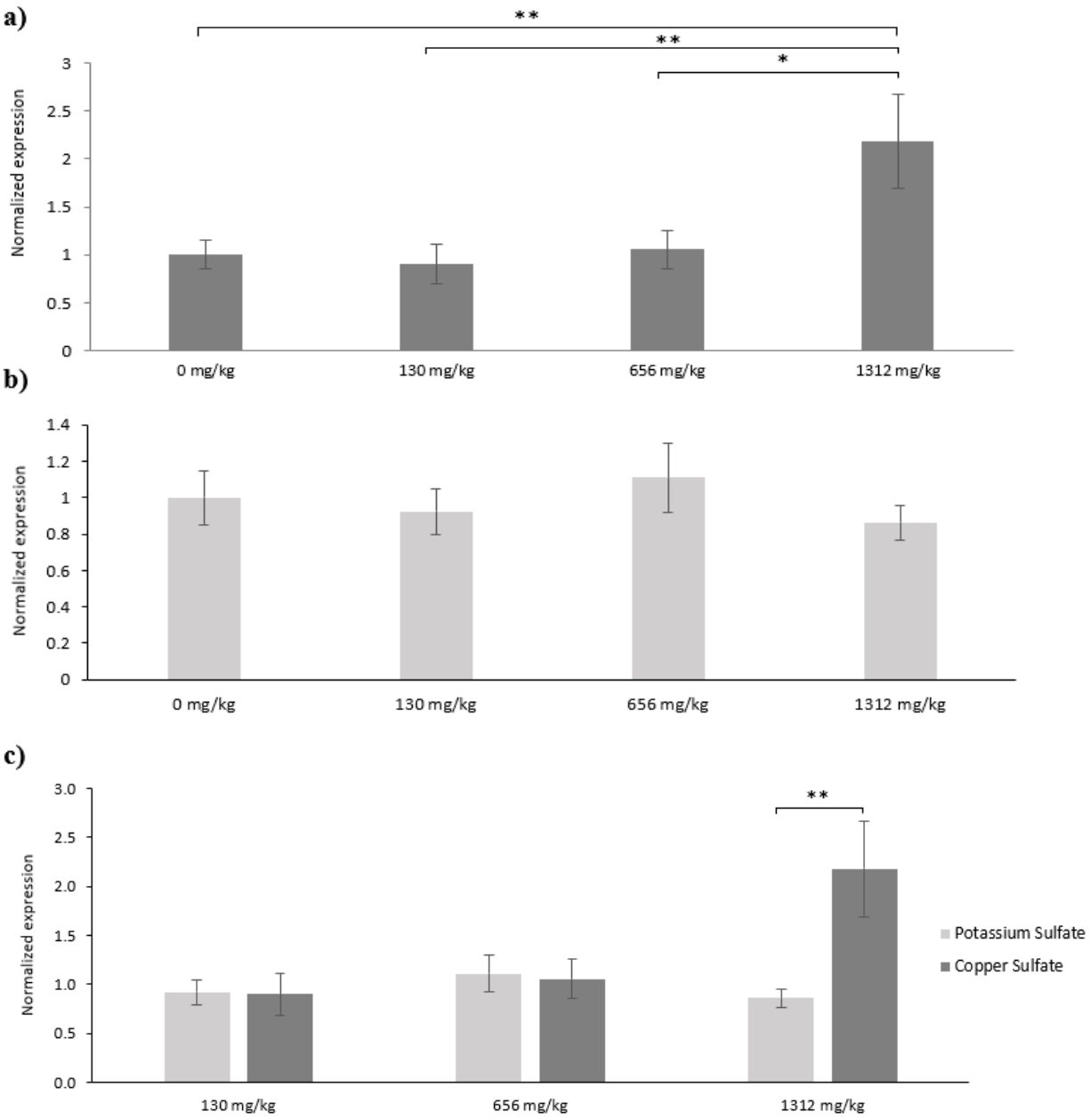

Figure 1. *MRP4* gene expression in white spruce (*Picea glauca*) roots treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.2. 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 when compared to water (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). The 1,312 mg/kg of copper sulfate treatment induced a higher gene expression compared to the two lower copper sulfate concentrations (656 mg/kg and 130 mg/kg) (Fig 2a). Likewise, *MT2b* expression was downregulated in samples treated with 656 mg/kg of copper sulfate compared to those treated with 130 mg/kg of copper sulfate (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 1312 mg/kg of potassium sulfate, *MT2b* expression was significantly decreased when compared to water and to samples treated with 130 mg/kg of potassium sulfate (Fig 2b). *MT2b* expression was downregulated in samples treated with 1312 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 1312 mg/kg of copper sulfate when compared to the potassium sulfate (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 comparing samples treated with 656 mg/kg of copper sulfate and potassium sulfate (Fig 2c).

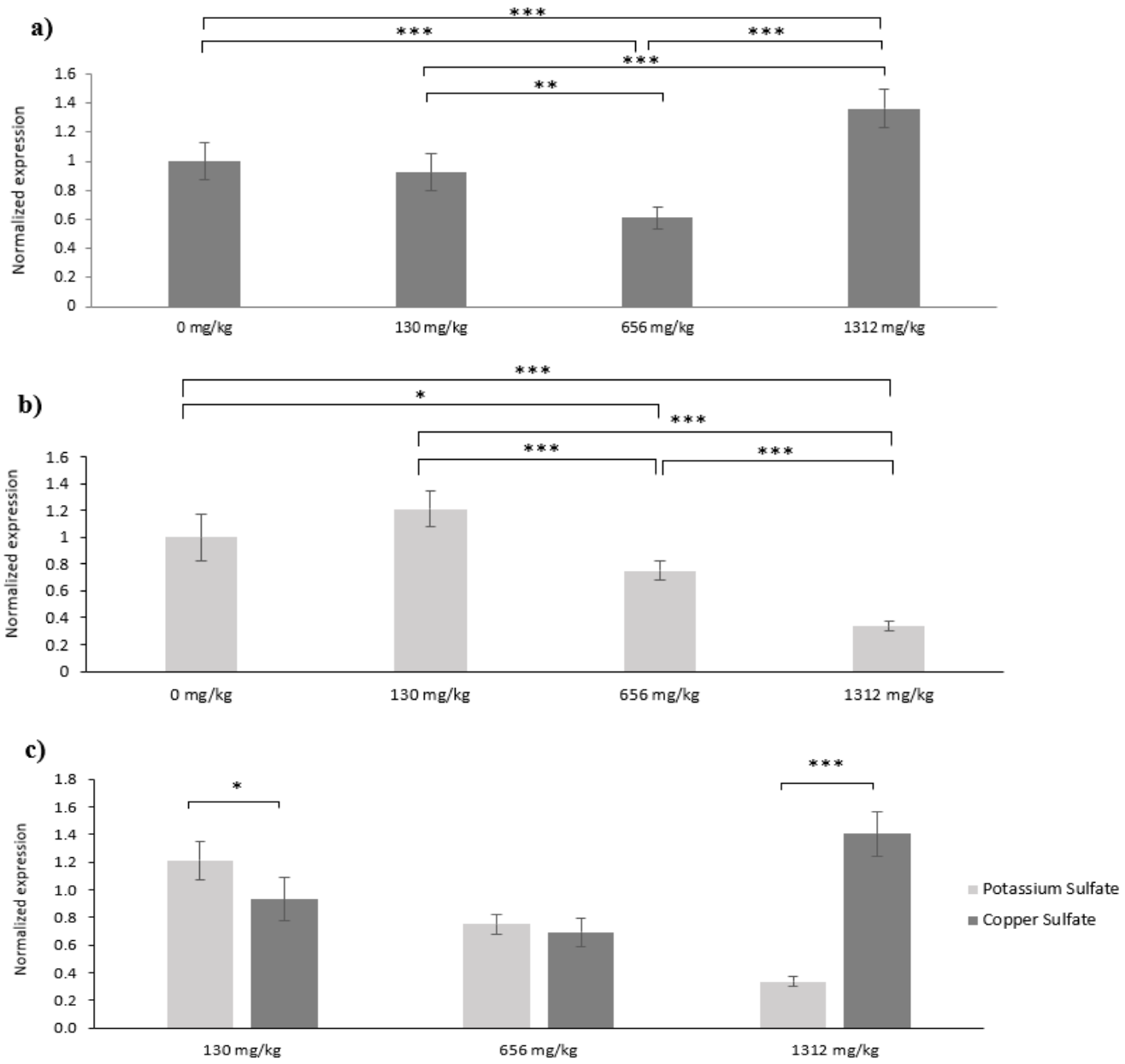

Figure 2. *MT2* gene expression in white spruce (*Picea glauca*) roots treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.3. RAN1 expression in roots

RAN1 expression was increased by 2-fold for samples treated with 1312 mg/kg of copper sulfate as compared to the water control, and significantly increased compared to the lower copper concentrations (656 mg/kg and 130 mg/kg) (Fig 3a). There were no significant changes to the expression of *RAN1* when samples treated with water, 130 mg/kg of copper sulfate, and 656 mg/kg of copper sulfate treatments were compared (Fig 3a). The 656 mg/kg of potassium sulfate treatment induced a significantly higher *RAN1* expression compared to the water control and to samples treated with 1,312 mg/kg of potassium sulfate (Fig 3b). On the other hand, the 1,312 mg/kg of potassium sulfate treatment induced a significantly decreased *RAN1* expression compared to the 130 mg/kg potassium sulfate concentration (Fig 3b). There was an increased *RAN1* expression for samples treated with 1,312 mg/kg of copper sulfate compared to the equivalent concentration of potassium sulfate (Fig 3c). The 656 mg/kg copper sulfate treatment induced a significant downregulation of *RAN1* expression when compared to the equivalent potassium sulfate concentration (Fig 3c). The expression of *RAN1* was not significantly different when samples treated with 130 mg/kg of copper sulfate and the equivalent potassium sulfate treatments were compared (Fig 3c).

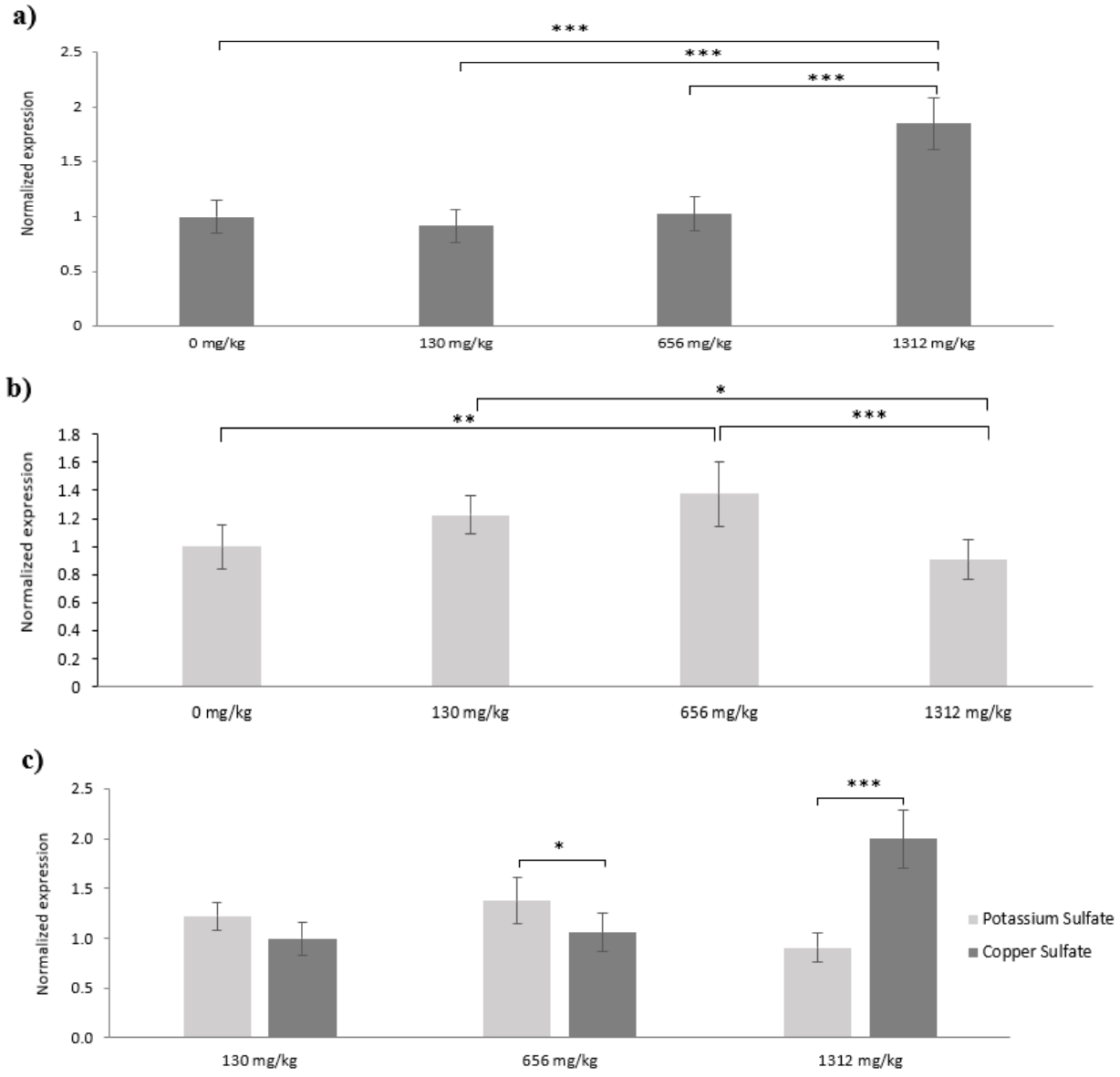

Figure 3. *RAN1* gene expression in white spruce (*Picea glauca*) roots treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.4. Gene expressions in copper susceptible and resistant genotypes

The expression of *MRP4* and *MT2b* was higher in the resistant genotype compared to water control (Fig 4ab). An increase of *MRP4* gene was also observed in the susceptible genotype compared to the water control (Fig 4a). However, the *MT2b* gene was downregulated in the susceptible genotype compared to the water control and resistant genotype (Fig 2b). The expression of *RAN1* was similar in resistant and susceptible genotypes compared to water (Fig 4c).

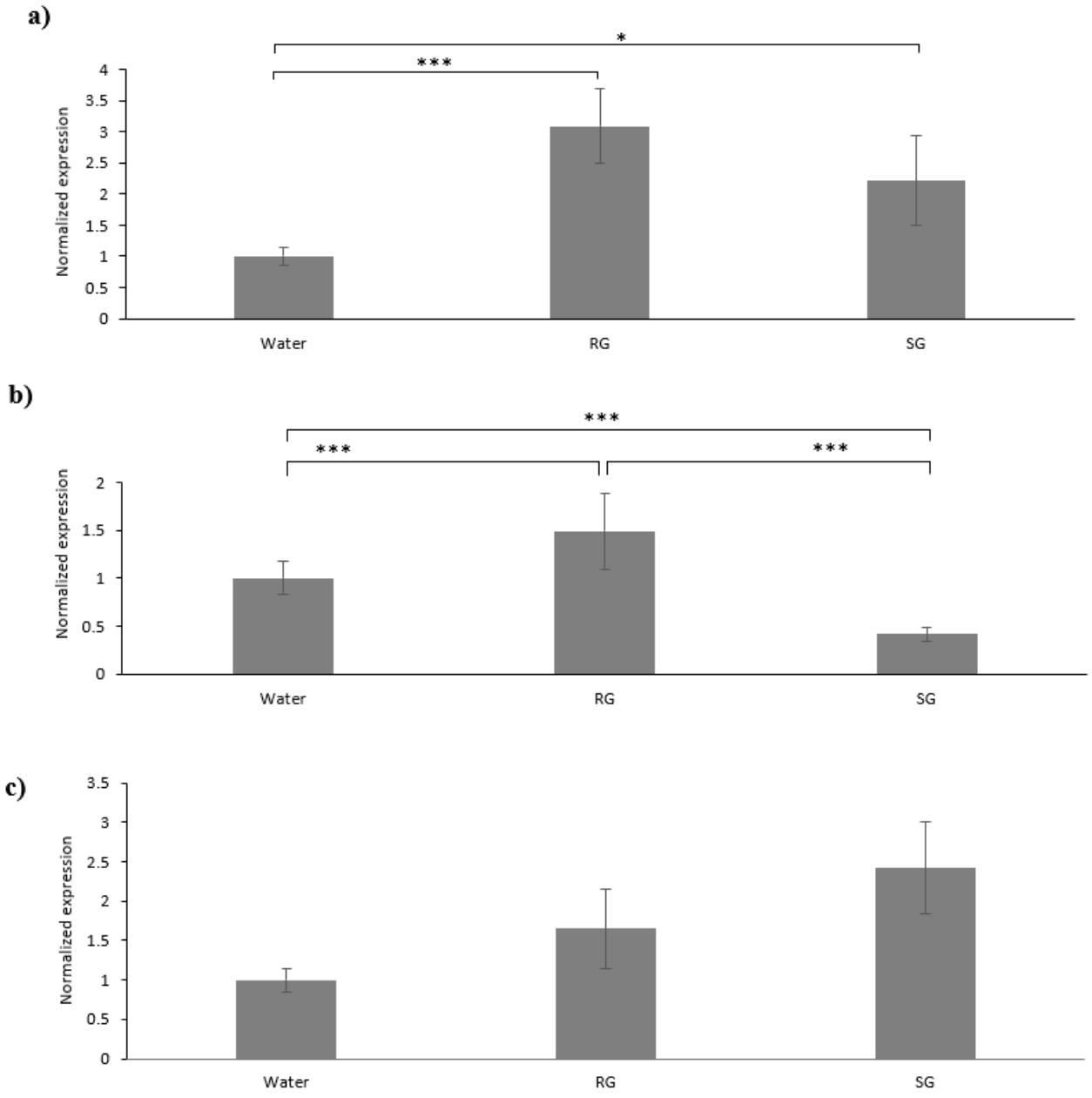

Figure 4. Gene expression in white spruce (*Picea glauca*) roots treated with 1312 mg/kg of copper sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. The phenotypic damage rating of the resistant genotype (RG) and susceptible genotype (SG) individuals was 2 and 6 respectively. Gene expression of a) *MRP4* b) *MT2* and c) *RAN1* are presented. Significant differences among the means of the treatments are marked using * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.5. MRP4 expression in needles

The expression of *MRP4* in needles increased by 2.8-folds in samples treated with 1312 mg/kg of copper sulfate compared to the water control, and was significantly higher compared to the other copper concentrations (656 mg/kg and 130 mg/kg) (Fig 5a). A significantly increased *MRP4* gene expression was observed in samples treated with 656 mg/kg of copper sulfate, compared to the water control (Fig 5a). There was also an increased *MRP4* expression in samples treated with 656 mg/kg of potassium sulfate, compared to the water control and the 130 mg/kg and 1312 mg/kg potassium sulfate concentrations (Fig 5b). However, for samples treated with 1312 mg/kg of potassium sulfate, there was a significant downregulation of *MRP4* expression compared to the water control (Fig 5b). As well, for samples treated with 1312 mg/kg of potassium sulfate, there was a repressed *MRP4* gene expression when compared to samples treated with 130 mg/kg of potassium sulfate (Fig 5b). Overall, there was an elevated *MRP4* gene expression for samples treated with 1312 mg/kg of copper sulfate when compared to the equivalent potassium sulfate treatment (Fig 5c). Samples treated with 130 mg/kg and 656 mg/kg of copper sulfate and potassium sulfate treatments had no significant differences in *MRP4* gene expression when comparing the equivalent concentrations with each other (Fig 5c).

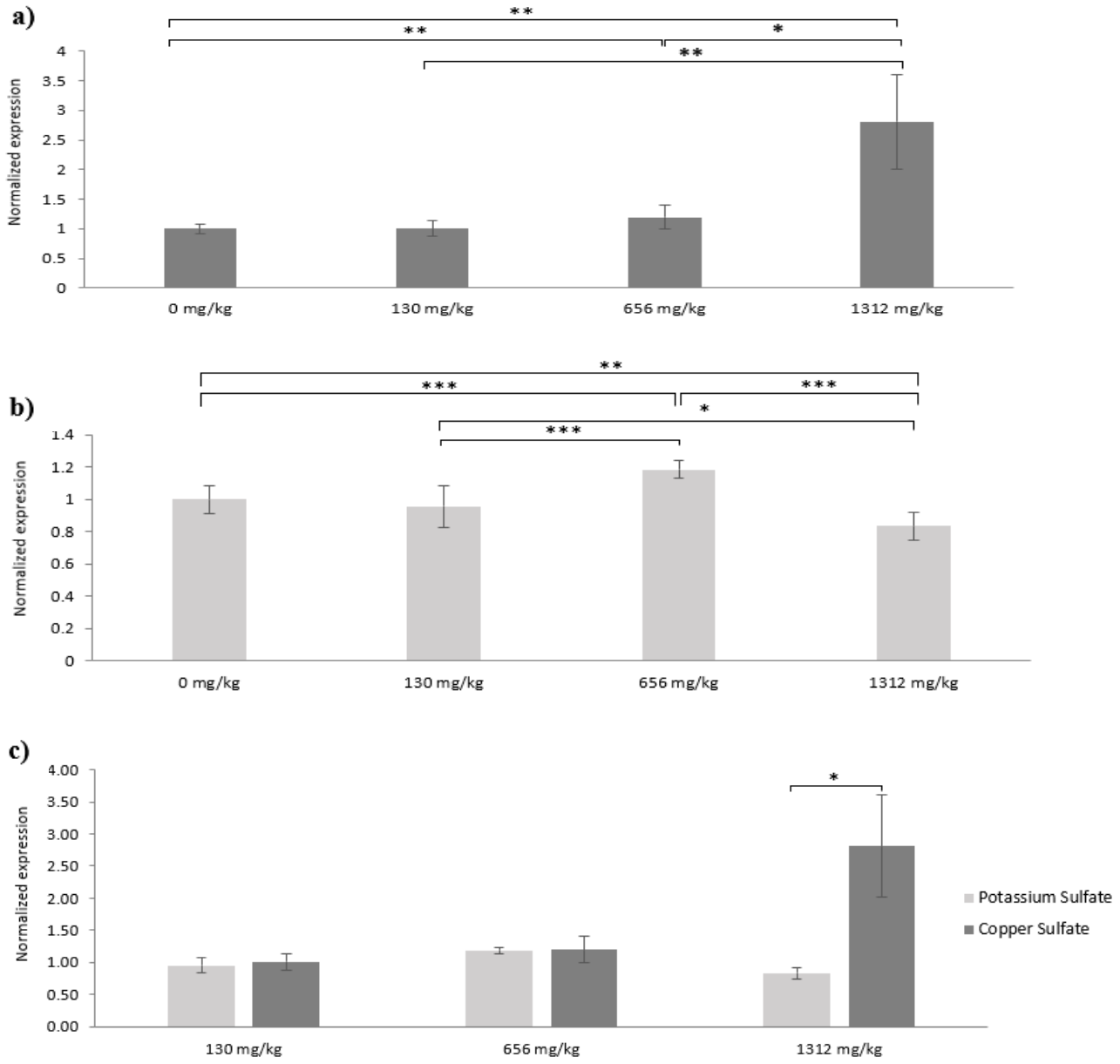

Figure 5. *MRP4* gene expression in white spruce (*Picea glauca*) needles treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.6. MT2b expression in needles

The *MT2b* gene expression was significantly downregulated in genotypes treated with 656 mg/kg of copper sulfate, when compared to samples treated with 1312 mg/kg of copper sulfate and the water control (Fig 6a). 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 6a). *MT2b* expression was significantly downregulated in samples treated with 130 mg/kg, 656 mg/kg, and 1312 mg/kg of potassium sulfate, when compared to the water control (Fig 6b). Overall, there were no significant differences in *MT2b* gene expression when the equivalent concentrations of potassium sulfate and copper sulfate treatments were compared (Fig 6c).

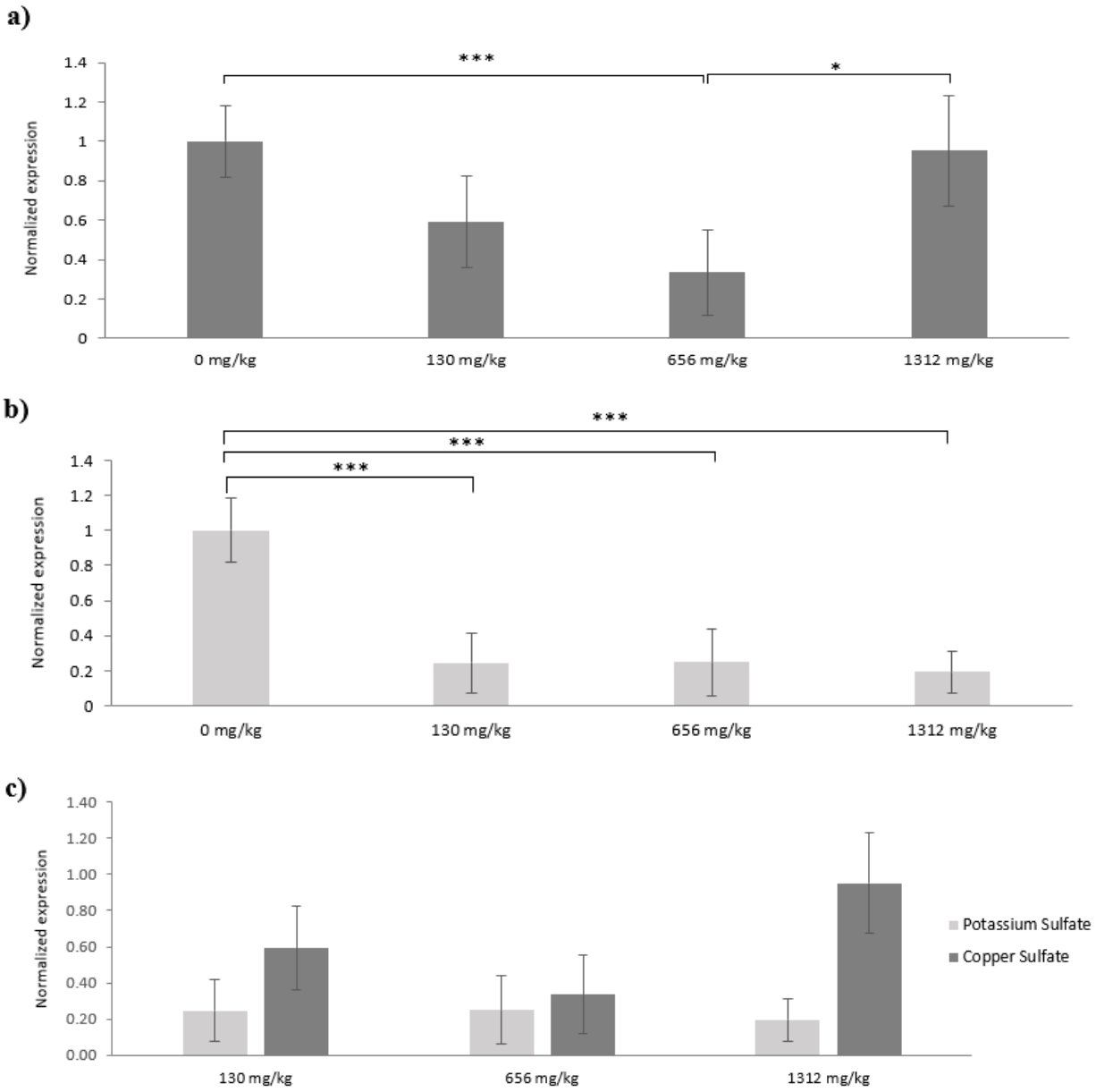

Figure 6. *MT2* gene expression in white spruce (*Picea glauca*) needles treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.7. RAN1 expression in needles

RAN1 gene expression was significantly higher in samples treated with 1,312 mg/kg of copper sulfate compared to genotypes treated with 656 mg/kg of copper sulfate (Fig 7a). No significant difference in RANI expression was observed between samples treated with 1,312 mg/kg and 130 mg/kg of copper sulfate or the water control (Fig 7a). A downregulation of RANI was observed in samples treated with 1,312 mg/kg of potassium sulfate, compared to the water control and lower potassium concentrations (656 mg/kg and 130 mg/kg) (Fig 7b). Overall, samples treated with 1312 mg/kg of copper sulfate induced a significant upregulation of *RAN1* compared to the corresponding potassium sulfate treatment (Fig 7c). However, the lower copper sulfate concentrations did not have a significantly different *RAN1* gene expression compared to the corresponding potassium sulfate controls (Fig 7c).

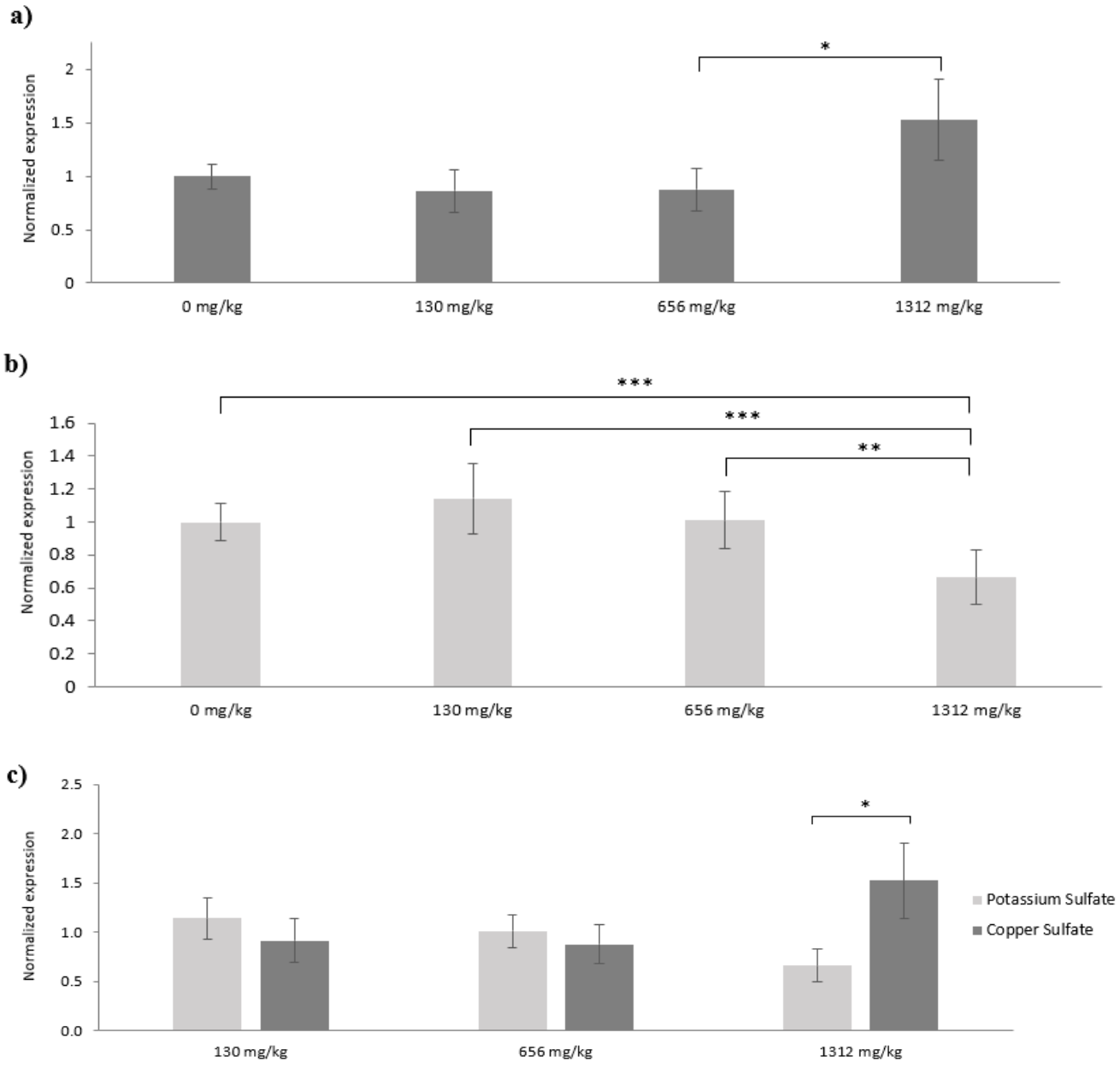

Figure 7. *RAN1* gene expression in white spruce (*Picea glauca*) needles treated with different concentrations of copper sulfate and potassium sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Gene expression of a) copper sulfate 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 * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.8. Gene expressions in copper susceptible and resistant genotypes

A significant upregulation of *MRP4* was observed between resistant and susceptible genotypes compared to the water control (Fig 8a). Likewise, *MRP4* expression was significantly higher in the susceptible genotype compared to the resistant genotype analyzed (Fig 8a). There were no significant changes in *RAN1* and *MT2b* expressions in needle tissues, when the water control and the resistant and susceptible genotypes were compared (Fig 8bc).

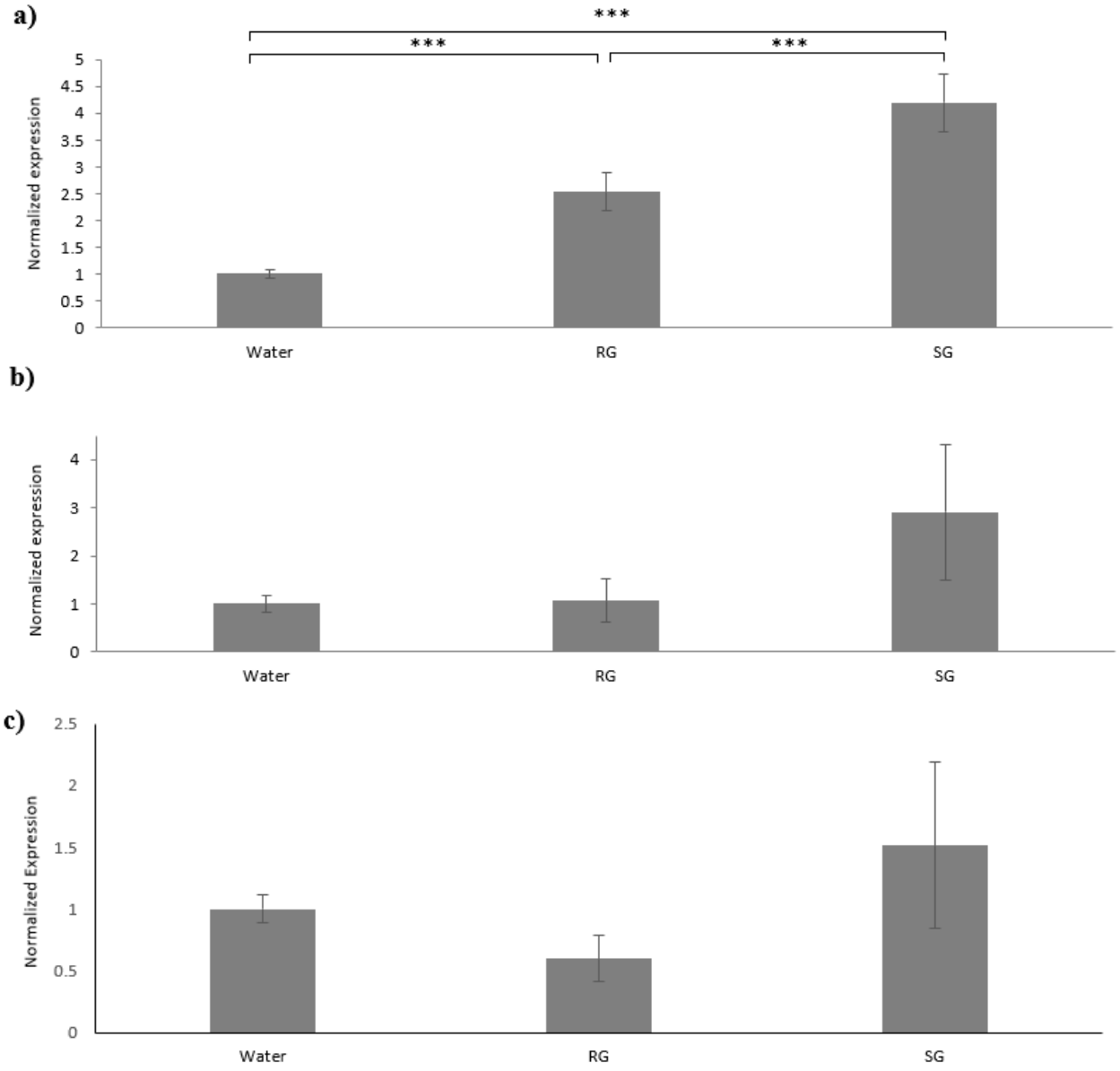

Figure 8. Gene expression in white spruce (*Picea glauca*) needles treated with 1312 mg/kg of copper sulfate. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. The phenotypic damage rating of the resistant genotype (RG) and susceptible genotype (SG) individuals was 2 and 6 respectively. Gene expression of a) *MRP4* b) *MT2* and c) *RAN1* are presented. Significant differences among the means of the treatments are marked using * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). Error bars represent the standard deviation of the treatment after being normalized to the reference gene and water.

2.3.2.9. MRP4 expression in needle vs. root

There were no significant changes in *MRP4* expression for all of the copper sulfate and potassium sulfate treatments when needle and root tissues were compared (Fig 9ab). However, analysis of the expression of *MRP4* in needles and roots revealed contrasting results for resistant and susceptible genotypes (Fig 9c). The *MRP4* gene expression was upregulated in roots of the resistant genotype and downregulated in roots of the susceptible genotype when compared to needle tissues (Fig 9c).

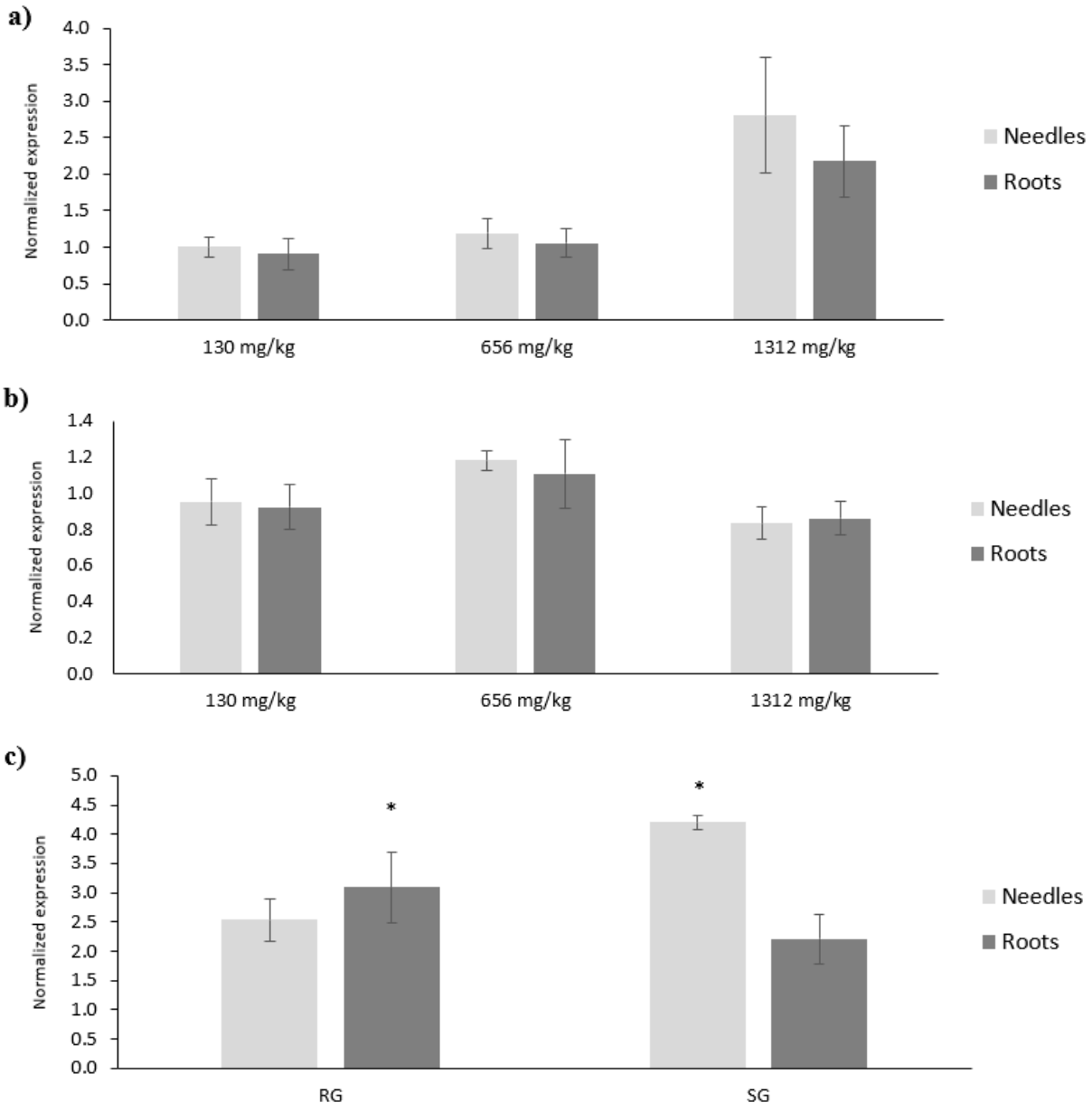

Figure 9. *MRP4* gene expression in white spruce (*Picea glauca*) tissues. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water which was used as the negative control. Comparison of the gene expression between needles and roots; a) copper sulfate treatments b) potassium sulfate treatments and c) resistant (RG) and susceptible (SG) genotypes treated with 1312 mg/kg of copper sulfate. Significant differences ($p \leq 0.05$) between tissues are represented with an asterisk (*).

2.3.2.10. MT2b expression in needle vs. root

While comparing *MT2b* gene expression for the root and needle tissues, it was found that the majority of the *MT2b* expression is significantly elevated in the roots (Fig 10ab). With the copper sulfate treatments, the *MT2b* gene expression was significantly elevated in the roots for all three concentrations (1312 mg/kg, 656 mg/kg, and 130 mg/kg) (Fig 10a). *MT2b* gene expression was significantly elevated in the roots compared to the needles, for samples treated with 130 mg/kg and 656 mg/kg of potassium sulfate (Fig 10b). For samples treated with 1312 mg/kg of potassium sulfate, results showed no significant differences in *MT2b* expression when comparing the root and needle tissues (Fig 10b). Interestingly, the susceptible genotype has a significantly elevated *MT2b* gene expression in the needles, when compared to the roots (Fig 10c). The *MT2b* gene expression was not significantly different between the roots and needles of the resistant genotype (Fig 10c).

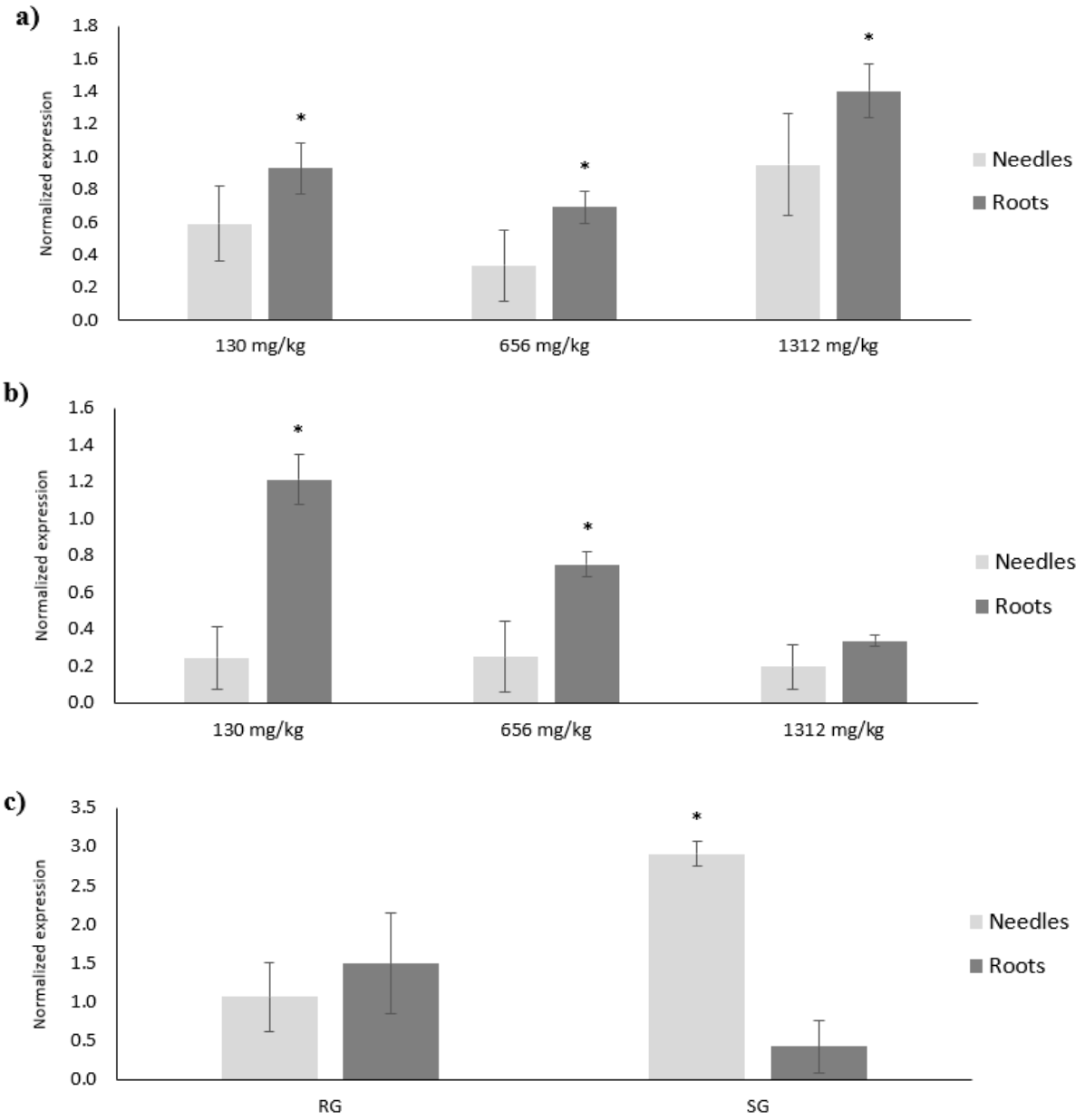

Figure 10. *MT2* gene expression in white spruce (*Picea glauca*) tissues. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Comparison of the gene expression between needles and roots; a) copper sulfate treatments b) potassium sulfate treatments and c) resistant (RG) and susceptible (SG) genotypes treated with 1312 mg/kg of copper sulfate. Significant differences ($p \leq 0.05$) between tissues are represented with an asterisk (*).

2.3.2.11. RAN1 expression in needles vs. roots

The *RAN1* gene expression was not significantly different between the roots and needles of the copper sulfate treatments (1312 mg/kg, 656 mg/kg, 130 mg/kg) (Fig 11a). The potassium sulfate treatment had an elevated *RAN1* gene expression in the roots of samples treated with 656 mg/kg and 1312 mg/kg of potassium sulfate, when comparing to the needle tissue (Fig 11b). *RAN1* gene expression was not significantly different between the roots and needles for samples treated with 130 mg/kg of potassium sulfate (Fig 11b). For both the resistant and susceptible genotypes, *RAN1* expression was significantly increased in the roots, when compared to the gene expression in the needles (Fig 11c).

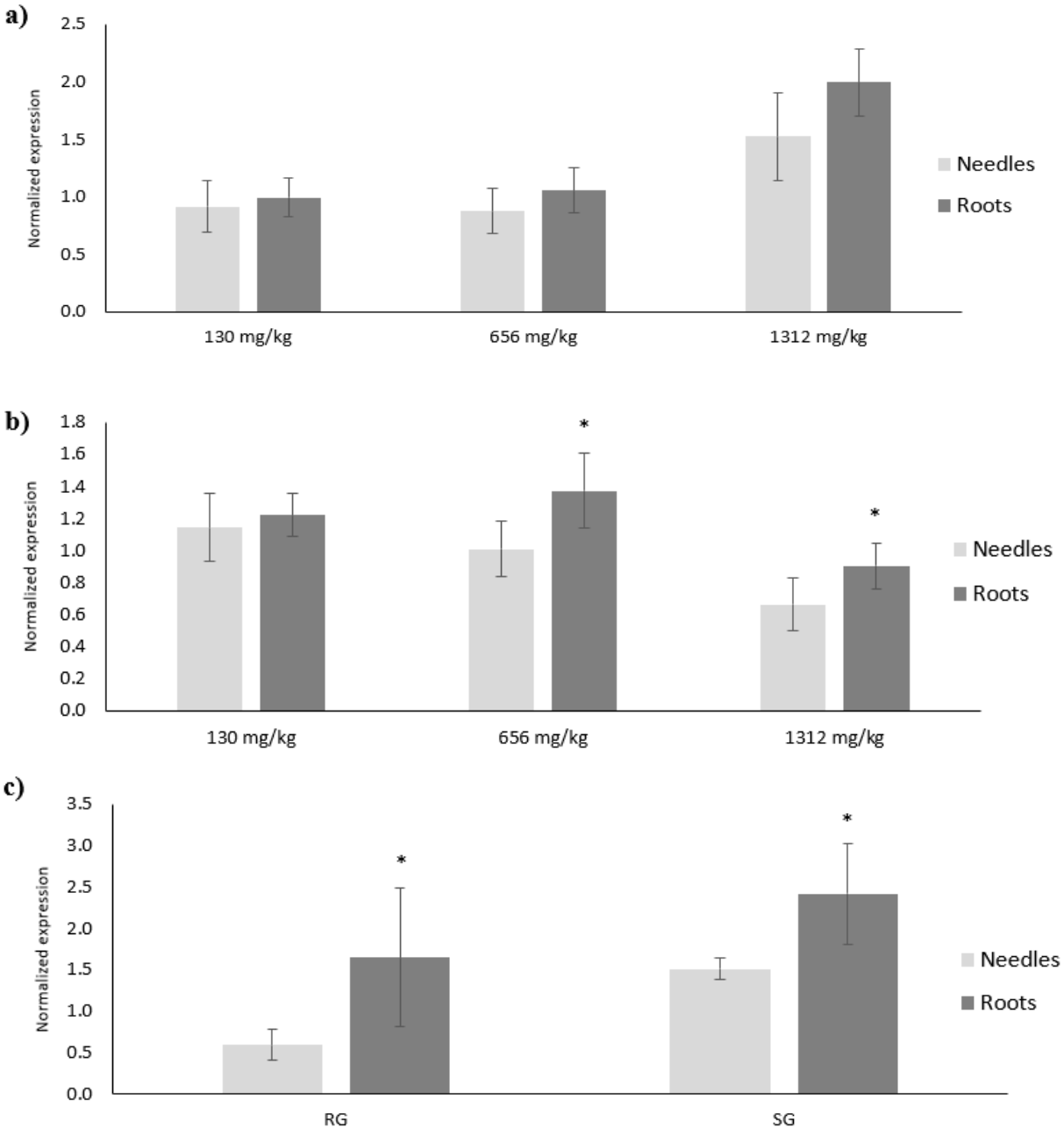

Figure 11. *RAN1* gene expression in white spruce (*Picea glauca*) tissues. The gene expression was normalized to the housekeeping gene (*EF1*- α) and water was used as the negative control. Comparison of the gene expression between needles and roots; a) copper sulfate treatments b) potassium sulfate treatments and c) resistant (RG) and susceptible (SG) genotypes treated with 1312 mg/kg of copper sulfate. Significant differences ($p \leq 0.05$) between tissues are represented with an asterisk (*).

2.4. Discussion

2.4.1. *Picea glauca* Response to Copper Sulfate Toxicity

The results of this study indicate that *Picea glauca* is tolerant to copper toxicity at lower concentrations of copper sulfate. The 130 mg/kg and the 656 mg/kg did not induce copper toxicity such as leaf browning or yellowing (Lombardi and Sebastiani 2005; Rout et al. 2013). However, the 1,312 mg of copper per kg of soil treatments exceeded the metal concentration threshold of the species, and caused toxicity symptoms such as leaf browning. Boyd and Nkongolo (2021) found that *P. glauca* was resistant to nickel toxicity for low and high concentrations of nickel nitrate tested. Proulx et al. (2017) showed that red oak (*Quercus rubra*) seedlings were resistant to 130 mg/kg, 656 mg/kg, and 1312 mg/kg of copper sulfate since there were no physical metal toxicity symptoms. Previous studies revealed copper toxicity symptoms caused by lower and higher concentrations of copper in *Rhododendron obtusum* (Delaware Valley Azalea) and *Cotoneaster divaricate* (spreading cotoneaster) (Kuhns and Sydnor 1976). Sardella et al. (2019) suggested that the accumulation of cysteine or reduced glutathione, which has previously been connected to the cell's sulfate uptake and sulfur pathway (Anderson 1980), could be responsible for the increased metal tolerance in *Scenedus acutus* (Sardella et al. 2019).

2.4.2. *MT2b* Gene Expression Associated with Copper Toxicity

An excess of copper or free copper ions are 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). Metallothionein's are a gene family which are 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 the two different isoforms of metallothioneins; *MT1* and *MT2* (Murphy and Taiz 1995). The isoforms may be differently expressed. For example, the *MT2* gene is expressed during metal treatments and the *MT1* gene remained unaffected, which was seen in *Arabidopsis* ecotypes (Murphy and Taiz 1995). Interestingly, the expression of *MT2* genes were not affected in the leaves of *Avicennia germinans* when the plant was 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 the roots and leaves after a copper sulfate treatment (Gu et al. 2015). When the *Iris lactea MT2b* gene construct was placed into *Arabidopsis* by a floral dip, the increased transgene expression showed greater root length growth compared to the non-transgenic plants after copper treatment (Gu et al. 2015). Since the *MT2* gene has been seen to be overexpressed in many different species once they undergo copper exposure, it is an indication that species are tolerant to copper when this gene is expressed (Guo et al. 2008; Zhao et al. 2012). This is consistent with the current study in *P. glauca*, where the highest concentration of copper sulfate had a significantly elevated *MT2b* gene expression in the roots, when compared to the water control. Plants treated with 656 mg/kg of copper sulfate had a repressed *MT2b* expression in the roots and needles when 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, and for all concentrations of potassium sulfate in needles. This indicates that the repressed *MT2b* gene

expression for 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 the leaves of *Quercus rubra* treated with 1312 mg/kg of potassium sulfate, when compared to the control (Proulx et al. 2017). Although the current study observed opposite 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). However, I propose that once the copper ion concentration reaches the metal toxicity threshold, the *MT2b* gene expression is increased rather than inhibited.

In other plant species, such as *Brassica campestris, MT2* expression 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 to deal with metal toxicity (Lv et al. 2013). When the *MT2* sequence from *Brassica campestris* was transfected to *Arabidopsis*, there was an increased tolerance to both the cadmium and copper metals, which was observed by increased root and shoot lengths when compared to the control plants (Lv et al. 2013). In the current study, comparisons of the *MT2b* gene expression in a metal susceptible and resistant genotype were performed to demonstrate possible differential *MT2b* gene expression between metal tolerant and metal susceptible genotypes. This comparison showed that *MT2b* was highly expressed in roots of copper resistant plants, but was inhibited in the susceptible genotype. Due to the possibility of apoptotic signalling to disrupt the *MT2b*

expression 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 which have not already begun the cell death process (Thomas et al. 2015).

When comparing the expression of *MT2* between the different tissues, it was found that *Brassica campestris* has a naturally high *MT2* expression in the leaves (Lv et al. 2013) and *B. napus* had a high *MT2b* expression in the cotyledons when compared to the gene expression in the hypocotyls (Pan et al. 2018). On the other hand, expression of *MT2b* in *Arabidopsis* was elevated within the vascular tissues, such as the phloem, in both the needles and roots (Guo et al. 2003). Expression of *MT2b* within the *Arabidopsis* MT family was increased in roots due to copper, whereas the young or maturing leaves showed no changes in *MT2b* expression (Guo et al. 2003). Expression of *MT2* in *Thlaspi caerulescens* shoot was neither induced or repressed when treated with copper sulfate (Roosens 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). *MT2b* expression was elevated in needles of the susceptible genotype, but was similar in the needles and roots of the resistant genotype.

2.4.3. *MRP4* Gene Expression Associated with Copper Toxicity

The multidrug resistance protein 4 (*MRP4*), also known as an ATP-binding cassette (*ABCC4*), has several roles in plants including the opening and closing of stomata, movement of methotrexate and possibly folates (Klein et al. 2004), and aiding in the transportation of crocin in *Crocus sativus* and *Nicotiana benthamiana* (Demurtas et al. 2019). The *MRP4* gene most commonly functions as

a transporter of glutathione conjugates and cysteine to aid in removing unwanted chemical substances or metals (Lai and Tan 2002; Morkunas et al. 2011; Koenderink et al. 2020). Glutathione S-transferase is required to unite glutathione with foreign contaminants and has been found to improve *Medicago sativa* L. metal tolerance when treated with mercury (Zhang et al. 2013). Mammalian *MRP4* reduces arsenic toxicity by transporting dimethylarsinic acid and a conjugate consisting of glutathione and monomethylarsonous acid, and possibly incorporating them into the urinary system (Banerjee et al. 2014). As well, plant glutathione has an important role for the synthesis of phytochelatins, and amino acid or reactive oxygen species transport (Grill et al. 1989; Mendoza-Cózatl and Moreno-Sánchez 2006).

MRP4 may work closely together with other ATP-binding cassette genes, such as the metal tolerance 1 (*HMT1*) gene, as it is involved in the transport of phytochelatins to yeast vacuoles in an attempt to increase cadmium tolerance (Ortiz et al. 1995; Sharma et al. 2016). Investigations on the function and expression of *MRP4* in plants is limited, but genes within the *MRP* gene family, such as *MRP1* and *MRP3*, have conserved regions in their transmembrane domain (Tommasini et al. 1998). They have also increased the transport of glutathione-conjugates in yeast and *Arabidopsis thaliana* resistant to cadmium (Tommasini et al. 1998). Specifically, the *MRP* genes are ATP dependent pumps which sequester toxins, such as metals, into cell vacuoles (Lu et al. 1997; Keinänen et al. 2007). *Arabidopsis MRP4* sequence data showed similarity to previous proteins involved with the glutathione-conjugate vacuolar membrane pumps with regards to the transmembrane domains (Sanchez-Fernandez et al. 1998).

An increased *MRP4* expression in *Arabidopsis* induced by superoxide oxidative stress caused by menadione was observed (Sanchez-Fernandez et al. 1998). However, exposure to a 20 micromolar concentration of cadmium had no significant changes to the *MRP4* gene expression (Sanchez-

Fernandez et al. 1998). This was also revealed in the current study where low concentrations of copper showed no significant changes in *MRP4* gene expression (Sanchez-Fernandez et al. 1998). However, *MRP4* gene expression has been up-regulated in a copper tolerant birch tree (*Betula pendula* Roth.) roots and shoots treated with copper (Keinänen et al. 2007). Based on several reports, changes to the *MRP4* gene expression may be species or metal dependant (Sanchez-Fernandez et al. 1998; Keinänen et al. 2007). Red Oak (*Quercus rubra*) has also demonstrated an increased expression of the *MRP4* gene once treated with a high concentration of copper (Proulx et al. 2017). In the current study, *P. glauca* root and needle *MRP4* was induced in samples treated with 1,312 mg/kg of copper sulfate. Since the potassium sulfate control did not induce *MRP4* changes in roots, it was concluded that the changes in *MRP4* expression was induced by copper ions. Surprisingly, potassium sulfate inhibited needle *MRP4* expression at the 1,312 mg/kg concentration, which shows that the potassium sulfate and copper sulfate treatments have opposing effects on *MRP4* gene expression. As well, in the needles, *MRP4* was induced for samples treated with 656 mg/kg of copper sulfate and potassium sulfate treatments, which indicate that the effects on the *MRP4* gene transcription may not be caused strictly by copper ions, but can also be caused by the sulfate ions. In *Quercus rubra*, it was also found that 656 mg/kg of potassium sulfate increased *MRP4* gene expression while 1,312 mg/kg of potassium sulfate inhibited its expression in leaves (Proulx et al. 2017).

Within the *MRP* gene family, the gene expression may vary, such as the *Arabidopsis MRP4* gene having a slight increase in expression and the *MRP3* gene being much more expressed under a cadmium chloride exposure (Bovet et al. 2003). Because of several roles of glutathione transport in plants, it is expected that the glutathione transporter gene, *MRP4*, would be upregulated under oxidative stress, amino acid transport, or metal toxicity (Mendoza-Cózatl and Moreno-Sánchez

2006; Chen et al. 2010). Interestingly, in both the resistant and susceptible genotypes treated with copper sulfate, the *MRP4* gene expression was induced in root and needle tissues. This is inconsistent with the apoptosis process observed in susceptible plants that would limit the transcription of many genes, other than the genes involved with the programed cell death (Thomas et al. 2015). This means *MRP4* may still be functioning during the apoptosis process (Thomas et al. 2015). The results from this study suggest that *MRP4* has a role in copper transport in roots and needles at higher concentrations of copper sulfate. This study also shows that *P. glauca* is tolerant to low concentrations of copper sulfate and will sequester copper ions in vacuoles at higher concentrations (Keinänen et al. 2007).

Studies on *Salvia miltiorrhiza* showed differential expression of genes within the *MRP* gene family in different tissues. *MRP4* was highly expressed in root tissues, whereas other *ABCC* genes were expressed in the flowers, stems, or leaves, but not the root (Yan et al. 2021). However, when methyl jasmonate (MeJA), a plant growth regulator, was induced, *Salvia miltiorrhiza* had an increased *MRP4* gene expression in the leaves (Yan et al. 2021). The current study shows no variation of the *MRP4* expression when roots and needles were compared in *P. glauca* treated with copper and potassium sulfate. Interestingly, the resistant and susceptible genotypes had contrasting results for *MRP4* gene expression, when comparing root and needle tissues. There are many reasons why *MRP4* is highly expressed in the needles of a susceptible genotype versus being repressed in the needles of the resistant genotype. Firstly, these differences with *MRP4* could be caused by the differential copper concentrations within different tissues. Similar results were reported in *Noccaea caerulescens* and *Noccaea japonica* that displayed differential expression of the *IREG2* gene under nickel exposure (Nishida et al. 2020). Previous nickel translocation data revealed similar concentration of nickel in roots, stems, and needles of *P. glauca* exposed to nickel

concentrations (Boyd 2020). Future analysis on *P. glauca* copper translocation may help to understand if differential copper concentrations in separate tissues will cause changes to *MRP4* expression in a metal-resistant and a metal susceptible genotype. Nishida et al. (2020) studying nickel hyperaccumulators, *Noccaea japonica* and *Noccaea caerulescens*, found that physical nickel toxicity symptoms were only visualized in the species which had a higher root to shoot nickel translocation. It was also found that the iron-regulated 2 gene (*IREG2*) was extremely elevated in roots of the species with a lower nickel translocation (Nishida et al. 2020). Hence, a high *IREG2* gene expression in the roots would hold the nickel ions within the root, instead of allowing the metal to move through the plant tissues (Nishida et al. 2020). The decreased *MRP4* expression in roots of the susceptible genotype, compared to the needles, could mean that there is a higher copper root to shoot translocation in the susceptible plant, compared to the resistant genotype with a high copper root sequestration (Nishida et al. 2020) Other factors may also explain this difference between susceptible and resistant genotypes. Some examples include the activation of root apoptotic signalling in susceptible genotypes causing *MRP4* mRNA degradation (Thomas et al. 2015), or plants needing needle *MRP4* activation for stomatal changes and transport of toxins into cell vacuoles (Klein et al. 2004; Keinänen et al. 2007).

2.4.4. *RAN1* Gene Expression Associated with Copper Toxicity

RAN1 is involved in several processes within a plant cell, including the growth of primordial meristems and lateral roots, mitotic cell division G2 phase advancement (Wang et al. 2006), as well as heavy metal resistance through copper transport and the ethylene hormone response pathway (Binder et al. 2010). *RAN1* is also called Heavy Metal ATPase 7 (*HMA7*), as it is a copper-transporting P-type ATPase (Baloun et al. 2014; Hoppen et al. 2019). Transporters in the *HMA* gene family have functions to transport zinc, cadmium, lead, silver, and copper ions (Wenli et al.

2020). Copper transport to the ethylene receptor is very important for plant growth functions, and is partly controlled by *RAN1* and the soluble copper transporter family, antioxidant-1 (*ATX1*) (Hoppen et al. 2019). However, some pathways bypass *RAN1* altogether and this was seen in *Nicotiana benthamiana*, where the *ATX1* and the copper transport protein *CCH* would transport the copper directly to the endoplasmic reticulum (ER) ethylene receptors without the intermediate use of *RAN1* (Hoppen et al. 2019). The ethylene hormone receptors need copper ions to allow ethylene to bind, resulting in ethylene signalling to complete downstream functions that protect plants against abiotic stresses (Rodríguez et al. 1999; Woeste and Kieber 2000). Ethylene receptors are found within both the ER and the Golgi apparatus (Dong et al. 2008). Although *RAN1* is localised within the ER-Golgi membranes, there are opposing theories that *RAN1* directly interacts with the ethylene receptors when bound to copper; either through the ER or Golgi apparatus, or both of these organelles in the endomembrane system (Hirayama et al. 1999; Woeste and Kieber 2000; Hoppen et al. 2019). Due to the similarities of amino acid sequences between *RAN1* and the human Menkes/Wilson proteins and yeast *Ccc2p*, it was first proposed that *RAN1* was localized in the trans-Golgi apparatus or post-Golgi vesicle (Dierick et al. 1997; Yuan et al. 1997; Hirayama et al. 1999). However, further studies found colocalization of *RAN1* with an ER marker, while there was no colocalization of *RAN1* with the Golgi marker (Hoppen et al. 2019). Due to the localization of *RAN1* in the secretory pathway, copper may enter the secretory vesicles to be transported out of the cell, as seen with the copper transporters *ATP7a/b* (Petris et al. 1996; Polishchuk and Lutsenko 2013). Since *RAN1* functions in more than just the copper transporting pathway, it is possible that under normal conditions, there will be *RAN1* bound to copper in the ER, but an abundance of copper will cause *RAN1* to transport copper to the post-Golgi apparatus

to remove it from the cell (Dierick et al. 1997; Yuan et al. 1997; Hirayama et al. 1999; Wang et al. 2006; Hoppen et al. 2019).

When different species were treated with different concentrations of copper sulfate, there were significant differences with the ethylene concentrations released into plants (Mertens et al. 1999; Groppa et al. 2003; Lequeux et al. 2010). *Helianthus annuus* and *T. aestivum* increased ethylene production in their leaves once they were treated with 0.5 mM of copper, but *A. thaliana* seedling ethylene production was not affected when treated with a lower concentration of copper (Groppa et al. 2003; Lequeux et al. 2010). On the contrary, younger seedlings of *A. thaliana* were treated with 0.025-0.5 mM of copper sulfate and had an increased ethylene production (Mertens et al. 1999). Interestingly, when there is increased ethylene in plants, the activity of enzymes found in the glutathione (GSH) pathway are increased, such as the ascorbate peroxidase and glutathione reductase, which have beneficial antioxidant functions (Grill et al. 1989; Mendoza-Cózatl and Moreno-Sánchez 2006; Khan and Khan 2014). Due to the increased GSH, *RAN1* and *MRP4* are intertwined within the copper transporting pathway in removing excess copper through ethylene signalling and glutathione transport (Khan and Khan 2014; Koenderink et al. 2020).

In the present study, *RAN1* expression in roots of *P. glauca* was induced at the highest concentration of copper sulfate, similar to the *MRP4* and *MT2b* genes. *RAN1* expression was also induced in samples treated with 656 mg/kg of potassium sulfate when compared to water. This is unexpected since the equivalent concentration of copper sulfate had no changes on the *RAN1* expression. However, since plants treated with 1,312 mg/kg of potassium sulfate showed no significant changes in *RAN1* gene expression, it can be concluded that *RAN1* expression is induced under high copper concentrations in *P. glauca* roots. This is consistent with the increased *RAN1* expression in *Silene vulgaris* roots after copper treatment (Baloun et al. 2014). Other studies

reported increased ethylene production due to copper sulfate, which may also be connected to increased copper-bound *RAN1* transporting the metal ion to the ethylene receptors (Mertens et al. 1999; Hirayama et al. 1999; Hoppen et al. 2019). In the current study, *RAN1* expression was unaffected in needles by the copper sulfate treatments, when compared to water. However, *RAN1* expression was inhibited at the highest concentration of potassium sulfate when compared to the water and other potassium sulfate concentrations. These differences between the *P. glauca RAN1* expression in roots and needles may be due to the sulfate ions having opposing effects to copper ions in needles at higher treatment concentrations. Previous analysis of *Silene vulgaris* revealed that *RAN1* gene expression was unaffected in shoots after copper exposure (Baloun et al. 2014). This is consistent with *P. glauca* increasing *RAN1* expression in roots, but not in the upper plant tissues. Proulx et al. (2017) reported that *Quercus rubra* leaf *RAN1* expression was repressed under copper exposure, but there were no comparisons to *RAN1* root expression. Although this study did not show an inhibited *RAN1* expression in needles, both *P. glauca* and *Quercus rubra* lack an overexpressed *RAN1* in the needles or leaves under copper exposure (Proulx et al. 2017). As well, the potential effects that copper translocation in *P. glauca* may have on the copper transporting genes may explain the different gene expression results for *RAN1* (Nishida et al. 2020). Analysis of nickel toxicity and *IREG2* nickel transporter showed that an elevated *RAN1* expression in roots may indicate that this metal is sequestered in roots before moving to shoots or needles (Nishida et al. 2020). This could explain why there wasn't an elevated *RAN1* expression in the needles, when compared to the water control (Nishida et al. 2020). No changes in *RAN1* expression in roots and needles of resistant and susceptible genotypes was observed when compared to water. This suggests that *RAN1* is not the major factor in copper tolerance in *P. glauca*.

In *Arabidopsis*, *RAN1* was found to be highly expressed in roots, compared to shoots (Andrés-Colás et al. 2006). *HMA5*, which is very closely related to *RAN1*, was also highly expressed in roots of *Arabidopsis*, and was found to interact with the *CCH* copper transporter, further proving that the *HMA* family has roles with diminishing copper stress (Andrés-Colás et al. 2006). In this study, *RAN1* expression was higher in roots of the two high concentrations of potassium sulfate, as well as both the resistant and susceptible genotypes treated with 1,312 mg/kg of copper sulfate, which is consistent with the previous study (Andrés-Colás et al. 2006). The current study did not show any variation to *RAN1* gene expression between roots and needles for the copper sulfate treatments. This is inconsistent with previous studies showing a significantly suppressed *RAN1* gene expression in the shoots of copper treated *Arabidopsis thaliana*, when compared to their control treatment (del Pozo et al. 2010). As well, there were no significant changes to *RAN1* gene expression in the roots of *Arabidopsis thaliana*, when compared to the control plant (del Pozo et al. 2010). The inconsistency could be caused by *RAN1* being activated or repressed due to its functions with other plant processes, or could be based on the gene variations among different plant species (Wang et al. 2006).

Chapter 3: DNA Methylation of *Picea glauca*

3.1. Introduction

There are many environmental and anthropogenic stresses which plants encounter and adapt to for survival. The ability of plants to survive these changes, such as an abundance of metal ions leaching into the soil from surrounding anthropogenic activities, can be attributed to changes to metal transporter gene expressions (Curie et al. 2000; Thomine et al. 2000; Keinänen et al. 2007; Kobayashi et al. 2008). Rapid alterations to gene expressions, as well as genome stability, can be performed through epigenetic mechanisms (Zhang et al. 2018). Changes to gene expression can be caused by DNA methylation, histone modification, among other epigenetic mechanisms (Weinhold 2006; Handy et al. 2011). Reports on transcription factors, such as nuclear respiratory factor 1 (*NRF1*), showed a competition between transcription factor binding and methylated DNA sequences (Domcke et al. 2015). In fact, it was found that the removal of methylated sequences using kinase inhibitors allowed *NRF1* to bind, but the transcription factor was quickly removed when the kinase inhibitors were removed and the DNA was re-methylated (Domcke et al. 2015). This shows the importance of hypo- and hypermethylation on gene transcription. These epigenetic changes allow the plant to adapt to stresses within the environment more readily, since there are no changes to the DNA sequence (Weinhold 2006; Thiebaut et al. 2019).

An RNA-directed DNA methylation pathway is crucial for de novo methylation in plants, but is less important in mammals (Zhang et al. 2018). DNA methylation within the *Arabidopsis* genome is partially dependent on the amount of transcription or size of the transcript for genes; Genes which are highly transcribed, or very rarely transcribed, have very little DNA methylation when compared to genes which are moderately transcribed and have a high percent of DNA methylation (Zilberman et al. 2007). To survive different stress factors, such as plant drought or salt stress, the

patterns of methylation will change in a gene-specific manner (Wang et al. 2016, 2021). If an organism is undergoing stress from an outside factor such as increased metal concentrations or drought, it was reported that genes within the stress signalling pathway may undergo hypomethylation to alleviate or adapt to stresses through a complicated gene regulation relationship (Wang et al. 2016, 2021). In fact, *Arabidopsis* active DNA hypomethylation on stress signalling genes, caused by ceasing the heat stress treatments, demonstrated one part of the pathway which allows the plant to adapt to heat stress (Korotko et al. 2021).

In regions where there are high metal concentrations, such as the City of Greater Sudbury, a large mining community, it would be beneficial to observe the effects that copper has on the plant species. Since the gene expressions of metal resistance genes may be affected due to epigenetic mechanisms, this research could provide knowledge on the epigenetic adaptations of *P. glauca* from the effects of copper toxicity. The objective of this component is to assess if copper or potassium ions induce changes in cytosine methylation in *P. glauca*.

3.2. Materials and Methods

3.2.1. Biological Materials and Copper and Potassium Sulfate Treatments

All plant treatments and growing procedures are described in section 2.2.1. Needle and root tissues were used for global DNA methylation analysis.

Four individual plants were chosen from the water control treatment, three individual plants were chosen for the highest concentration of copper sulfate treatment (1,312 mg of copper per kg of soil), and two individual plants from the potassium sulfate treatment (1312 mg/kg). Needles and roots were harvested from *P. glauca* seedlings and were stored in -20 degrees Celsius.

3.2.2. DNA Extraction and Global DNA Methylation Analysis

Total DNA was extracted from root and needle samples using the DNeasy Plant Mini Kit from Qiagen. The quality check for the eighteen extracted DNA samples was done using a 1% agarose gel. Quantification of the DNA samples was performed using the DNA Quantitation Kit (#1702480) by Bio-Rad Laboratories which utilized the 96-well plate reader (Fluostar Optima) from BMG Technologies and Hoechst staining. The quantification of DNA was calculated using the standard curve from control samples contained within the quantification kit. DNA samples were then diluted to a working stock concentration with sterilized water to be 50 ng of DNA per uL of water. The working stock of DNA was then used in the Global DNA Methylation Assay Kit (5 Methyl Cytosine, Colorimetric) kit from Abcam (ab233486) to measure global 5 methyl cytosine. In total, 100 ng of DNA was added to the wells of the plate, along with 100 uL of binding solution (Appendix 7). In addition to the DNA and binding solution, a detection complex which consisted of a 5-methyl cytosine antibody, signal indicator, and enhancer, was used to detect the DNA methylation. DNA washes were performed to verify purified DNA samples, and then a

developer solution and stop solution were used to start and stop color development, respectively. To calculate the percent of global DNA methylation, a standard curve was produced using methylated DNA control samples within the kit and the absorbance of all samples was read at 450 nm with the plate reader (Fluostar Optima) from BMG Technologies.

3.2.3. Statistical Analysis

Statistical analyses were conducted using the SPSS version 20 for windows (IBM, NY, USA). The Shapiro-Wilk test ($P \leq 0.05$) was performed and indicated unnormal data. A Kruskal-Wallace ($P \leq 0.05$) non-parametric test was performed to determine any significant differences between the ranks of the global DNA methylation between the treatments. A Dunn's Post Hoc Test ($P \leq 0.05$) was then performed for a pairwise comparison. Individual Mann-Whitney non-parametric tests ($P \leq 0.05$) were performed to analyze the significant differences between the ranks of the DNA methylation expressed in percentage for roots and needles.

To test any significant differences between the damage ratings of the white spruce trees involved in the global DNA methylation analysis, a Kruskal-Wallace ($P \leq 0.05$) non-parametric test was performed along with a Dunn's Post Hoc Test ($P<0.05$) for a pairwise comparison.

3.3. Results

3.3.1. *P. glauca* Global DNA Methylation

A decrease of global cytosine methylation (hypomethylation) in roots induced by the potassium sulfate treatment was observed when compared to the water control (Fig. 12a). The level of global methylation was also lower in roots treated with potassium sulfate, compared to the copper sulfate treatment in roots (Fig. 12a). Interestingly, the copper sulfate and water control treatments showed similar levels of global cytosine methylation in roots and needles (Fig. 12ab).

Analysis of needle samples showed that the potassium sulfate treatment induced a lower, but not significant, level of global cytosine methylation compared to the water control (Fig. 12b). As observed in roots, the potassium sulfate treatment significantly decreased the level of global cytosine methylation in needles when compared to the copper sulfate treatment (Fig. 12b).

The levels of global cytosine methylation were higher in roots, compared to needles, in genotypes treated with water or copper sulfate salts (Fig. 12c). Interestingly, the potassium sulfate treatment induced no significant changes in global cytosine methylation between roots and needles since both tissues show similar low DNA methylation (Fig. 12c)

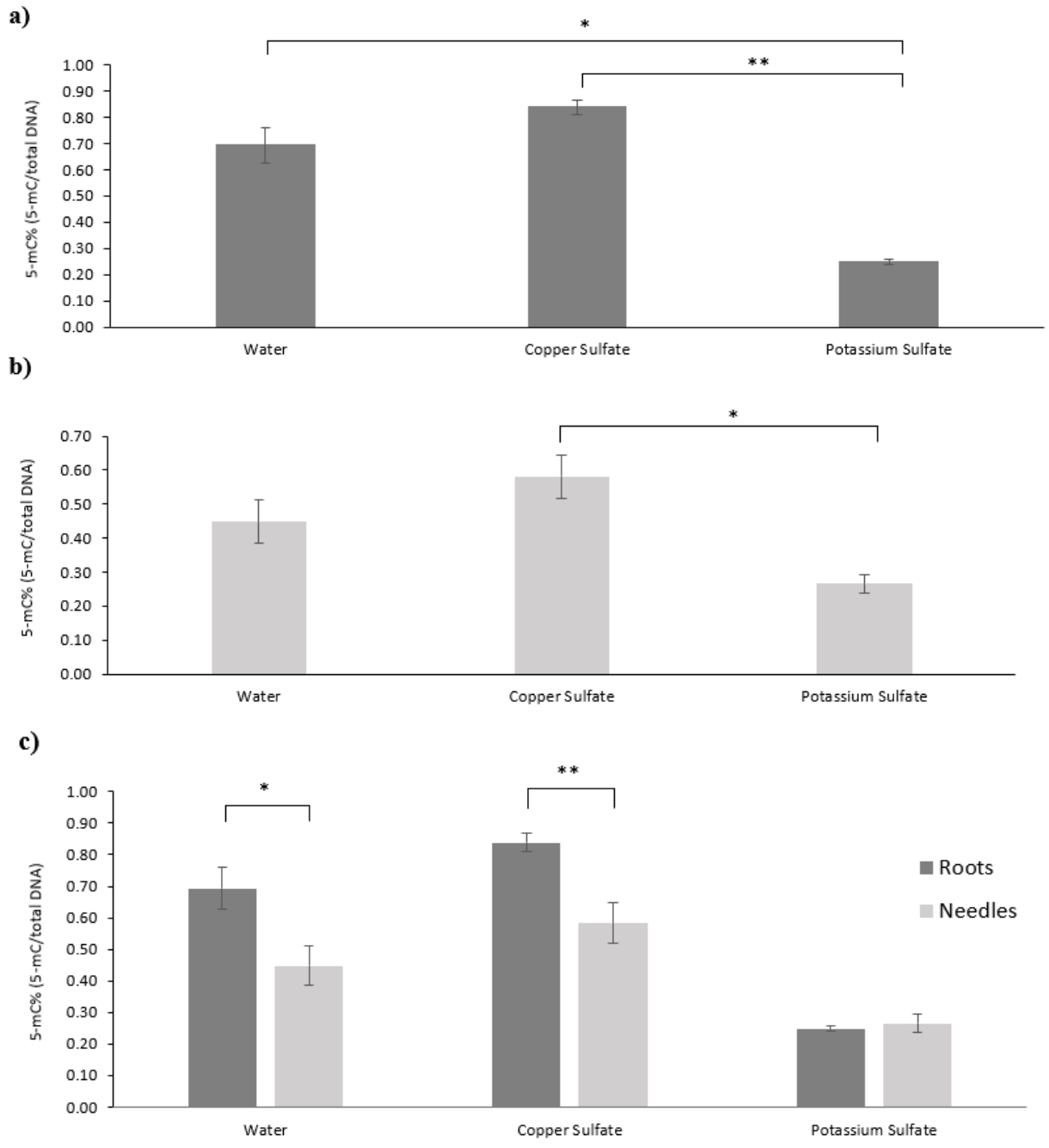

Figure 12. Percent of 5 methyl cytosine (5-mC%) in white spruce (*Picea glauca*) a) roots and b) needles treated with 1312 mg/kg of copper sulfate and potassium sulfate. Water was used as a negative control. C) Represents significant differences found between the percent of methylation in the roots and needles of the three treatments. Significant differences among the treatments are marked using * ($p \leq 0.05$), ** ($p \leq 0.01$). Error bars represent the standard error of the treatment.

3.3.2. Copper Sulfate Toxicity

The individual genotypes used in the global DNA methylation analysis presented variations in damage ratings. A low damage rating accounts for the plants which are resistant to the copper metal, and a high damage rating accounts for the plants which are susceptible to the copper metal based on their physical appearance after the treatments. The current study shows that the average damage rating of plants within the copper sulfate treatment is significantly higher than the average damage rating of the plants within the water control (Fig. 13). The study did not find any significant differences between the average damage rating for the plants treated with copper sulfate and potassium sulfate, but it is visually noticeable that the damage rating of potassium sulfate was much lower (Fig. 13).

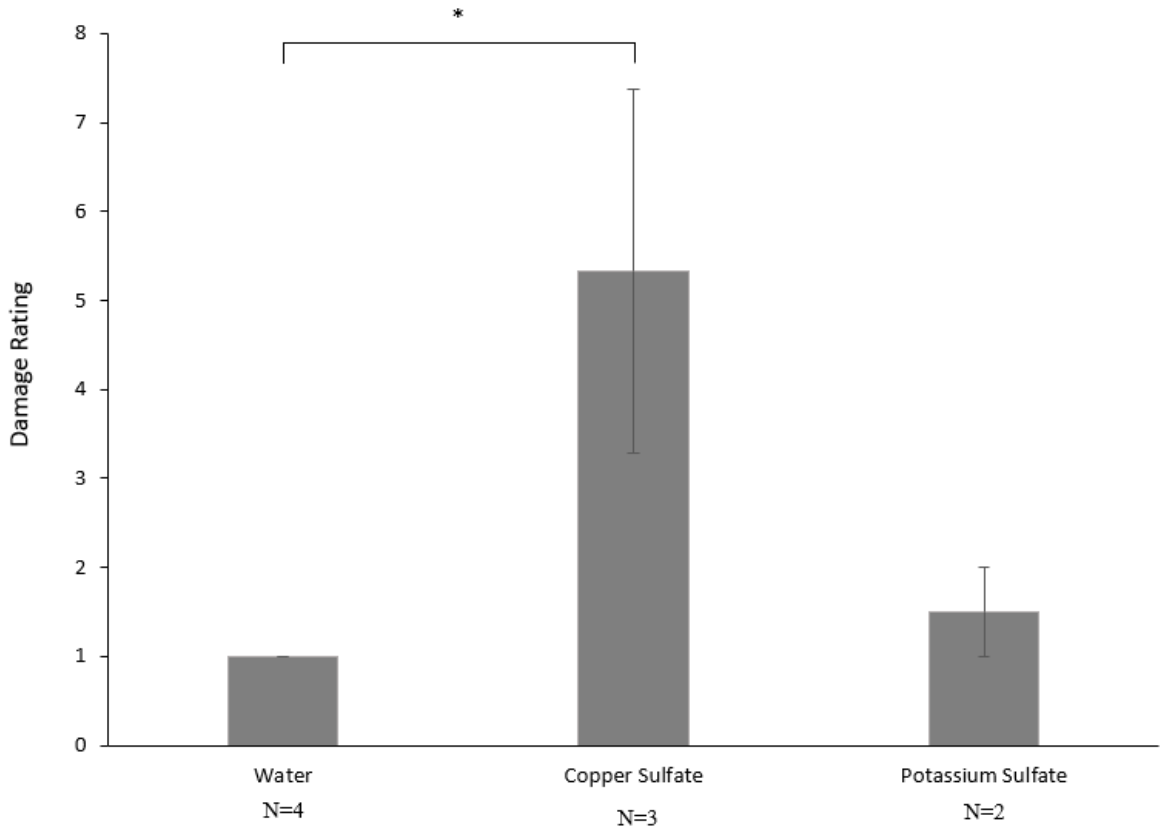

Figure 13. The phenotypic damage rating of white spruce (*Picea glauca*) trees treated with 1312 mg/kg of copper sulfate and potassium sulfate. Water was used as a negative control. The damage scores of 1 to 3 were considered healthy, 4 to 6 were moderately healthy, and 7 to 9 were damaged. Significant differences among the treatments are marked using * ($p \leq 0.05$). Error bars represent the standard error of the treatment.

3.4. Discussion

DNA methylation is an epigenetic mechanism that has been seen to affect all plant processes (Wang et al. 2016; Bednarek and Orłowska 2020). In barley, it was observed that copper and silver ions, which can be interchanged with one another, decreased the demethylation process, compared to the level of DNA methylation (Bednarek and Orłowska 2020). As well, it was determined that the copper and silver metal ions, DNA methylation, and the regenerative ability of barley were connected with one another, as seen by barley regeneration occurring during increased DNA methylation weeks after the metal treatments (Bednarek and Orłowska 2020). Plants which have been used for regreening, such as *Hydrilla verticillata* (L.f.) Royle, have demonstrated changes to the level and type of DNA methylation after being treated with a high concentration of copper (Shi et al. 2017). Specifically, it was found that copper causes a decrease in the level of double stranded DNA methylation (Shi et al. 2017). However, there was an increase of the total level of DNA methylation which accounts for single strand and double strand methylation (Shi et al. 2017). Interestingly, for both clover and hemp plants, the addition of nickel, cadmium, and chromium decreased the global DNA methylation (Aina et al. 2004). This hypomethylation was dependent on the metal concentrations, and appeared to be connected to the increase in reactive oxygen species which heavy metals induce (Dixit et al. 2002; Boominathan and Doran 2002; Aina et al. 2004).

Metal deficiencies are just as important as toxic metal concentrations. When *A. thaliana* underwent zinc deficiency and normal zinc concentrations, it was found that there was a higher CG methylation compared to the CHG and CHH methylation under both conditions with small, although not significant, differences between the level of methylation in the two zinc treatments (Chen et al. 2018). While the amount of global methylation did not drastically change, many

differentially methylated regions (DMR) were found, and it was discovered that CG methylation sites were different in promoters and gene bodies, whereas the CHG and CHH methylation sites were different in gene promoters and transposable elements (Chen et al. 2018). However, when *A. thaliana* was mutated to have deficient domains rearranged methyltransferases (DRM) and chromomethylase (CMT), it was found that the overall shoot and leaf growth of the mutant was affected under zinc deficiencies, showing that changes in DNA methylation have a large impact on the growth in zinc deficient areas (Chen et al. 2018). The current study does not determine the impact of DMRs, but it is interesting to note that the percent of global DNA methylation may not be altered by the copper sulfate treatment, while the potential for DNA methylation site changes could alter the growth of *P. glauca* in the copper stress treatment.

Interestingly, an untreated nickel resistant mouse cell line and a metal-tolerant plant (hemp) both exhibited higher levels of DNA methylation when compared to the nickel susceptible cell line and plant (clover) (Lee et al. 1998; Aina et al. 2004). After nickel treatment, the percent of global DNA methylation was lowered for the nickel-resistant mouse cell line, clover, and hemp, but not the nickel-sensitive mouse cell line (Lee et al. 1998; Aina et al. 2004). Interestingly, it was observed that the metal-resistant mouse cell line eventually increased the DNA methylation levels and cytosine 5-methyltransferase activity after stopping the stress treatment (Lee et al. 1998). In the current study, *P. glauca* global DNA methylation was unaffected by treatment with a high concentration of copper (1,312 mg/kg). Since this study was not measuring the effect of copper on global DNA methylation based on temporal conditions, it is unknown whether *P. glauca* has a quick recovery period after the metal treatment. *Arabidopsis* also demonstrated temporal differences with DNA methylation changes, since a decrease in DNA methylation did not occur in early stages of heat stress, but were only discovered in later stages where the heat stress treatment

was removed (Korotko et al. 2021). On the other hand, it may be possible that plants were undergoing apoptosis due to the toxicity levels of the copper (Appendix 3 and 6). The DNA quality check gel electrophoresis demonstrates a DNA laddering effect which is common with endonucleases during apoptosis and extreme stress conditions (Wyllie 1980). In previous studies, there was increased 5-methyl cytosine DNA methylation, as well as increased activity of methyltransferases, while mice neuronal cells underwent apoptosis (Chestnut et al. 2011). However, it has also been found that tumors bypass apoptotic signalling by methylating the promoters of apoptotic signalling genes, such as Apoptosis-associated speck-like protein containing a CARD (ASC), preventing cell death (Collard et al. 2006). Due to the possibility of apoptotic signalling to change the level of global DNA methylation occurring in the plant cells under stress treatments, it must be understood that the *P. glauca* results from this study should be compared to results with plants that are not currently undergoing apoptotic signalling. The connections between apoptosis signalling and DNA methylation is lacking. This study demonstrates a regulation of the level of global DNA methylation in *P. glauca* undergoing apoptosis due to copper exposure.

Other growth conditions may alter the DNA methylation patterns such as drought stress (Wang et al. 2016). Although previous studies have found differences between differentially methylated regions of DNA in a drought sensitive and drought tolerant rice cell line, the current study avoided the possibility of drought stress in *P. glauca* seedlings by keeping the soil wet during the growth period (Wang et al. 2016). The DNA methylation patterns are altered using methyltransferases such as chromomethylase-3 and DNA (cytosine-5)-methyltransferase-1, or demethylase enzymes such as *ROS1* or other glycosylases (Gong et al. 2002; Stroud et al. 2013; Li et al. 2018). There were differential gene expressions of specific methyltransferases in the chickpea plant after

drought, salt (sodium chloride), and cold stress (Garg et al. 2014). As well, when soybeans were treated with NaCl, it was found that there was a slight decrease from 63.7% CpG, 43.6% CHG, and 4.0% CHH methylation in control plants, to 61.2%, 39.7%, 3.2% of respective methylation in salt treated plants (Chen et al. 2019). *P. glauca* was kept within the growth chamber with a steady temperature and day-night cycle, which would remove any cold stress. However, to treat the plants with an aqueous form of copper, the seedlings were treated with copper sulfate and potassium sulfate salts. It is possible that methyltransferases were affected by the salt or potassium ions in *P. glauca*, which could contribute to the significant decrease in global DNA methylation when the seedlings were treated with potassium sulfate.

Sulfate has many roles within the plant cells, such as being a donor of sulfur to create cysteine, methionine, glutathione, and other important plant cell compounds (Anderson 1980). Ferrari et al. (2020) analyzed a chromium tolerant and a chromium susceptible *S. acutus* to discover a relationship between the DNA methylation patterns of the different strains, including the methylation of genes related to the sulfate pathway. After bisulfite sequencing, differences in the CG, CHG and CHH methylation, as well as changes to DMRs, within the chromium resistant and susceptible *S. acutus*, were discovered (Ferrari et al. 2020). The sulfate transporter (*SaSULTR1*) had a hypomethylation in the promoter region of the chromium tolerant strain, which consequently created increased *SaSULTR1* gene expression (Ferrari et al. 2020). Interestingly, the genes which were involved in sulfate assimilation had opposite outcomes, where there was hypermethylation resulting in lower gene expressions (Ferrari et al. 2020). It is possible that the level of global DNA methylation was significantly decreased during potassium sulfate treatments due to the sulfate or potassium ions, and could be responsible for the increased expression of genes involved with the transport or assimilation of potassium and sulfate ions, as supported by the *S. acutus* results (Ferrari

et al. 2020). More understanding between the specific DNA methylation sites in *P. glauca* is needed to analyze specific methylation changes caused by the copper sulfate and potassium sulfate treatments, and to determine what gene regulations are being affected by DNA methylation.

Chapter 4: General Conclusions

The objectives of this study were to 1) determine the effects of different concentrations of copper and potassium sulfate on the level of expression of genes associated with copper resistance (*RAN1, MT2b, MRP4*) and 2) evaluate the level of DNA methylation *P. glauca* exposed to copper. The results showed that the lower concentrations of copper sulfate did not cause damages to plants. Although copper is a micronutrient which is needed for plant survival, it was determined that the highest concentration of copper (1,312 mg /kg) caused severe damages to some genotypes. Copper resistant genotypes were identified in the segregating populations analyzed.

The expression of the three genes in roots were induced by copper sulfate treatments compared to the water control. *MT2b* in roots was repressed in plants treated with 656 mg/kg of copper sulfate, compared to the control. *MT2b* gene expression was repressed in plants treated with 656 mg/kg and 1,312 mg/kg of potassium sulfate, while *RAN1* expression was increased by 656 mg/kg treatments of potassium sulfate when compared to the control in root tissues.

MRP4 was upregulated in both the copper resistant and susceptible genotypes, while the *MT2b* expression was only significantly higher in the roots of the resistant genotype, when compared to water. On the other hand, *RAN1* expression showed no significant changes when susceptible and resistant genotypes were compared to the water control and between themselves.

The *MRP4* gene is the only gene that was highly expressed in needles of plants treated with 656 mg/kg and 1312 mg/kg of copper sulfate. However, *MRP4* expression was also significantly increased in plants treated with 656 mg/kg of potassium sulfate and significantly decreased in genotypes treated with the 1,312 mg/kg of potassium sulfate. Thus, the increased *MRP4* expression in the needles of plants treated with 656 mg/kg of copper sulfate may be caused by the sulfate ions.

MT2b expressions in needles was significantly decreased in plants treated with 656 mg/kg of copper sulfate but were severely repressed in plants treated with the three concentrations of potassium sulfate. Thus, sulfate ions are inhibiting *MT2b* expression in needles, even during copper sulfate treatments.

Expression of *RAN1* was not affected by the copper sulfate treatments in the needles. However, the *RAN1* expression was significantly decreased in plants treated with 1,312 mg/kg of potassium sulfate, indicating that the sulfate ions may be causing *RAN1* inhibition in needles.

MRP4 expression was significantly increased in both the resistant and susceptible genotype needles. *RAN1* and *MT2b* showed no significant changes between the two genotypes (resistant and susceptible) when compared to water.

MRP4 expressions showed no significant differences in needles versus roots, meanwhile almost all treatments of potassium sulfate and copper sulfate increased *MT2b* expression in roots. *MRP4* expression was significantly higher in roots of the copper resistant genotype, and significantly higher in the needles of the susceptible genotype compared to water. The susceptible genotype showed an increased *MT2b* expression in needles compared to roots. *RAN1* gene expression was significantly higher in roots of plants treated with 656 mg/kg and 1,312 mg/kg of potassium sulfate, when compared to the needles. As well, the resistant and susceptible genotypes both had an elevated *RAN1* gene expression in roots, when compared to the needles.

Epigenetic analysis revealed an hypomethylation induced by potassium ions when copper sulfate and potassium sulfate treatments were compared. No difference in global cytosine methylation was observed between the copper sulfate treatment and water.

Future Studies

The current study presents findings of white spruce being resistant to lower concentrations of copper sulfate, and inducing copper resistant mechanisms at higher concentrations of copper. To better understand the connection between a high concentration of copper sulfate and increased gene expression, it would be beneficial to perform a transcriptome analysis on the white spruce seedlings treated with copper sulfate. Field studies would also be beneficial to determine if the plants involved in the regreening program, such as white spruce and other conifer species, have elevated gene expressions due to other factors such as the temperature, soil pH, organic soil matter composition. Since the plants in the current study were shocked with a copper sulfate or potassium sulfate treatment after six-months of growth, it may also be reasonable to analyze *P. glauca* seedlings grown in a copper contaminated soil from day one to analyze the potential mechanisms that allow this species to grow and reproduce in a copper contaminated region.

Since there were variations in gene expressions after plants were treated with potassium sulfate, it may be reasonable to treat plants with another control, such as potassium nitrate, to demonstrate if the changes to the gene expressions are occurring due to the potassium ions or the sulfate ions. This will help us to better understand the changes in gene expression in plants treated with copper sulfate, as we would be able to better describe contrasting effects from the copper ions versus the sulfate ions.

To analyze the differences in metal-tolerant versus the metal-susceptible plants which were treated with 1,312 mg of copper per kg of soil, it would be useful to perform a temporal study to determine if the genes are activated before the plant undergoes apoptosis signaling, and if there is a specific time after the copper sulfate treatment that the genes are activated. It would also be relevant to increase the sample size to limit the effects of any variation within individual plants.

To better understand changes in DNA methylation within copper resistant gene promoters, it would be beneficial to perform DNA bisulfite sequencing PCR to determine if there is a methylation site change in the gene body or promoter region, compared to analyzing the methylation within the entire genome. Once the methylation sites are detected with the bisulfite sequencing, corresponding gene expression analyses from genes within differentially methylated regions could be performed to determine if hypo or hyper-methylation is affecting gene expressions within the copper resistance pathway.

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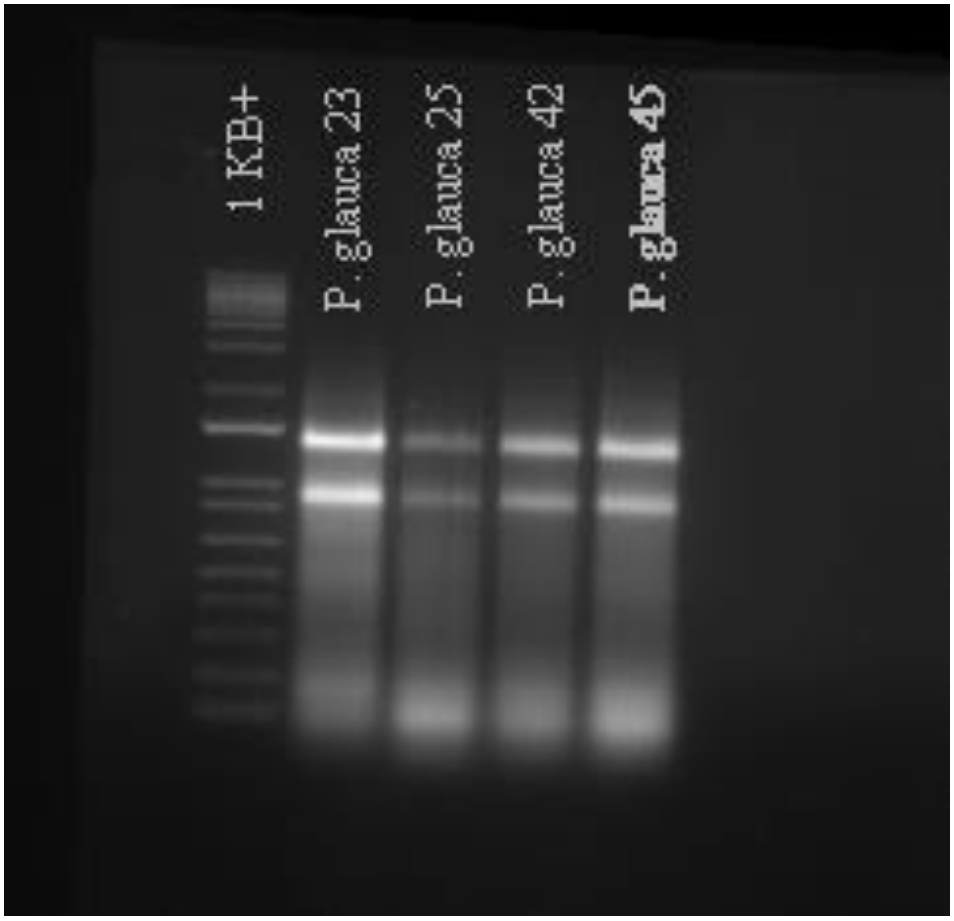
Appendices

Appendix 1. White spruce (*Picea glauca*) in a growth chamber (blue = water, green = 130 mg/kg, yellow = 656 mg/kg, red = 1312 mg/kg)

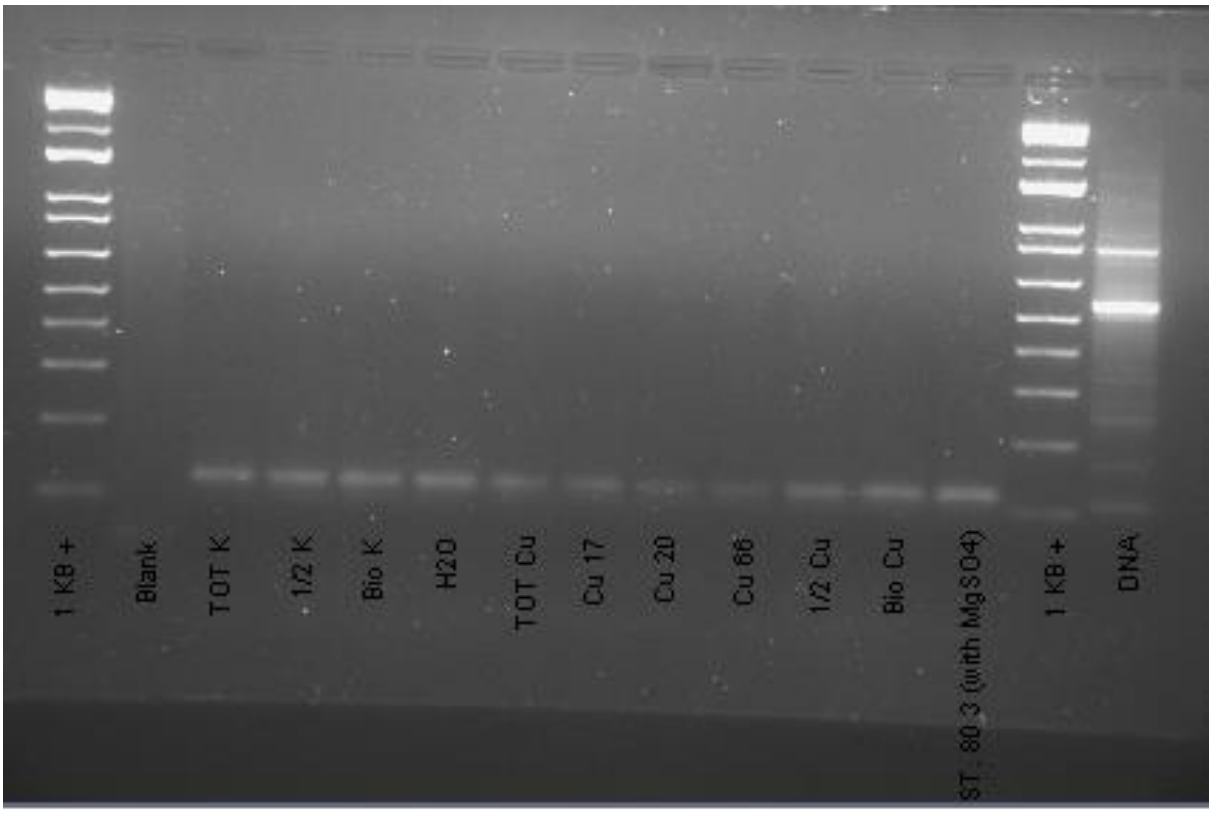
Appendix 2. Plant treatment tools: Using a pipette to treat each plant with 50 mL of the selected treatment (Water, potassium sulfate, or copper sulfate)

Appendix 3. A resistant (Left) and susceptible (Right) white spruce (*Picea glauca*) plant after being treated with 1312 mg of copper per kg of soil

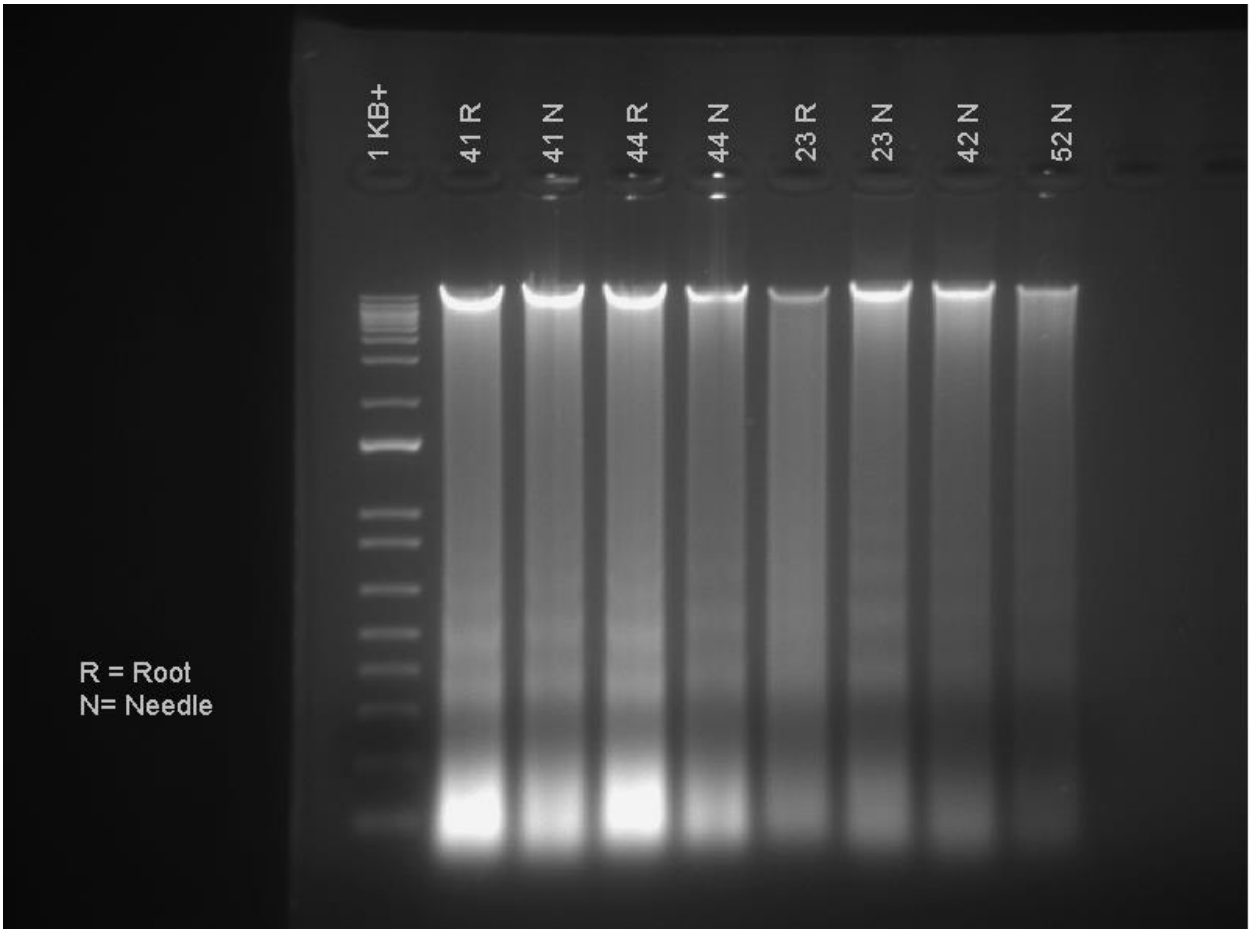
Appendix 4. RNA quality check using gel electrophoresis. Lane 1 contains 1-kb ladder, lane 2-5 contains extracted RNA samples of *Picea glauca*



Appendix 5. cDNA quality check using gel electrophoresis after performing PCR. Lane 1 and 14 contain 1-kb ladder, lane 2 contains a blank control, lanes 3-13 contain cDNA samples after PCR amplification, and lane 15 contains a *Picea glauca* DNA control sample



Appendix 6. DNA quality check using gel electrophoresis. Lane 1 contains 1-kb ladder, lanes 2-9 contain extracted DNA samples of *Picea glauca*



Appendix 7. Colorimetric assay to analyze the percent of global DNA methylation in *Picea glauca*. The plate contains extracted DNA samples, along with the positive and negative controls, and a standard curve