Effects of nickel toxicity on seed germination and expression of genes associated with nickel resistance in *Populus tremuloides*

by

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Abstract:

Although Nickel is an essential nutrient for plant growth in low concentrations, its excessive amounts in soil above threshold values can result in toxicity. The main objectives of the present research were to determine the effects of different doses of nickel on a) trembling aspen (*Populus tremuloides*) seed germination and b) gene expression. This study revealed that nickel in agarose media even at a low dose inhibits seed germination. In soil however, only the highest dose of 1, 600 mg of Ni per 1 kg of soil has detrimental effects on germination of *P. tremuloides* seeds. *P. tremuloides* seedlings were resistant to 150 mg /kg, 400 mg /kg, and 800 mg /kg of Ni doses in growth chamber screening tests. At high Ni dose of 1, 600 mg /kg, differential responses were observed as Ni- resistant, moderately resistant, and susceptible genotypes were identified. Expression of the *AT2G16800* gene was repressed with increasing nickel concentration and this effect was most significant at the 800 mg/kg Ni dose. Surprisingly, the study also revealed that the higher concentration of potassium nitrate without nickel (800 mg/kg and 1, 600 mg/kg) induced a significant upregulation of the *NAS3* and *NRAMP4* genes.

Key Words: Trembling aspen (*Populus tremuloides*); Nickel toxicity; Seed germination; Gene expression; RT-qPCR.

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Chapter 1: Literature review

1.1. Metal toxicity in plants

Metal toxicity in plants has become an increasing problem seen in disturbed metal-contaminated environments due to mining industries, coal-burning, smelting and other anthropogenic activities. These processes often lead to metals like zinc (Zn), cadmium (Cd), copper (Cu), lead (Pb), chromium (Cr), mercury (Hg), and nickel (Ni) leaking into the surrounding air, water and soil and becoming major environmental pollutants (Winterhalder, 1996). Most plants species have developed mechanisms to uptake metals from the environment because small amounts of some metals are crucial for plant metabolism. However, when soil concentrations of metals in the bioavailable form exceed the thresholds levels that plants can tolerate, toxicity symptoms appear. Plants stressed by metal toxicity ultimately have altered metabolism, physiological and biochemical processes and biomass production (Mehes-Smith *et al.*2013). These effects can negatively impact the sustainability and health of the ecosystems in the vicinity, agricultural crops, and water supply.

Typically, metals in the soil are found in trace amounts, and this is normally sufficient for plants that require them for essential redox reactions and as an integral part of some enzymes. For instance, copper (Cu) has an integral role in photosynthetic proteins like plastocyanin in higher plants such as rice and cauliflower (Mahmood and Islam 2006; Chatterjee *et al*. 2000). It is a cofactor for oxidases, mono- and di-oxygenases, and other enzymes that are important for ROS defense (Demirevska-Kepova *et al*. 2004). Copper is also a micronutrient needed in ATP synthesis and CO_2 assimilation (Thomas *et al*. 1998). Despite its essential role in many plant processes, excessive Cu in the soil from industrial and mining activities has been shown to cause many metal toxicity symptoms in plants. Exposure to toxic levels of Cu can cause plant growth

reductions, oxidative stress (Stadtman and Oliver 1991), and disturbances in metabolic pathways (Hegedus *et al.* 2001). When Cu and Cd are present in combination at toxic levels, this can cause further symptoms like inhibited germination and seedling growth such as seen in *Solanum melongena* (Neelima and Reddy 2002).

Zinc (Zn) is also another metal that is an essential component of enzymes like superoxide dismutase and RNA polymerase. It is also essential for ribosome integrity, and is a catalyst for oxidation reactions. Excess levels of Zn and Cd caused phytotoxicity symptoms like oxidative damage, impaired metabolism and decreased growth in green bean (*Phaseolus vulgaris)* (Cakmak and Marshner 1993), and brown mustard plants (*Brassica juncea)* (Prasad *et al*. 1999). The catalytic efficiency may also be affected for the key enzymes that need Zn as a cofactor (Van Assche *et al*. 1998; Somasekharaiah *et al*. 1992; Romero-Puertas *et al*. 2004). Plants that are exposed to high Zn concentrations in soil can develop chlorosis in their leaves (Ebbs and Kochian 1997). This chlorosis effect may be caused in part by an induced Fe-deficiency because hydrated Zn^{2+} and Fe^{2+} ions compete for plant uptake through the roots (Marschner 1986). Manganese, copper, and phosphorus (P) deficiencies have also been observed in some plants exposed to excess Zn, each with their own consequences to plant metabolism (Lee *et al*. 1996; Ebbs and Kochian 1997).

Iron (Fe) is another significant metal that plants cannot survive without, because of its multiple biological roles in several processes. Fe is a major component of heme-containing proteins such as hemoglobin, catalase, peroxidase, and leghemoglobin (Marschner 1995). Fe is also a part of the heme proteins, cytochromes which are essential for photosynthesis, chlorophyll biosynthesis, and chloroplast development (Marschner 1995). Most mineral soils are iron-rich but the majority of this metal is in an insoluble Fe^{3+} or insoluble Fe^{2+} form so plants are not adversely affected.

Excess amount of iron in its soluble form will lead to high Fe^{2+} uptake through the roots into the plant causing toxicity symptoms in species such as tobacco, canola, soybean, and water thyme (Sinha *et al.* 1997). A decrease in photosynthesis and an increase in oxidative stress due to Fe-induced free radical production are also seen in plants stressed by Fe toxicity (Arora *et al.* 2002; de Dorlodot *et al.* 2005; Sinha *et al.* 1997)

Lead (Pb) on the other hand, has no essential function in plants but it will still be absorbed through the roots when it is present in its phytoavailable form in soil water. For centuries, lead has been an important and useful metal in human activities. But the inefficient and pollutive ore mining processes used to extract lead has resulted in surrounding environments becoming contaminated with excess concentrations of this metal. Known to have toxic effects in other organisms like animals, lead can also be highly toxic to plants and may cause serious health problems if it enters the food chain in an ecosystem (Porrut *et al*. 2011). Germination inhibition after exposure to excess lead has been observed in plants such as *Spartiana alterniflora (*Morzck and Funicclli 1982) and *Pinus helipensis* (Nakos 1979). This may be caused by an inhibition of endospermic protease and amylase activities which are essential for mobilizing resources needed for germination as seen in rice seedlings (Mukherji and Maitra 1976). Lead toxicity can also inhibit seedling growth in soya bean (Huang *et al.* 1974), rice (Mukherji and Maitra 1976), maize (Miller *et al*. 1975), and other crops. Morphological abnormalities are commonly seen in many plants (Paivoke 1983) and other physical symptoms like chlorosis can appear (Hewilt 1953). Similar to other metals, excess lead in a plant system can induce oxidative stress (Reddy *et al.* 2005), inhibition of important enzymes in photosynthesis, and altered membrane permeability (Sharma and Dubey 2005).

Soluble proteins levels are often reduced due to metal stress because synthesis rates go down and the hydrolysis and breakdown of proteins may be accelerated. For instance, sugar beet plants exposed to Ni and Cd had a significant decrease in soluble protein content (Kevresan *et al*, 1998). However, in some cases, higher levels of soluble proteins have been reported in species such as rice sedge (*Cyperus difformis)* after exposure to Cd, Ni and Pb. (Ewais 1997). Proteins can be directly damaged by reactive oxygen species arising from metal-induced stress (Baccouch *et al* 1998, 2001; Gajewska *et al*. 2006). This oxidative stress can be further increased as the concentration of low-molecular-weight proteins is depleted (Rao and Sresty 2000; Kukkola *et al.* 2000.). Metals can also bind to side protein groups. SH-groups are common, and disrupt the protein function (Seregin and Kozhevnikova 2006). Furthermore, metal ions can directly damage proteins via oxidation of amino acid residues and free amino acids. The oxidative ability of metals often modifies amino acids like histidine, arginine, lysine, proline, methionine, and cysteine near the metal binding side of a protein. This can impact protein function or unintentionally target some proteins for protease degradation (Roseman and Levine 1987).

Increased protein degradation can result in decreased protein concentrations and an accumulation of free amino acids within plant cells. A species-specific effect has been observed in terms of accumulation of free amino acids as a response to metal stress. This is seen particularly in hyperaccumulator or metal tolerant plants, which may use specific free amino acids as an important detox mechanism (Ahmad and Ashraf, 2011**).** For instance, asparagine has been reported to be an agent of chelating Cd, Pb, Ni, and Zn to form metal-ligand complexes that can immobilize the metal (Homer *et al.* 1997). Bhatia *et al.* (2005) studied the Ni hyperaccumulator species *stackhousia tryonii* and found slight decreases in some amino acid levels such as glycine in plants exposed to Ni. However, concentrations of alanine, asparagine, and glutamine

significantly increased in the xylem in response to the Ni stress suggesting that they may contribute to Ni complexation in these species. Proline is also a prominent amino acid whose levels may increase in response to Ni stress and can potentially be used as a metabolic indicator for Ni tolerant plants. Furthermore, it may act as an osmoprotectant (Paleg *et al.* 1984), membrane stabilizer (Bandurska 2001; Matysik *et al.* 2002), metal chelator (Cobbett 2000), ROS scavenger (Alia *et al.* 2001), and enzyme protector (Sharma and Dietz 2006). Histidine has also been reported as potentially having the highest association to form metal-ligand complexes with Ni (Smith and Martell 1989).

An important part of the defense activated in response to metal stress are proteins and polypeptides (phytochelatins) with N-, O- or S-ligands(Van Assche and Clijsters 1990; Clemens 2001; Vacchina *et al.* 2003; Montarges-Pelletier *et al.* 2008.) which bind Ni to immobilize metals from interfering in other reactions. Some proteins that can aid in complexing and binding Ni are permeases (Wolfram *et al*.1995; Eitginer and Mandrand-Berthelot 2000), metallothioneins (Schor-Fumbarov *et al.* 2005), and metallochaperones (Hausinger 1997; Olson *et al* 1997; Watt and Ludden 1998). These can all mitigate the oxidative stress caused by metal toxicity.

Metal toxicity can also cause genotoxic effects and plants can be damaged at the DNA level. Metal cations that have passed the nuclear membrane into the nucleus can readily interact with the negatively-charged DNA and cause direct conformational changes leading to mutations, DNA strand breaks, rearrangements, and other abnormalities (Eichhorn *et al.* 1985; Duguid *et al.* 1993; Kasprzak 1995). Metals like iron and copper act as catalysts for oxygen reduction reactions which give rise to hydroxyl radicals at a rate higher than a plant's ROS defense can tolerate. ROS can cause direct DNA sequence alterations when they come in contact and remove or add H^+ atoms to the nucleotides or the DNA backbone (Pryor, 1998). Metals such as Cr^{3+} in

complexes can bind to the double helix and can impede access or deform the binding site for transcription factors that need to bind to DNA to initiate gene expression (Raja and Nair 2008). The increase in reactive oxygen species due to metal toxicity is well documented and they can also directly induce DNA damage and mutations (Cunningham 1997). Nickel particularly has been observed in model systems to outcompete magnesium ions for DNA binding and cause chromatin condensation in cells treated with excess Ni. Chromatin that is condensed and hypermethylated in normally coding regions of DNA results in the silencing of essential genes such as anti-oncogenic genes (Lee *et al*. 1995). Plants are not as susceptible to carcinogenicity because plant cells remain contained within the cell wall and cannot proliferate and metastasize as seen in animal systems. However, effects of metal toxicity on plant's genetic material can lead to an increased mutational load, increased DNA damage, and gene silencing which will ultimately negatively affect plant survival and health.

Overall, there is a wide variety of negative effects seen in plants exposed to excess metals. Some toxicity symptoms can vary based on the metal, but overall metal toxicity can inhibit growth, disrupt essential physiological processes, and lead to reduced plant survival.

1.2. Nickel toxicity in plants

Once absorbed by the root system, nickel and its compounds can be transported to the shoots and the leaves via the xylem (Peralta-Videa *et al.* 2002) (Krupa *et al.* 1993). In its bioavailable form, nickel can be translocated within the plant quite easily because it is essential in plant processes. Some proteins can bind nickel and facilitate its transportation (Hausinger 1997; Colpas and Hausinger 2000). Metal-ligand complexes such as nicotianamine/ histidine also play a regulatory role (Vacchina *et al.* 2003; Kim *et al* 2005; Pianeli *et al.* 2005; Haydon and Cobbett 2007). Normally, about 50% of nickel will be retained within the plant roots system (Cataldo *et al.*

1978). Some accumulator species (including *Populus tremuloides*) are hypothesized to retain a greater amount of nickel in the shoots and leaves in response to excess nickel concentrations to prevent its toxic effects from affecting the rest of the plant biomass.

Nickel can be found in fruits and seeds of some plants because it can be translocated via the phloem (McIlveen and Negustanti 1994; Welch 1995; Fismes *et al.* 2005; Page e*t al.* 2006). As a result, some plants may develop coping strategies to limit the effects of nickel on subsequent seed germination. For example, nickel was found partitioned mainly in the pericarp (fruit wall) in seeds of *Stackhousia tryonii* and not in the endospermic and cotyledonary tissues. This suggests that this Ni hyperaccumulator species may have molecular processes that can limit nickel toxicity in the tissues that are essential for healthy germination and plant growth. (Bhatia *et al.* 2003)

Nickel's role as an essential plant nutrient in plant metabolic processes was first reported by Dixon *et al.* (1975). Their work with the Jack-bean showed that nickel is an integral component for proper functioning of the metalloenzyme urease. Since then, about 500 proteins and peptides have been discovered in living systems that are able to bind Ni, including other metalloenzymes. The major enzymes found in organisms that Ni is an integral component of include urease (Klucas *et al.* 1983), superoxide dismutase (Ragsdale 2009), NiFe hydrogenases (Lubitz e*t al*. 2007), methyl coenzyme M reductase (Ragsdale 2003; Jaun and Thaeur 2007), carbon monoxide dehydrogenase (Lindahl and Graham 2007), acetyl coenzyme-A synthase (Doukov *et al.* 2008), other hydrogenases (Küpper and Kroneck 2007), and RNase-A (Ragsdale 2009).

Ni deficiency is uncommonly found in plants because they require only small amounts that are naturally present in most soils. A major consequence of Ni deficiency is the disturbance in urease function which normally catalyzes the reaction of urea into ammonium in the N assimilation

process. Thus, a buildup to toxic levels of urea may result such as seen in Ni-deficient soybean leaf tips may result (Eskew *et al.* 1983). This can lead to plant toxicity symptoms such as chlorosis and necrosis.

Although nickel at low concentrations is a vital micronutrient needed for healthy plant function, many toxic effects can be seen when plants are exposed to excess concentrations of nickel. Some physical effects of nickel toxicity observed in plants include decreased shoot and root growth, branching system not fully formed, deformation of other plant parts such as irregular flower shape, decreased plant biomass, leaf spotting, chlorosis, and foliar necrosis (Nedhi *et al* 1990).

Roots are typically the first plant part to be in direct contact with soil, so this is often where symptoms of Ni toxicity may first appear. Roots will show reductions in growth and increased shape abnormalities (Wong and Bradshaw 1982; Yang *et al.* 1996; Kopittke *et al.* 2007). For instance, white birch (*Betula papyrifera)* and honey suckle (*Lonicera tatarica*) exposed to high Ni concentrations had a reduction in new root hairs forming and deformation of existing ones (Patterson and Olson 1983). Inhibited lateral expansion of root growth due to Ni toxicity has been seen in crops like maize (Seregin *et al.* 2003). It is hypothesized that this Ni-induced effect occurs because Ni can pass through the endodermal barrier with ease and accumulate at toxic concentrations in pericycle cells which are crucial for cell division and proliferation (Seregin *et al.* 2003**;** Seregin and Kozhevnikova 2006).

Plants cells undergoing excessive Ni stress can experience increased permeability of cell membrane due to the damage caused by Ni-induced oxidative stress. Reactive Oxygen Species arise in excess amounts and can degrade the membrane lipids and proteins through lipid peroxidation (Dat *et al.* 2000; Verma and Dubey 2003). Plants can utilize enzymes such as

catalase (*CAT*), peroxidase (*POD*), superoxide dismutase (*SOD*), and glutathione reductase (*GR*) to minimize damage caused by ROS. Non-enzymatic compounds such as antioxidants, ascorbic acid, phenolics, tocopherols, reduced glutathione antioxidants can also be produced in plants to seek out and remove or neutralize ROS (Pandolfini *et al.* 1992; Noctor and Foyer 1998; Alscher *et al.* 2002; Verma and Dubey 2003; Freeman *et al.* 2004; Maheshwari and Dubey 2009). At high levels of nickel exposure these mechanisms often fail to adequately deal with the increase in ROS and plant membrane damage occurs (Howlett and Avery 1997; Zhang *et al.* 2007; Wang *et al.* 2008). Increased membrane permeability can cause electrolyte leakage from cells and a loss of water which decreases cell turgor pressure (Wang *et al.* 2008; Llamas *et al.* 2008). Lipid peroxidation may also occur because some antioxidant enzymes cannot be activated with the appropriate metal cofactor such as Fe (i.e. catalase and peroxidase) (Ranieri *et al.* 2003) when high Ni levels in the soil are outcompeting these other elements for plant uptake. Ni also competes with metals that are needed for membrane stability like Ca and Zn (Valko *et al.* 2005; Taiz and Zeiger 2006).

Nickel toxicity affects photosynthesis and gas exchange processes in many ways resulting in an overall inhibition of photosynthesis (Nedhi *et al.* 1990; Bishnoi *et al.* 1993). A decreased photosynthetic rate (Sheoran *et al.* 1990; Bushnoi *et al.* 1993; Krupa and Baszynski 1995), transpiration rate (Bishnoi *et al.* 1993; Pandey and Sharma 2002), stomatal conductance (Heath *et al.* 1997) and efficiency of water-use (Bishnoi *et al.* 1993) have been observed in plants undergoing nickel stress. These effects are seen regardless if Ni enters the plant system through uptake via roots or through direct application of Ni on isolated guard cell chloroplasts (Tripathey *et al.* 1981; Singh *et al.* 1989; Molas 2002; Boisvert *et al.* 2007).

Chloroplasts are sensitive to Ni toxicity and their structure can be damaged or deformed possibly due to the lipid peroxidation occurring from a Ni-induced increase in oxidative stress (Heath *et al*. 1997; Hermle *et al.* 2007). For instance, Molas (1997) observed alterations in the photosynthetic apparatus such as improperly formed grana and thylakoid membranes, smaller chloroplasts, and changed composition of the lipid membranes in wild cabbage (*Brassica oleracea*) that is exposed to excess Ni. As a result, photosynthesis reactions can be disrupted including the electron transport chain (Singh *et al.* 1989; Tripathy *et al.* 1981) and the Calvin Cycle via enzyme inhibition. The opening and closure of stomata may be affected as well and can lead to a CO_2 deficit which further inhibits photosynthesis (Sheoran *et al.* 1990). The electron transport chain in plants is known to be inhibited by Ni during the light reaction through a similar mechanism as other metals (Mohanty *et al.* 1989; Krupa and Baszynski 1995). This starts in Photosystem II where nickel can cause the reduction of thylakoid membrane pigments including cyt b6-f and b559, ferredoxin and plastocyanin. The dark reactions may also be suppressed by nickel in some way considering there is evidence of other metals inhibiting key enzymes such as rubisco, 3-PGA kinase, F-1,6 biphosphotase, aldolase, and phosphoglyceraldehyde (Sheoran *et al.* 1990**)**. In this case, an imbalance can be created and the accumulation of light reaction products (ATP and NADPH) can ultimately create a pH gradient across the thylakoid membrane that is too high and impedes PSII activity (Krupa and Baszynski 1995).

1.3. Effects of metals on seed germination

Metals are released into the environment over time through natural means such as from volcanoes or weathering of rocks. However, industrial activities have caused an increase of metal concentrations and pollutants to toxic levels in the surrounding environment where they take

place. A large amount of this contamination is present in soil, so plants which are the primary producers at the beginning of the food chain are often the first to be affected. Plants already established in an area that experiences an increase in metals concentration experience many toxic side effects (Nedelkoska and Doran 2000). Some species may develop mechanisms to tolerate excess metals but many cannot survive in these metalliferous habitats. The process of germination and the early establishment of seedlings is sensitive to many factors including temperature (McDonough, 1979), light availability (Mott 1974), water status (Chachalis and Reddy 2000), and soil's mineral composition (Zlesak 2007). Many of these factors can be negatively affected by environmental stressors. For instance, salinity and drought can negatively impact water status and inhibit germination **(**Peralta-Videa *et al.* 2001**;** Houlam and Fares 2001). The mineral composition of soil is also very important for seeds to uptake the correct balance of micronutrients and metal contamination has been shown to have inhibitory effects on germination in many plant species.

A range of metals including Hg, Zn, Cd, Co, Pb and Ni have already been shown to decrease germination rates in lentil, radish, mustard, and rice seeds (Ayaz and Kadioglu 1997; Espen *et al.* 1997; Fargasova 1994; Mishra and Choudhuri 1998; Munzuroglu and Geckil 2002). Furthermore, excess levels of metals can inhibit the early developmental processes that occur in the germinating embryo and are needed to establish a growing seedling. Metals like Cu, Cd, Ni, and Pb have been shown to negatively impact the seedlings height, root establishment, shoot elongation, total plant biomass, and other characteristics of rice and mungbean growth (Zhang *et al.* 2009; Sfaxi-Bousbih *et al.* 2010; Ahmad *et al.* 2007; Pourrot *et al.* 2001).

A study by Munzuroglu and Geckil (2002) measured the effects of Cd, Co, Cu, Pb, Zn, and Mg on germination in wheat (*Triticum aestivum*) and cucumber (*Cucumis sativus*) seeds. They found

significant decreases in germination for all increasing toxic levels of metals applied to the seeds in both species. However, each metal did not inhibit germination at the same rate and the two species were also affected differently by the same metal. For instance, cucumber seeds were completely inhibited from germinating when exposed to 1.1 mM concentrations of Hg, while wheat seeds could tolerate higher Hg concentrations and germination was never completely inhibited. In contrast, the other metals tested showed much higher germination inhibition in wheat seeds than cucumber seeds. This suggests that the ability of a metal in excess amounts to inhibit germination may be species-specific. This could be ascribed in part to seed structure among species. For instance, the seed coat provides the main barrier to prevent metal uptake into the seed reaching the germinating plant embryo. Differences in seed coat have been observed among plant species. This may be a factor that affects the ability of a plant to tolerate excess metals during germination (Weirzbicka and Obidziniska 1998).

There are multiple underlying causes for the inhibitive effects of excess metals on seed germination and seedling growth. First, metal ions can impede the activation of key enzymes that are needed in the metabolism of carbohydrate and protein reserves stored in the endosperm of the seed. A number of enzymes such as α-amylases, β-amylases, acid invertases, and acid phosphatases normally digest the main carbohydrate source starch and activate the mobilization of these molecules to the growing embryonic axis. Metals including Cd, Cu, and Pb (Sfaxi-Bousbih *et al.* 2010; Pena *et al.* 2011; Singh *et al.* 2011) have been shown to inhibit the activities of these enzymes and the lack of resources in the correct location inhibits further seedling growth.

An increase in Reactive Oxygen Species (ROS) that arises from excess metals increases plant stress and often resources within cells must be diverted to upregulate the transcription of ROS

defense enzymes. For example, Pb toxicity has been shown to induce an upregulation in antioxidant enzymes superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase (APX) as a means to combat increased ROS (Wang *et al.* 2010). Cu toxicity can also induce oxidative stress and an upregulation in antioxidant and stress-related proteins including glyoxalase I, peroxiredoxin, and aldose reductase (Zhang *et al.* 2009). Cu toxicity can further disrupt metabolic processes through the upregulation of regulatory proteins like DnaK-type molecular chaperone and Ulp1 proteases in response to the oxidative stress (Zhang *et al.* 2009). Some of these proteins are consistently upregulated in response to a specific metal and they may be considered as markers for metal stress in plants.

One major consequence of oxidative stress is the increase in plant cell membrane permeability. Cell membrane damage is often caused by ROS-induced lipid peroxidation resulting in loss of turgor pressure from water loss and excessive leakage of osmolytes (Singh *et al.* 2011). Photosynthesis can also be affected because of damage to the thylakoid membranes causing obstructions in the electron transport chain. Inhibition of enzymes in other parts of photosynthesis such as the Calvin cycle and alterations to photosynthetic pigments have also been observed in plants experiencing metal stress (Pourrot *et al.* 2011).

Ultimately, metal contamination inhibits seed germination and seedling growth. Seeds and early developing seedlings are very vulnerable to toxic effects caused by an uptake of excess metals from soil. Many alterations can occur throughout genomic, transcriptomic, and proteomic activities in plant cells in response to metal induced oxidative stress, membrane damage, and metabolic irregularities (Sethy and Ghosh 2013). Seeds and seedlings can tolerate these effects but the ability to adequately detoxify metals has not yet been established.

1.4. Gene expression associated with metal contamination

Environmental stressors can cause changes in gene expression at the gene transcriptional level in order for plants to adapt. In particular, metals, heat shock, reactive oxygen species, and drought, have already been implicated in alterations of gene transcription. (Matters and Scandalios 1986).

Changes in gene expression in response to excess metal concentrations are commonly seen in metal tolerant plants like accumulators and hyperaccumulators. Genes that have been identified to possibly play a role in metal tolerance can be species-specific and metal-specific. However, it appears that this group of plants use genes that have a similar function and are commonly involved in metal transport.

For instance, *Thlaspi caerulescens* is well known to be able to hyperaccumulate Cd and Zn (Brown *et al.* 1995a,b) and some ecotypes may be able to accumulate Ni and Co as well (Baker, 1981; Baker and Brooks, 1989; Brown *et al.* 1995b). Kochian *et al.* (2002) found that the *ZNT1* gene which codes for a zinc transporter protein is upregulated in *T.caerulescens* roots. The increase in *ZNT1* expression and in the Zn^{2+} root uptake suggests that this gene is essential for transport and Zn hyperaccumulation in the shoots. *ZNT1* has similarly been shown to be involved in Cd^{2+} uptake as well, suggesting that it is a Zn^{2+}/Cd^{2+} transporter (Lasat *et al.* 1996).

The *ZNT1* gene is closely related to the *ZIP* (*ZRT/IRT*-like Protein) gene family, a group of metal transporter protein genes that can bind and transport metal cations including cadmium, iron, manganese, and zinc (Guerinot 2000). These genes are potential candidates for metal tolerance in hyperaccumulator plants such as seen in *Arabidopsis halleri* (Eng *et al.* 1998; Grotz *et al.* 1999). In fact, Kochian *et al.* (2002) found an increased expression of at least two other metal transporter genes in the *ZIP* family and an increased *NRAMP* expression, another metal transporter family, in *T.caerulescens* compared to the non-accumulating *T.arvense.* The low metal specificity of the *ZIP* and *NRAMP* genes suggests that some homologues may be involved

in Ni-accumulation in a similar way as Zn (Mizuno *et al.* 2005). It is known that plants are not limited to being able to accumulate only one type of metal and a gene that plays a role in tolerance to one metal may provide cross resistance to other metal ions. Considering that *Populus tremuloides* were able to accumulate high concentrations of Zn and Ni in the leaves (Kalubi *et al.* 2015), it is likely that they do so using similar genes and mechanisms.

The most current literature review considered both model and non-model plants and identified 11 genes associated with nickel resistance. These genes include 1-aminocyclopropane-1-carboxylic acid deaminase (*ACC*), high affinity nickel transporter family protein (*AT2G16800*), iron-regulated protein (*IREG*), glutathione reductase (*GR*), glutathione-s-transferase, Metal transporter (*NRAMP 1,2,3,4*), Nicotianamine synthase (*NAS3*), Putative transmembrane protein (*TMP*), Serine acetyltransferase (*SAT*), Thioredoxin family protein, Zn finger protein of *Arabidopsis thaliana* (*ZAT11*), and *MRP4* (Freeman *et al*. 2004; Lemaire *et al*. 2004; Stearns *et al.* 2005; Mizuno *et al*. 2005; Schaaf *et al*. 2006; Mari *et al.* 2006; Visioli *et al*. 2012; Liu *et al*. 2014; Theriault *et al.* 2016).

The *NRAMP* (Natural resistance associated macrophage protein) transporters is a family of genes whose main function is to bind and transport divalent metal ions. This is a highly conserved gene family during evolution and homologues have been identified in a large range of organisms including bacteria, yeast, mammals, and higher plants (Williams *et al.* 2000). The metal ions binding is dependent on the species and the protein. Some divalent cations that can be bound by *NRAMP* metal ion transporters include Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} (Supek *et al.* 1997; Liu *et al.* 1997; Chen *et al.* 1999, Nevo *et al.* 2004). Mizuno *et al.* (1990) were the first to identify the association between *NRAMP4* and Ni in a yeast study. They found that expression of *NRAMP4* in yeast cells exposed to nickel increased Ni^{2+} sensitivity and Ni^{2+} concentration in

cells. In plants, Oomen *et al.* (2008) found that the metal hyperaccumulator *Thlaspi caerulescens* highly expressed *NRAMP3* and *NRAMP4* which can bind Fe, Mn, Cd and for both Zn for *NRAMP4*. Theriault *et al* (2016) further investigated the *NRAMP* genes implicated in Ni transport in white birch (*Betula papyrifera*), a Ni-accumulator species. They found that the plants that showed resistance to excess nickel may do so partially via the downregulation of genes associated with binding and transporting activity like *NRAMP1-2*.

The Nicotianamine synthases genes (*NAS1, 3* and *4*) are a group of enzymes that synthesize the metal chelator nicotianamine (NA). This nonproteinogenic amino acid can bind to some transition metals like Fe, Cu, Zn, Mn, and Ni and immobilize them for transport. NA has already been implicated in transporting Fe, Cu, and Zn for long distances within some plant species (Wintz *et al.* 2003). An elevation in *NAS* gene expression has been observed in hyperaccumulator plants *Arabidopsis halleri* and *Noccaea caerulescens* (Deinlein *et al.* 2012; Visioli *et al.* 2014). It appears that an increase in nicotianamine contributes to metal tolerance in hyperaccumulator plants via metal chelation. This facilitates metal translocation (Weber *et al*. 2004; Deinlein *et al*. 2012). In contrast, *NAS* genes seem to play a role in metal homeostasis (i.e. Fe, Cu, Zn and Mn) only in non-accumulating plants such as *Arabidopsis thaliana* (Curie *et al.* 2009).

The ATP Binding Cassette (*ABC*) transporters is a superfamily of genes that is represented in a wide range of organisms from archaea to humans (Higgins, 1992). The existence of some of these transporters in plants was first discovered in *Arabidopsis thaliana* with the identification of a multidrug resistance (*MDR*)-like gene (At*PGP1*) (Dudler and Hertig 1992). Since then, other *MDR* homologues have been found in *A. thaliana.* These membrane-associated proteins are Mg^{2+} ATPases and act as membrane pumps to mitigate the transport of molecules across extra- and

intra-cellular membranes (Rea 1999). The *MRPs* are glutathione-S conjugate pumps typically in vacuolar membranes and are important for the detoxification of harmful compounds within the cell and for metal vacuolar sequestration (Martinoia *et al.* 2002). Altered gene expression of *ABC* transporters genes has been observed in plants species exposed to different metal contamination. For example, Keinäen *et al.* (2007) found an increased expression of an *ABC* transporter homologue *MRP4*, and *DnaJ*, a vacuolar sorting receptor-like protein, in Cu-tolerant birch (*Betula pendula)* compared to Cu-susceptible birch. Zientara *et al.* (2008) observed an increased expression of the At*MRP3* gene transformed into *Arabidopsis thaliana* when seedlings were exposed to excess concentrations of Cd, Ni, As, Co and Pb but not to Zn and Fe. Thus, some members of this gene family are thought to facilitate metal tolerance via metal transport into sequestered areas like vacuoles.

Metal stress can affect the expression and activity of important antioxidant enzymes needed to deal with Reactive Oxygen Species. For instance, though nickel does not directly generate ROS because it is a redox-inactive metal (Boominathan and Doran, 2002), it has been reported to stimulate antioxidant enzymes such as superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*), and glutathione s-transferase **(**Gabbrielli *et al.* 1999**;** Rao and Sresty 2000**;** Baccouch *et al.* 2001**;** Gajewska and Skłodowka 2008**)** in plants. The activities of these genes appeared to be reduced based on a transcriptome analysis of white birch (*Betula papyrifera*). This result is possibly due to enzyme inactivation from direct binding of Ni^{2+} to a -SH group or histidine, Ni displacement of other metals in the active binding site, or indirectly (Theriault *et al.* 2016).

Kalubi *et al.* (2016) investigated how gene expression is affected by metal contamination in one Ni accumulator tree species, trembling aspen (*Populus tremuloides)* and one tree species [red

maple (*Acer rubrum*)] that deals with excess Ni using the avoidance strategy. The transcript level was measured for target genes associated with nickel resistance including *NRAMP4*, *NAS3*, *AT2G16800* and *MRP4*. They found that *P. tremuloides* samples from both contaminated (Kelly Lake) and uncontaminated (St.Charles) areas in the Greater Sudbury Region had significantly higher gene expression than found in the *A. rubrum* samples. Expression of *AT2G16800*, and *MRP4* was significantly increased in the *P. tremuloides* samples from the contaminated site compared to the uncontaminated. *NAS3* had a high upregulation in *P. tremuloides* for both sites compared to *A. rubrum* samples. Finally, *NRAMP4* expression was also higher in *P. tremuloides* samples from both sites compared to *A. rubrum* but this was the smallest difference. Though the downregulation of the *AT2G16800* and *MRP4* genes in *A. rubrum* samples from a metal-contaminated site was apparent, the direct environmental factors could not be established yet. It is hypothesized that *P. tremuloides* may be more sensitive to the abiotic stressors in the contaminated site which may trigger the change in the gene expression of these stress related genes to tolerate the excess nickel.

A gene expression study on northern red oak (*Quercus rubra)* conducted by Djeukam *et al.* (2017) found that other genes associated with metal-stress including ACC, SAT, and *NAS3* could be upregulated in response to high doses of nickel nitrate (800 mg/kg, 1,600 mg/kg). The red oak is another important species for the GSR because it is classified as a Ni/Zn accumulator and has the ability to store these metals in its leaves at high concentrations (Tran *et al.* 2014).

Overall, changes in gene expression induced by metal toxicity have been observed in many plants. It is likely that genes associated with metal resistance mechanisms in tree species such as *P. tremuloides* and *Q. rubra* will be identified in near future.

1.5. Species of Interest: trembling aspen

Trembling aspen (*Populus tremuloides)* is a fast growing species and it is the most widely distributed tree in North America. It belongs to the Populus *genus* within the Salicaceae family and composed of 40 species of poplar, cottonwood, aspen and hybrids (Ye *et al*. 2011). Many members of this genus, including *Populus tremuloides*, are commonly used in the forestry industry, particularly for pulp products like books and newsprint. Some species have been proposed as potential low-cost and low-maintenance bioenergy crops that could be used to produce biofuels (Ye *et al*. 2011). Trembling aspen also has an ecologically important role in forest ecosystems because it is a foundation species (Rai *et al.* 2013). Furthermore, it is considered as a good model plant species for adaptive responses to climate change because it already thrives across many climatic zones with high adaptability (Rai *et al.* 2013).

P. tremuloides is a good candidate for land reclamation and phytoremediation for numerous reasons. It is dioecious; meaning male and female reproductive organs develop in separate individuals (Cox, 1988). Though it can sexually reproduce, the main mode of propagation is through root suckering. This is favorable for the species in disturbed soil environments because once one tree is successfully established, it can go on to propagate numerous clones through root suckering and populate an area (Elliott and Baker 2004).

Furthermore, there is evidence that *P. tremuloides* is a metal accumulator species. This means it can translocate excess metals away from the roots and store them in the shoots or leaves at high concentrations. Kalubi *et al.* (2015) found that nickel (Ni) and zinc (Zn) accumulated in the leaf tissues as opposed to the roots in *Populus tremuloides* samples growing in Northern Ontario. This means that this species can be useful in removing contaminants from soils, sediments and water, through the biotransformation of metals into their inert forms and storing them at high concentrations in the plant leaves (Kalubi *et al.* 2015). The large biomass production of

trembling aspen is also beneficial because larger sized trees have more area to store excess metals so they may be able to tolerate more heavily contaminated soils. Finally, the extensive root systems of *P.tremuloides* may enable them to survive in disturbed soils because their roots can reach deeper volumes of the soil where there is less contamination (Radwanski *et al*. 2017). Overall, *Populus tremuloides* is a hardy and adaptable tree species that is important for the Greater Sudbury Region and across the continent North America.

1.6. Rationale and objectives

Seed germination is one of the most sensitive processes to metal contamination in plants. Without a sufficient rate of seed germinations, the ability of plant species to naturally establish themselves in a contaminated environment through seed dispersal will be significantly impeded. This study will help assess how best to use *P. tremuloides* for bioremediation efforts. Practically, knowledge of seed germination in a species such as *P. tremuloides* under metal contamination will be useful in assessing long term sustainability of populations of this species in a mining region such as the City of Greater Sudbury with a long history of soil metal contamination.

Moreover, a number of studies have reported the regulation of metal transport genes and other genes in the presence of metals in a number of plant species. Gene expression in trembling aspen (*P. tremuloides*) in the presence of metals is lacking. Likewise, there is no study that investigated metals dosage effects on seed germination and gene expression.

The main objectives of the present research are to determine the effects of different doses of nickel on a) *P. tremuloides* seed germination and b) gene expression under controlled conditions.

Chapter 2: Effect of nickel treatments on trembling aspen (*Populus tremuloides*) seed germination and plant growth.

2.1. Introduction

Nickel is an essential micronutrient for plant growth so it is readily absorbed through plant roots but there are negative consequences when it is present in toxic concentrations. Excess Ni often competes with other necessary micronutrients for uptake into plant tissues from soil. As a result, nutrient deficiencies may arise within seeds. These nutrients are important cofactors and enzyme activators involved in the metabolic processes/events needed to ensure successful germination and seedling growth. Nutrient deficiencies result in imbalances and suppression of those metabolic processes, thus inhibiting plant growth (Valko *et al.* 2005; Taiz and Zeiger 2006).

Exposure to high Ni levels can alter the protease and RNAse activity resulting in altered RNA and protein levels during seed germination (Kirchgessner and Schnegg 1979; Booker 2004). For instance, toxic Ni exposure caused the most reduced protease activity in sunflower plants (Pena *et al.* 2006). Changes in these enzymatic activities may result in the lack of key biomolecules at the site of the growing embryonic axis due to the delay in the mobilization of endospermic energy reserves (Maheshwari and Dubey 2007, 2008). The disruption of these events by excess Ni leads to reduced germination (Maheshwari and Dubey 2007, 2008).

Studies on effects of nickel on *P. tremuloides* seed germination and plant growth is lacking. The main objectives of this study is to assess the effects of different doses of nickel nitrate on *P. tremuloides* seed germination. Toxicity of nickel on plant growth was also investigated.

2.2. Materials and Methods:

2.2.1. Germination of *P. tremuloides* seeds in nickel-treated media.

Populus tremuloides seeds were provided by the Canadian Forest Services seed bank in Fredericton. These seeds were collected in Woodstock, NB (seedlot# 20061003.0) and stored at 4°C. To assess the effect of Ni on seed germination in media, four doses of commercial nickel nitrate ($Ni(NO_3)_2$) salts were tested including 4.28 mg Ni/ per mL, 2.14 mg/mL, 1.07 mg/mL, and 0.401 mg/mL of media solution. These doses correspond to the nickel concentrations used in the assays on seed germination in soil (1,600 mg of Ni per 1kg of dry soil, 800 mg/kg, 400 mg/kg and 150 mg/kg respectively). Commercial potassium nitrate (KNO_3) salts were used to control for each concentration of nitrates present in the nickel dosages. The 4.28 mg/mL (588.72 μmol of Ni), 2.14 mg/mL (294.36 μmol of Ni), 1.07 mg/mL (147.18 μmol of Ni) and 0.401 mg/mL (55.20 μmol of Ni) contained 1177.44 μmol, 588.72 μmol, 294.36 μmol, 110.4 μmol of nitrate, respectively. The salts for each treatment were dissolved in 1/4X media (Murashige and Skoog basal salt mixture) with 1% agar added. Jars containing each treatment were autoclaved and agar media solidified before adding approximately 20-25 disinfected *P. tremuloides* seeds to each jar. Each treatment was conducted in triplicate. Finally, sterile water was added and then the jars were covered with lids and kept under controlled settings in a growth chamber over a few weeks. The growing conditions were 18 h of light at 26°C followed by 6 h of darkness at 18°C. The percentage of germinated seeds were recorded for each treatment.

2.2.2. Germination of *P. tremuloides* seeds in nickel-treated soil.

To assess the effect of Ni on seed germination in soil, four doses of Ni were tested including 1,600 mg/kg (1,600 mg of Ni per 1 kg of dry soil), 800 mg/kg, 400 mg/kg and 150 mg/kg. The

dosage of 1,600 mg/kg represents the level of total nickel found in contaminated sites in the GSR (Greater Sudbury Region) as described by Kalubi *et al*. (2016)**.** The 150 mg/kg dosage correspoinds to the bioavailable amount of Ni from the total dose found in contaminated sites (Kalubi *et al*. 2016**)** Commercial nickel nitrate (Ni$(NO_3)_2$) salts were dissolved in distilled water and added to each replicate pot. Commercial potassium nitrate (KNO_3) salts were used to control for each concentration of nitrates administered. The 1,600 mg/kg (301.69 μmol of Ni), 800 mg/kg (150.85 μmol of Ni), 400 mg/kg (75.42 μmol of Ni), and 150 mg/kg (28.27 μmol of Ni) dosages contained 603.38 μmol, 301.69 μmol, 150.85 μmol, and 56.54 μmol of nitrate, respectively. Distilled water was used as the negative control. Two seeds were planted in each pot in a 50:50 mix of sand and soil. Nine rows of twenty pots each were kept under controlled settings in a growth chamber. The growing conditions were 18 h of light at 26°C followed by 6 h of darkness at 18°C. Seeds were watered as needed. Every two days, the numbers of germinated seeds were recorded.

2.2.3. Germination of *P. tremuloides* seeds in metal contaminated soil from a local site.

Two soil samples were collected from a metal contaminated area on the Laurentian university campus. The total average nickel dose in these samples were 1, 600 mg Ni per kg dry soil (Kalubi *et al*. 2016). Samples were transferred to small pots and about 10 *P. tremuloides* seeds were planted in pot soil. The pots were watered as needed and observations of seed germination were recorded over six days.

2.2.4. Assessment of nickel toxicity on trembling aspen (*P. tremuloides*) seedlings.

The same seedlot (# 20001001) described in section 2.2.1. was used for this study. Seeds were germinated in a Petawawa germination boxes and seedlings were grown in a deep tray with soil. Four month-old seedlings were transplanted into pots containing a 50:50 sand/soil mixture and left to grow for an additional month and a half in a growth chamber. Plants were watered as needed and fertilized twice a week with equal amounts of nitrogen, phosphorus and potassium (20-20-20**)**.

Ni toxicity was assessed by treating seedlings with an aqueous solution of nickel nitrate salt [$Ni(NO_3)_2$] at the following concentrations: 150 mg, 400 mg, 800 mg, and 1,600 mg of nickel per 1 kg of dry soil. These doses represent the bioavailable fraction of total nickel available to biota, quarter total, half total, and total nickel, respectively found in metal-contaminated soils in the GSR. (Nkongolo *et al*. 2013; Kalubi *et al.* 2016). These levels correspond to 301.69 μmol, 150.85 μmol, 75.42 μmol, and 56.54 μmol of Ni, respectively. To control for any possible toxic effects due to the increase in nitrate ions (NO_3) in the plants, an aqueous solution of commercial potassium nitrate (KNO_3) salts was used for controls in equal molar amounts to each dose of the nickel salts. The nitrate controls for 1,600 mg/kg, 800 mg/kg, 400 mg/kg and 150 mg/kg corresponds to 603.38 μmol, 301.69 μmol, 150.85 μmol and 113.08 μmol of nitrate respectively. Salt-free water was used as a negative control (0 mg Ni per 1 kg of dry soil). The experimental design was a completely randomized block design with 12 replications per each nickel treatment, 11 for the water control, and 5 per nitrate control.

After treatment, damages to plants were assessed every two days based on a damage rating scale of 1 to 9, 1 representing no visible toxicity symptoms and 9 dead plants as described in (Theriault *et al.* 2016a). Plants were rated individually and a genotype with a score of 1 to 3 was

considered nickel resistant, 4 to 6, moderately resistant/susceptible, and 7 to 9 susceptible. Plant height was recorded on the day of treatment and on the day 7 after treatment before harvest. On this day 7, roots and leaves were harvested from seedlings, frozen in liquid nitrogen and stored at -20°C. Total RNA was extracted from these samples and was used later for the gene expression analysis discussed in Chapter 3.

2.2.5. Statistical analysis

Kruskal-Wallis and one way ANOVA **(**Post-hoc Dunnett's T3) statistical tests were performed using SPSS 20 for Windows to determine significant differences among means ($p \leq 0.05$) for seed germination, and plant growth.

2.3. Results:

2.3.1. Germination of *P. tremuloides* seeds in nickel-treated media.

No germination was observed in any of the nickel treatments for the media experiment after six days (Figure 1a). All four concentrations of nickel nitrates dissolved in the media from 0.401mg Ni per mL of media to 4.28 mg/mL, inhibited completely seed germination. However, germination and seedling development was observed at about an equal percentage (fifty percent of seeds germinating) in all the remaining treatments consisting of the media used as negative control, and the four nitrate control doses (Figure 1b). These results are summarized in Table 1.

2.3.2. Germination of *P. tremuloides* seeds in nickel-treated soil.

Almost all the seeds planted in soil treated with water and in the four nitrate control germinated by the experiment's end (Figure 2). Over the course of the germination test, there was a decrease

in germination as nickel concentration in the soil was increased (Figure 3). The highest potassium nitrate dose of 1,600 mg/kg resulted in the lowest germination rate, but by day 8 more than 50% of seeds germinated. Seeds successfully germinated at a high rate in the soil treated with the lowest nickel dose, but this trend did not continue. A small amount of seeds germinated in the 400 mg/kg Ni dose, but these seedlings were unhealthy/deformed and showed limited development. No seed germination was observed at the higher dose of nickel (800 mg/kg or 1,600 mg/kg). Figure 4 shows the average amount of germination for each treatment on the final day (day 8). Significant differences in the average number of seeds germinated per pot were found among the three highest nickel concentrations of 400 mg/kg, 800 mg/kg and 1,600 mg/kg and the six remaining treatments.

2.3.3. Germination of *P. tremuloides* seeds in metal contaminated soil from a local site.

All *P. tremuloides* seeds successfully germinated in soil samples obtained from two local sites with known metal contamination (Figure 5). Ni or any other reported metal in these soils did not inhibit seed germination.

2.3.4. Assessment of nickel toxicity on *P. tremuloides* seedlings.

Significant differences were observed among the average damage ratings on day 6 after the treatments. Table 2 summarizes the damage ratings over time for plants treated with nickel. No significant damage was observed for the 150 mg/kg (bioavailable amount of Ni), 400 mg/kg, and 800 mg/kg doses. At the 1,600 mg/kg dose, significant damage was observed over time and some plants were dead or nearly dead by the end of the experiment. This treatment had the highest

variation in plant reactions since after day 8 there were 3 resistant, 2 moderately susceptible, and 7 susceptible plants.

a) 

b) 

Figure 1: Trembling aspen (*Populus tremuloides)* seeds germination in 1% agar media treated with a) different doses of nickel nitrates and b**)** either an aqueous solution of potassium nitrates or water.

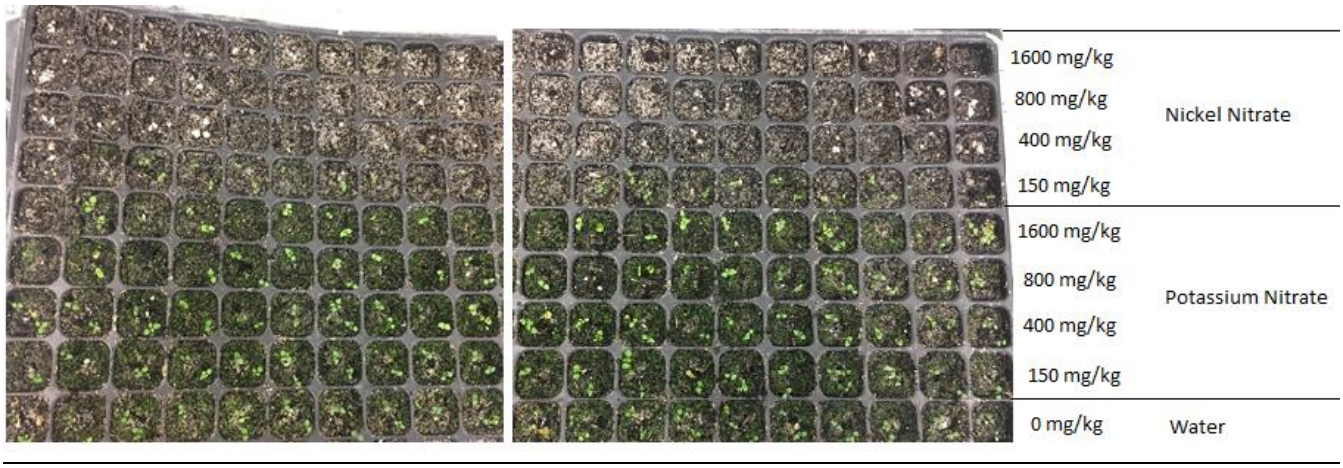


Figure 2. *Populus tremuloides* seed germination in soil samples treated with different doses of nickel nitrate and potassium nitrate (2 seeds per pot).

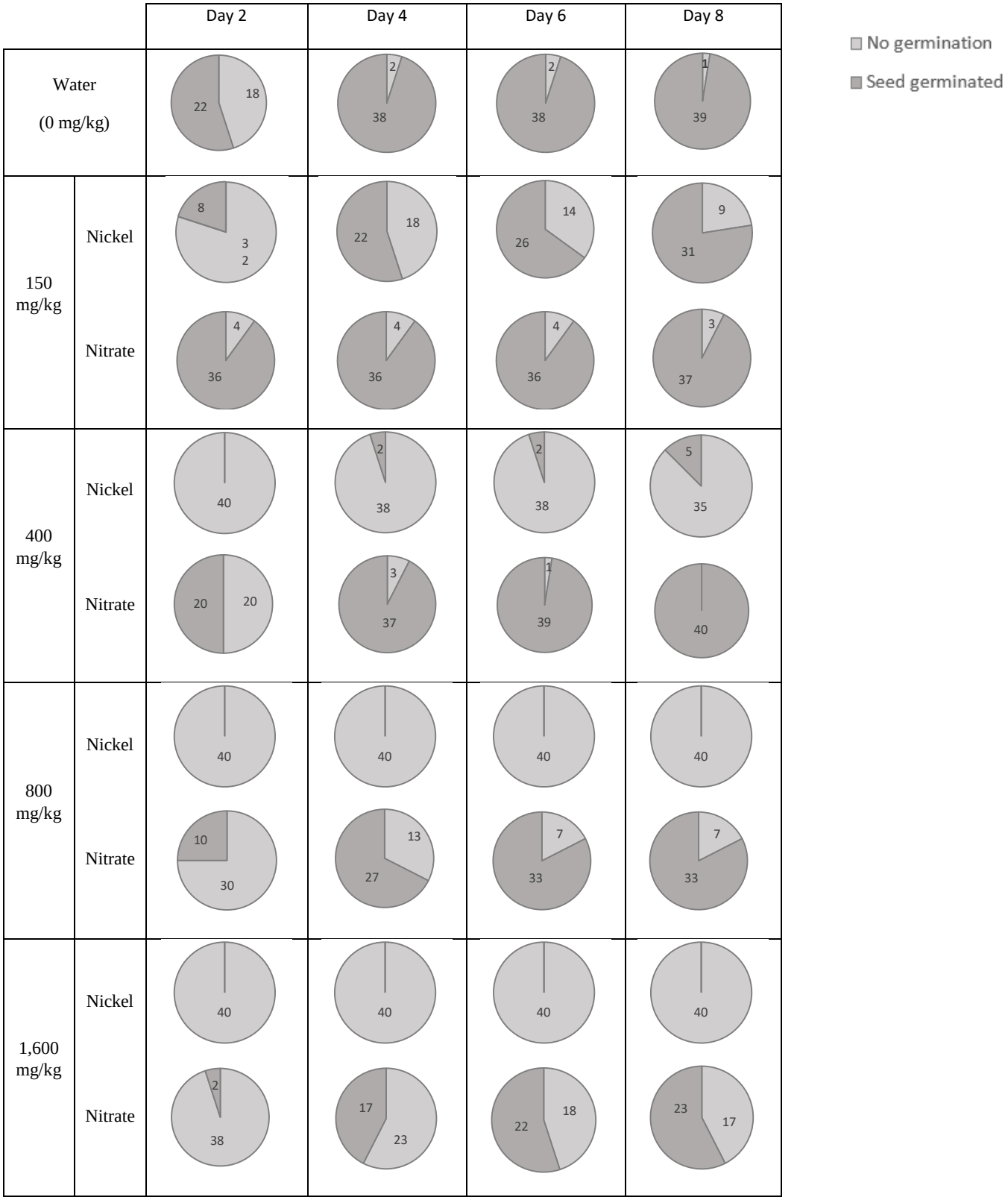


Figure 3: Germination of Trembling Aspen (*Populus tremuloides*) seeds in soil after treatment with varying concentrations of a nickel dose, the corresponding nitrate control dose, and water. The number of seeds that germinated and began to develop cotyledon was recorded every two days after soil treatment. (n = 40 per treatment)

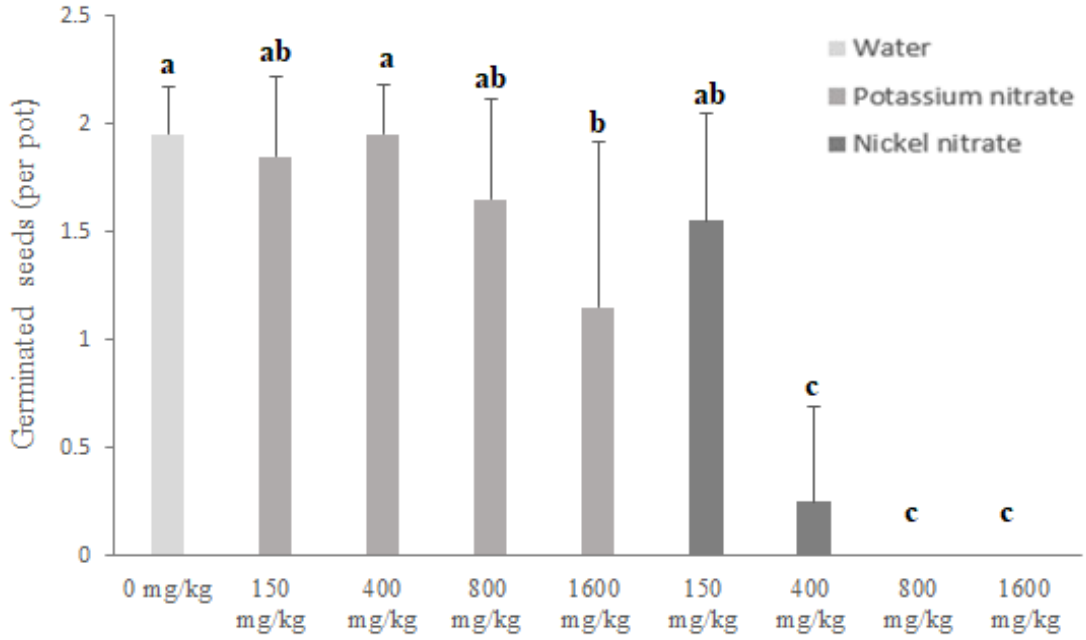


Figure 4: Germination of Trembling Aspen (*Populus tremuloides*) seeds in soil after treatment with varying concentrations of nickel, the corresponding nitrate control dose and water. Eight days after treatment the number of seeds that germinated and began cotyledon development was recorded. Kruskal-Wallis test was performed to determine difference among treatments ($p<0.05$). Bars with different indices represent significant differences among means($p \leq 0.05$) from ANOVA-one way test (Dunnett's T3 post-hoc) .

Figure 5: *Populus tremuloides* seed germination in soil from Laurentian University site with high levels of total metal content. Most seeds germinated after 8 days.

Table 1: Percentage of *Populus tremuloides* seeds that germinated after 7 days in media treated with different doses of nickel nitrate and potassium nitrate (three triplicates per treatment).

Treatment	**Concentration (mg/mL)**	**Total # of seeds**	**# germinated**	**% of seeds germinated**
Media control	0	74	38	51.4
Nitrate	0.401	67	46	68.7
	1.07	70	38	54.3
	2.14	71	33	46.5
	4.28	70	35	50.0
Nickel	0.401	68	0	0.0
	1.07	67	0	0.0
	2.14	71	0	0.0
	4.28	66	0	0.0

Table 2: Damage rating of *Populus Tremuloides* treated with different nickel nitrate and potassium nitrate doses

Treatment	**Concentration (mg/kg)**	**Damage rating**		
		Days after treatment		
		Day 2	Day 4	Day 6
Water control	0	1	1	1**a**
Nitrate	150	1	1	1**a**
	400	1	1	1**a**
	800	1	1	1.5 ± 0.71**ab**
	1,600	1	1	1**a**
Nickel	150	1.11 ± 0.33	1.11 ± 0.33	1.11 ± 0.33**ab**
	400	1.11 ± 0.33	1.44 ± 0.53	1.44 ± 0.53**ab**
	800	1.22 ± 0.44	1.56 ± 0.68	1.89 ± 0.60**b**
	1,600	3.08 ± 2.25	5.75 ± 2.75	6.83 ± 2.32**c**

*Kruskal-Wallis test performed to determine if a difference exists across treatments on day 6 ($p<0.05$). Average ratings with different indices represent significant differences among means ($p \leq 0.05$) based on ANOVA-one way test (Post hoc Dunnett's T3) ($p < 0.05$ n= 49).

2.4. Discussion

Effects of nickel on *P. tremuloides* seed germination

Evidence of metal toxicity causing inhibition in germination has been well documented for numerous metals and across many plant species. The concentrations at which an excess metal will induce this toxic effect varies with metal and the plant species tested. The purpose of this research was to determine the potential effect of Ni on *P. tremuloides* seed germination at different doses.

The results of the present study showed that nickel nitrate inhibited completely seed germination. In contrast, nitrate controls without nickel showed a similar germination rate as the water control. This suggests that the germination inhibition seen in the nickel nitrate treatments is likely due to a nickel effect.

Unexpectedly, no seeds germinated when treated with the lowest dose of 0.401 mg Ni per mL corresponding to the bioavailable amount of Ni (150 Ni mg per kg of dry soil) found in local contaminated soils in the GSR. Ni is most bioavailable and ready for root uptake when it is in an aqueous form dissolved in a neutral salt solution. In this study, the bioavailable Ni added to the media may have all been in direct contact with the seeds and was readily absorbed, increasing the seeds' sensitivity to this lower level of Ni concentration. However, when the same concentration of nickel (150 mg Ni per 1kg of dry soil) was added to soil samples, almost all of the seeds germinated. This suggests that added nickel was not completely absorbed by seeds through their membranes and some Ni ions remained attached to soil colloids or matrix (Kim *et al.* 2015). This would limit the direct exposure of seeds to Ni.

The partial inhibition of germination seen at the 400 mg/kg dose and the complete inhibition observed at the 800 mg/kg and 1,600 mg/kg concentrations suggests that the tested seeds are very sensitive to levels of nickel over the bioavailable concentration (150 mg /kg). The small amount of seeds that germinated at the 400 mg/kg concentration resulted in deformed seedlings with impeded growth suggesting that these seedlings would likely not survive for a long time. The toxic effects of nickel on germination have been seen for many plant species in the literature and many are similar to the toxicity observed in *P. tremuloides*. For example, nickel interferes and inhibits metabolic activities of alpha and beta amylases and proteases which are needed for digesting and mobilizing the endospermic reserves to the growing embryonic axis. Nutrient deficiencies due to nickel outcompeting other metals for root uptake, and changes in RNA and protein levels all can contribute to the inhibition of germination by excess nickel.

The two soils samples used in this study were collected from a metal- contaminated area (Laurentian University site) with an average concentration of 1,600 mg/kg total nickel in soil and the bioavailable portion of Ni was 150 mg/kg (Kalubi *et al.* 2016). All the seeds planted in these soils germinated resulting to healthy seedlings. This suggests that the bioavailable portion of nickel in the soils from these contaminated areas with a high total nickel concentration is low enough that seeds can still tolerate it and germinate. Thus, some *P. tremuloides* trees may still be able to survive and germinate in high nickel concentrations because most nickel present is not bioavailable.

Effect of nickel toxicity to trembling aspen seedlings

The six-month old *P. tremuloides* seedlings showed a much higher tolerance to nickel than seeds. Table 2 shows that no damage occurred to seedlings that were treated with the nitrate solution controls at low and high doses or with water suggesting that excess nitrate is not impacting plant

health. Furthermore, the significant increase in plant damage seen as physical symptoms of Ni toxicity for plants treated with the highest nickel dose of 1, 600 mg/kg is likely due to the excess of nickel and not nitrate because the corresponding nitrate control dose did not cause any significant damage. It appears that 1,600 mg/kg is the threshold for nickel concentration in soil where nickel toxicity symptoms begin to arise in the trembling aspen. Slight changes in damage appeared when Ni doses was increased from 150 mg/kg to 800 mg/kg.

The 1,600 mg/kg nickel treatment had the widest range of damage amongst the samples tested. In addition to two moderately susceptible, and seven susceptible plants, three of the genotypes were classified as resistant because they had a damage rating of 1 or 3 at the end of the experiment. This supports the hypothesis that high genetic variability exists within *P. tremuloides* trees from metal uncontaminated or contaminated areas (Hamrick *et al.* 1979; Kirkey *et al.* 2012). This type of variability was seen in a similar analysis of *Betula papyrifera* populations. This species is widespread in Northern Ontario and can survive in metal contaminated areas in the GSR. Like *Populus tremuloides*, this species has been found to accumulate nickel at high concentrations in the aerial parts of the plant (Kalubi *et al.* 2015; Theriault *et al.* 2015).

It should be pointed out that an inhibition of seed germination and a decrease in seedling growth have been observed in many plant species exposed to high concentrations of nickel (Espen *et al.* 1997; Leon *et al.* 2005). Overall, changes in the levels of proteins, sugars, amino acids, and nucleotides in the seed as a response to toxic levels of metals results in failure to germinate. Nickel toxicity can also severely impede seed germination and seedling growth via the inhibition of alpha and beta amylases, and proteases (Bishnoi *et al.* 1993a; Lin and Kao 2006; Maheshwari and Dubey 2007). Upon seed imbibition (uptake of water necessary to initiate germination),

alpha and beta amylases (starch digestion) and proteases (protein digestion) are activated. These enzymes are responsible for the digestion of food reserves stored in the endosperm and subsequent mobilization to the cotyledons. The formation of the embryonic axis and cotyledon is a critical step in plant establishment and without enough energy reserves at the site of growth, seedling development is inhibited. Other enzymes such as starch phosphorylase and invertase (acid and alkaline) can also be inhibited due to nickel toxicity effects. Without this enzymatic activity, the breakdown of starch carbohydrates in the endosperm is impeded and sugar levels are found to be decreased (Bishnoi *et al.* 1993a; Yang *et al.* 2001; Van den Ende *et al.* 2002).

The translocation of broken down starch reserves to the cotyledon is also reliant on proteolytic and ribonucleolytic enzymes that are activated during germination to regulate RNA turnover (Gomes-Filho *et al.* 1999). These enzymes are also important in regulating protein turnover through protein degradation and recycling (Palma *et al*. 2002). For instance, protease degrades the protein stored in the endosperm to mobilize those amino acids needed in forming the embryonic axis (Yamauchi 2003). Ni may have a toxic effect on these enzymes required for the metabolic processes in germinating seeds.

In conclusion, it is apparent that the germination of *P. tremuloides* seeds can be significantly inhibited when exposed to Ni concentrations as low as 400 mg/kg in soil. The germination process is very sensitive to metal exposure because metals like Ni can disrupt the metabolic events needed to establish a growing seedling. Established *P. tremuloides* saplings treated with nickel appear to be able to tolerate the metal at higher concentrations with significant damages only seen starting at 1,600 mg/kg.

Chapter 3: Expression of genes associated with nickel resistance induced by different doses of nickel and nitrate in trembling aspen (*Populus tremuloides*)

3.1. Introduction

Thanks to the development of a detailed genome sequence of *Populus trichorpa*, the genus *Populus* (poplar) has become the most studied for woody plants in term of genomics and transcriptomics (Dharmawardhana *et al.*, 2010; Qiu *et al.*, 2011; Foster *et al.*, 2015). Most of the investigations are related to gene expression at different developmental stages, growing weather conditions, and during infections. Analysis of poplar species exposed to environmental stressors is limited. Moreover, coping mechanisms of *Populus* spp. to soil metal contamination is lacking. The main objective of this research was to assess the regulation of genes associated with Ni resistance in model and non –model plant species in *P. tremuloides* under different doses of Ni treatments.

3.2. Materials and Methods

Seedlings treated with different doses of Ni described in section 2.3.4 were used for this study.

3.2.1. RNA Extraction and RT-qPCR

Total RNA was extracted from the root samples using the protocol described previously by Theriault *et al.* (2016b) with some modifications. Only 0.3 g of root material were used to extract RNA. The chloroform phase separation steps were carried out with 1 ml of CTAB solution: 1ml phenol chloroform. RNA was precipitated in 100 µl of SDS extraction buffer and chloroform steps were scaled down accordingly. Extracted RNA from 49 samples was run on a 1% agarose gel to verify quality. It was quantified with the Qubit® RNA BR assay kit by Life Technologies

(Carlsbad, United States). Samples from the same treatment were pooled together in equal amounts resulting in a total amount of 10 micrograms of root RNA per treatment.

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

RT-qPCR was performed for the targeted genes (Table 3) associated with nickel resistance in other plant species. BLAST search for these genes was performed based on the *Populus trichocarpa* genome in the NCBI database. Primers flanking the gene sequences were designed to span the exon-exon border of the gene, when possible. The OligoAnalyzer 3.1 program by IDT (https://www.idtdna.com/calc/analyzer) was used to check primer sequences for potential hairpins, self and hetero-dimers**.** Then, the cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit by Life Technologies. PCR was performed on cDNA and DNA. Reactions were run on a 1% agarose gel to verify the amplicon size. Primer pairs were used for RT-qPCR only if they showed a strong reproducible single band of the expected cDNA transcript size for the gene target. The RT-qPCR was performed according to the manufacturer's protocol using the Dynamo HS SYBR Green Kit (Life Technologies). Each reaction was run in triplicates using the MJ Research PTC-200 Thermal Cycler. The set program includes (1) initial denaturing at 95^{o}C for 15 min; 2) denaturing at 94°C for 30 sec; 3) 30 sec at 55 ^{o}C annealing; 4) elongation at 72^{o}C for 30 sec; 5) read 6) repeat step 2 –6 for 41 cycles; 7) final elongation at 72°C for 7 min; 8) melting curve 72 –95°C, every 1°C, hold for 10 sec; and 9) final elongation at 72°C for 3 min as described in Djeukam *et al*., (2017). The RT-qPCR was performed two separate times per gene target and samples were loaded in triplicates. The result

was six quantitation data points per bulked sample. Outliers among the triplicates were excluded in further analysis.

3.2.2. Data Analysis

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

3.3. Results:

Primer pairs used to amplify the housekeeping and the target genes are listed in Table 1. Based on the PCR amplification data, high affinity nickel transporter family protein (*AT2G16800*), *MRP4*, Nicotianamine synthase (*NAS3*) and metal transporter (*NRAMP4*), along with α-tubulin were selected for RT-qPCR and further gene expression analyses.

A decrease of *AT2G16800* expression was observed as the nickel nitrate concentration increased with the lowest level of expression observed in samples treated with 800 mg/kg (Figure 6a). No significant differences in *AT2G16800* expression was observed in samples treated with water compared to those treated with different doses of potassium nitrates (Figure 6b). There was no significant difference in *AT2G16800* expression level between the nickel nitrate treatments and their corresponding potassium nitrate controls with the exception of the 800 mg/kg dose for

which a significant reduction in this gene expression caused by nickel was observed. A significant repression of the *AT2G16800* gene was observed for the 400 mg/kg, 800 mg/kg, and 1,600 mg/kg of nickel nitrate compared to water control (Figure 6c).

A significant increase of *NAS3* expression was induced by the 400 mg/kg and 800 mg/kg of nickel nitrate doses compared to water. But no significant difference was observed between the 1, 600 mg/kg dose and the water control (Figure 7a). There was no significant difference in gene expression when the 150 mg/kg dose of potassium nitrate was compared to water (Figure 7b**)**. Surprisingly, *NAS3* expression was significantly upregulated at the 400 mg/kg, 800 mg/kg, and 1,600 mg/kg dose with a 1.5X, 3X, and 3X fold increase compared to water, respectively (Figure 2b and 2c). Significant differences between nickel nitrate and potassium nitrate were observed at 150 mg/kg, 800 mg/kg and 1,600 mg/kg (Figure 7c**).**

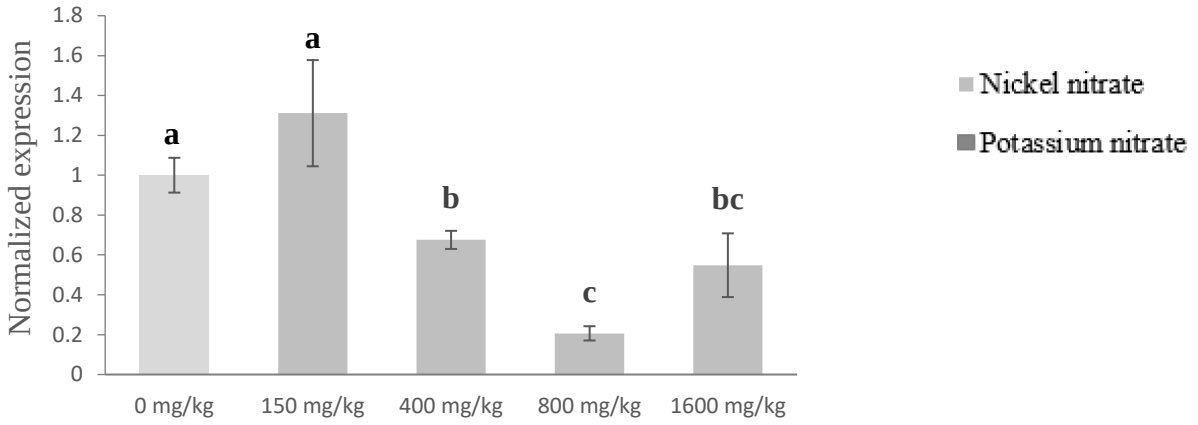
No significant difference in *NRAMP4* expression was observed for any of the four nickel nitrate doses compared to water (Figure 8a). On the other hand, there was an unexpected trend of increased of *NRAMP4* expression when samples were treated with increasing concentrations of potassium nitrate (Figure 8b). The nitrate controls for the 800 mg/kg dose and the 1,600 mg/kg dose were significantly different from the water control, and the 150 mg/kg dose. The 400 mg/kg dose was significantly different from the 1,600 mg/kg dose as well. When nickel nitrate and potassium nitrate were compared, there were a significant upregulation of *NRAMP4* induced by 150 mg/kg of nickel nitrate compared to potassium nitrate used as control; and a significant downregulations for the 800 mg/kg and the 1,600 mg/kg (Figure 8c).

No data were generated with the primers targeting *MRP4* gene since no amplification was observed even after a series of optimization of the RT-qPCR processes

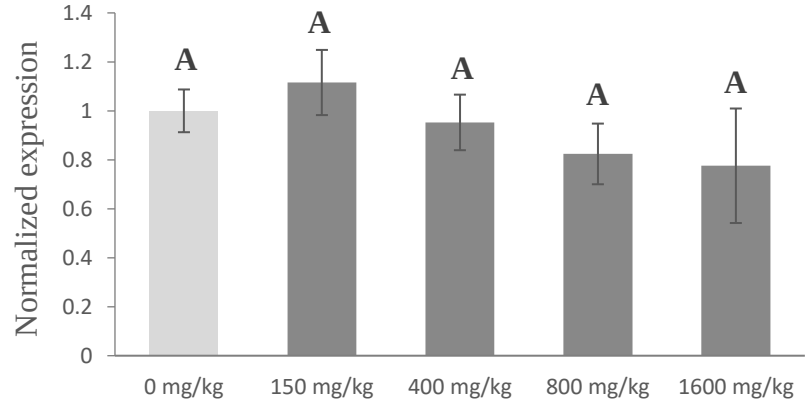
Table 3: Sequences of trembling aspen (*Populus tremuloides*) primers used for RT-qPCR

Target	Melting temp (° C)	Primer	Expected amplicon (bp)	PCR product in cDNA (bp)
MRP4	F: 60.00	F:TGTTGGCTTAGCGCTCCTAT	158	158
	R: 60.01	R:TTGCCTTCATCCTTGAATCC		
NAS3	F: 60.02	F:AAAGTTGCGTTTGTGGGTTC	232	232
	R: 59.98	R:CTGCCAAGAAGACGACATCA		
NRAMP4	F: 59.96	F: CCTTGTAAATGCAGGGCAAT	292	292
	R: 60.02	R: TGACTGCAGCACATTTAGCC		
AT2G16800	F: 59.97	F:AAAGGACCGACTCCACATTG	239	239
	R: 60.21	R:GTAGCCCATGGACAATACCG		
Housekeeping				
α-tubulin	F: 60.21	F: GGCAAGCAGGGATTCAAGTA	150	150
	R: 60.36	R: GGCACATGTTTTCCAGAACC		

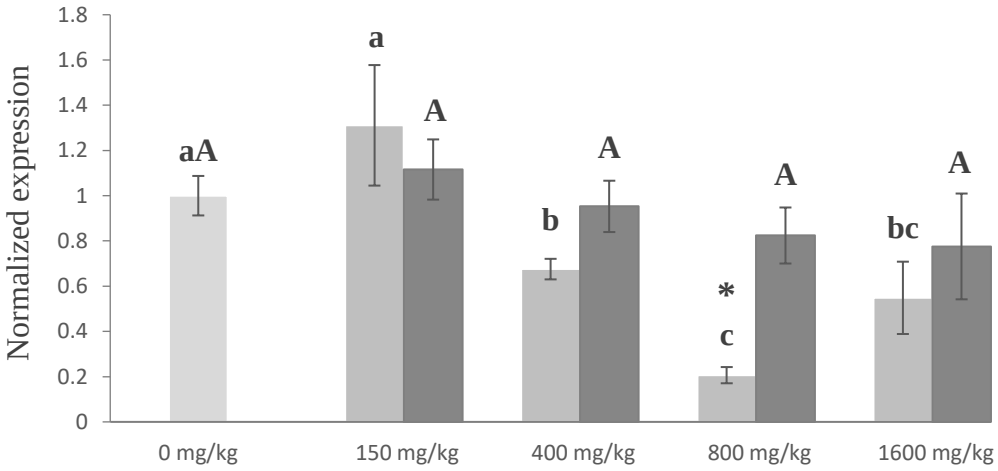
*Primers were designed by matching gene sequences to the *Populus trichocarpa* genome. When possible primers were designed to span the exon-exon border of the gene.



a)

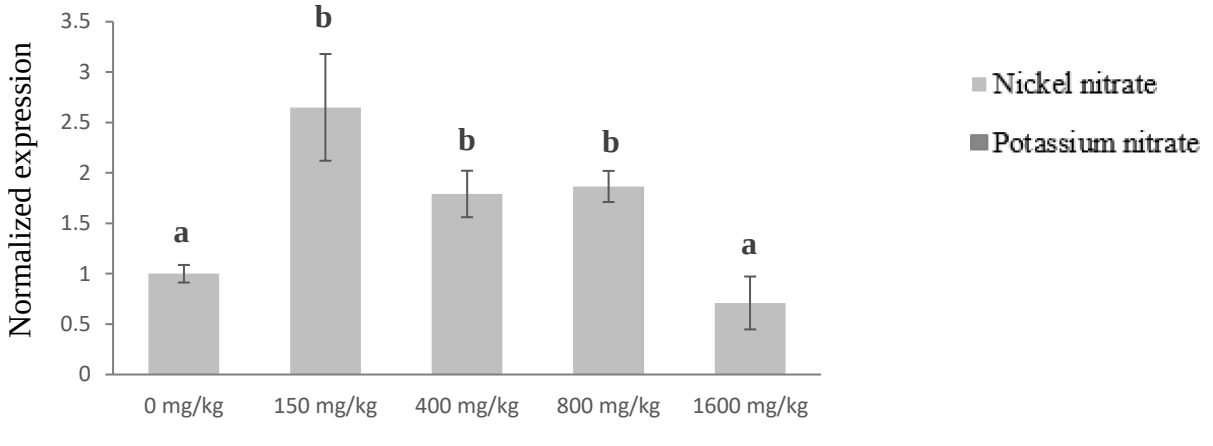


b)

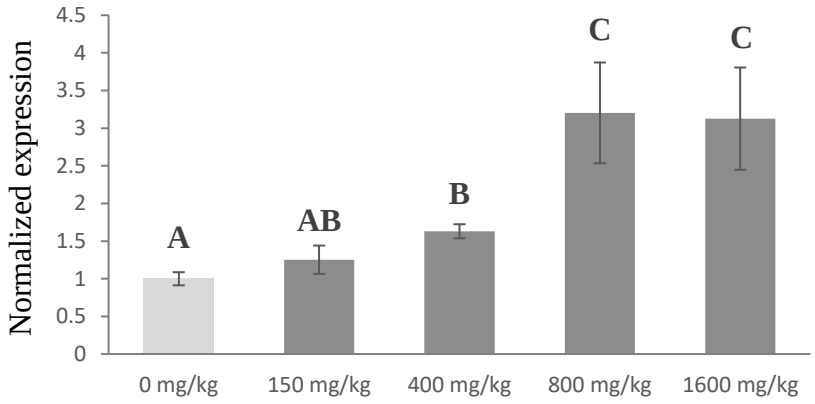


c)

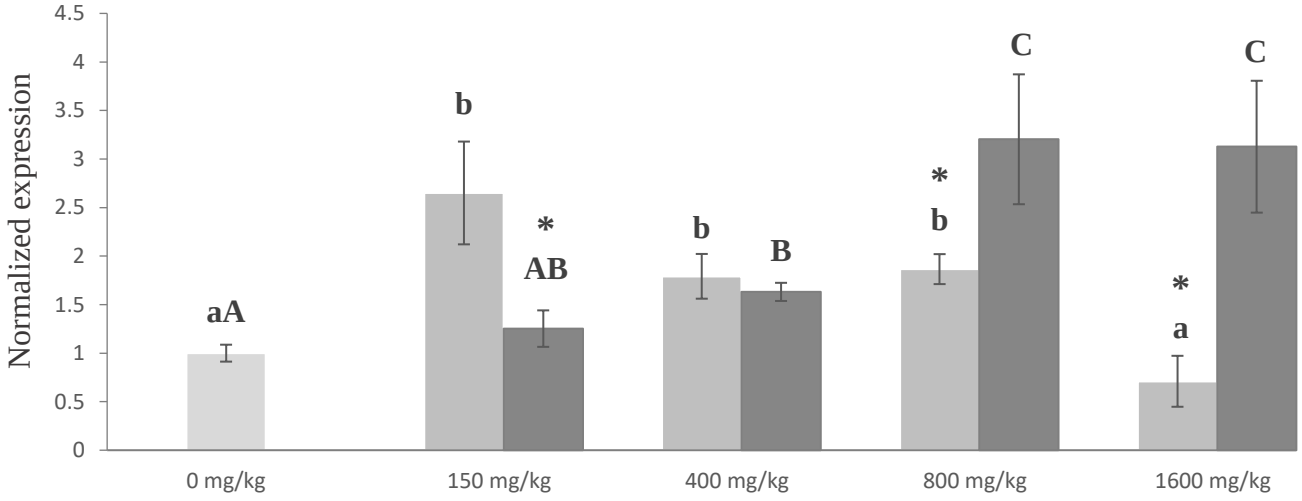
Figure 6: *AT2G16800* gene expression in trembling aspen (*Populus tremuloides*) treated with different doses of a) nickel nitrate and b) potassium nitrate. The gene expression was normalized to housekeeping gene (α-tubulin) and water was used as the negative control. c) Gene expression of all the treatments combined. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water. Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*****)



a)



b)



c)

Figure 7: *NAS3* gene transcription in trembling aspen (*Populus tremuloides*) treated with different doses of a) nickel nitrate and b) potassium nitrate. The gene transcription was normalized to housekeeping gene α-tubulin and water was used as the negative control. c) Gene transcription of all the treatments combined. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water. Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk.

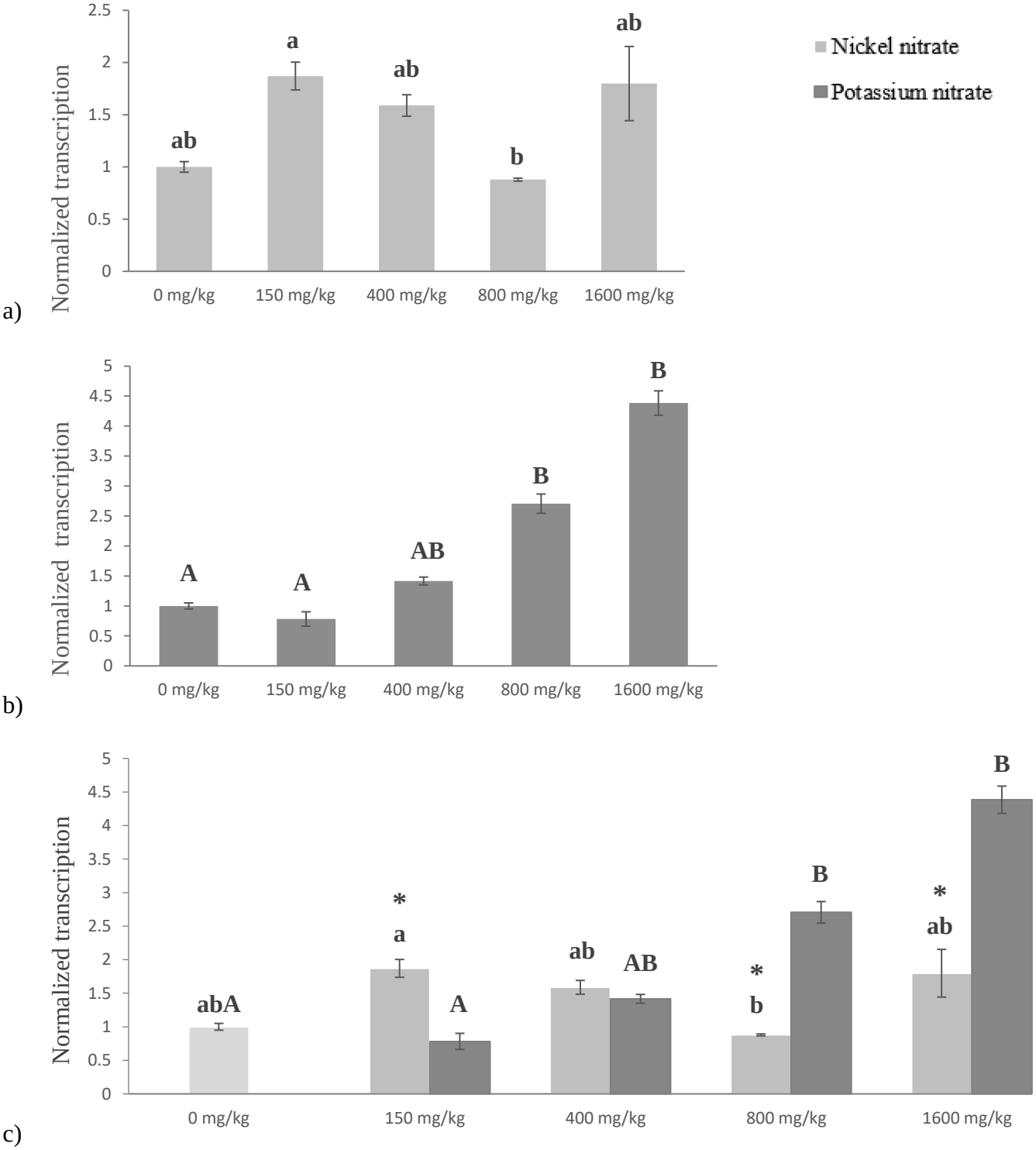


Figure 8: *NRAMP4* gene transcription in trembling aspen (*Populus tremuloides*) treated with different doses of a) nickel nitrate and b) potassium nitrate. The gene transcription was normalized to housekeeping gene α-tubulin and water was used as the negative control. c) Gene transcription of all the treatments combined. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water.

Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*****)

3.4. Discussion:

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

AT2G16800 is a known nickel/cobalt ion transporter from the NiCoT protein family. This protein utilizes the proton-motive force to drive the uptake of Ni^{2+} and Co^{2+} ions into the plant root system (Rodionov *et al.* 2006). This family of transporters is well characterized in prokaryotes that use these genes for nickel bioaccumulation (Deng *et al.* 2013). Though knowledge of the Ni/CoT is limited in plants, it is a good candidate gene involved in metal resistance in accumulators. Theriault *et al.* (2016) investigated the effects of the nickel treatment at the high dose of 1,600 mg/kg on *AT2G16800* expression in the accumulator species *Betula papyrifera*. They found that Ni significantly repressed *AT2G16800* expression suggesting that excess Ni does affect *AT2G16800* activities. However, no difference was found in the expression level at this dose among the susceptible, moderately susceptible and resistant genotypes. This suggests that *AT2G16800* is not directly involved in nickel resistance in *B. papyrifera*. A similar result was found in the present study where the expression of *AT2G16800* in *P. tremuloides* was significantly repressed with increasing nickel concentrations compared to the water reference. This repressive effect was most strongly seen at the 800 mg/kg dose. The significant difference in *AT2G16800* expression seen in this nickel treatment compared to the water control and its corresponding nitrate control is assumed to be a nickel effect because the 800 mg/kg nitrate control treatment was similar to water.

In most studies using nickel nitrate, the potential effects caused by these nitrate anions is ignored (Freeman *et al.* 2004, Douchkov *et al.* 2005 and Merlot *et al.* 2014). However, there is evidence that high nitrate concentrations can be toxic to plants when they exceed the thresholds that plants can tolerate (Parker *et al.* 1983; Goyal and Huffaker 1984). In fact, in the present study, all the *P. tremuloides* genotypes treated with potassium nitrates showed no significant toxicity symptoms or damage. Furthermore, the nitrate controls did not noticeably affect the germination of *P. tremuloides* seeds except for a slight decrease in germination rate seen at the 1,600 mg/kg dose. The gene expression of the target metal transport genes were expected to be unaffected by the nitrate doses as seen in *AT2G16800*. Unexpectedly, both *NAS3* and *NRAMP4* expression had a trend of upregulation with increasing concentration of potassium nitrate used as controls. This difference was most significant at the 800 mg/kg dose for both genes. This highlights the importance of including control treatments when using metal salts to assess the toxicity of the specific element.

The Nicotianamine synthase (*NAS*) genes are important for the synthesis of the cross-metal chelator protein nicotianamine (NA) which can bind numerous transition metals. Recently, Ni has been identified to have high binding affinity to NA, particularly in the Ni and Zn hyperaccumulator species *T. caerulescens* (Vacchina *et al.* 2003). The *NAS3* gene was found to be overexpressed and high levels of NA were produced in *T. caerulescens* and in another hyperaccumulating species *A. halleri* exposed to Ni (Vacchina *et al.* 2003; Becher *et al.* 2004). This implication with nickel tolerance suggests that *NAS3* gene expression would also be affected in *P. tremuloides*. Overall, our results show a trend of higher *NAS3* expression in samples treated with both nickel nitrate and potassium nitrate. This suggests that nitrate affects the expression of *NAS3* and that the use of nitrate control should be required in any study

assessing effects of Ni using nickel nitrate salts. The role of this gene in *P. tremuloides* nickel tolerance is unclear.

The *NRAMP* transporters gene family is conserved in many organisms including plants. These genes code for *NRAMP* proteins that can bind and complex heavy metal ions for transport. The transcriptome analysis conducted by Theriault *et al.* (2016) associated the gene expression of two *NRAMP* transporters with nickel resistance and nickel accumulation in *B. papyrifera*. They found a downregulation of five genes involved in metal transport including *NRAMP1* and *NRAMP2* for plants that were nickel resistant. It was hypothesized that the expression of *NRAMP* transporters may have a similar role in nickel resistance in *P. tremuloides*. The *NRAMP4* primers used in RT-qPCR confirmed the presence of this gene in *P. tremuloides*. The results showed no significant differences in *NRAMP4* expression in any of the nickel treatments compared to water. However, high concentrations of potassium nitrates at the 800 mg/kg and 1,600 mg/kg dose did significantly increase *NRAMP4* expression in comparison with the water treatment. The direct comparison of nickel nitrate and potassium nitrate suggest that *NRAMP4* gene expression seems to be more affected by potassium than the nickel and these effects increase as the concentration increases. For the *MRP4* gene, the failure of its amplification using the designed primer pair may be a result of the lack of primer binding sites or weak primer binding.

Overall, the results of the present study confirmed data reported by Kalubi *et al.* (2018) in a field study indicating that the low level of bioavailable nickel in metal–contaminated soils (< 150 mg / kg) cannot induce differential expression of *AT2G16800*, *NAS3*, and *NRAMP4*.

Chapter 4: General Conclusions

Nickel is a micronutrient required for plant growth and physiological functions. An excess amount will cause Ni toxicity that is detrimental to plant development. Hence, with the increasing levels of Ni contamination in the environment, it is essential to understand the functional roles and toxic effects of Ni in plants. The main objectives of the present research were to determine the effects of different doses of nickel on a) *P. tremuloides* seed germination and b) gene expression under controlled conditions.

All four concentrations of nickel nitrates tested ranging from 0.401 mg of Ni per mL of media to 4.28 mg/mL, inhibited completely seed germination. However, germination and seedling development were observed at about an equal percentage (fifty percent of seeds germinating) in negative water (agarose without nickel) controls, and nitrate control (without nickel).

Almost all the seeds planted in soil samples treated with water and nitrate controls germinated by day 8 of the trials. Over the course of the experiment, there was a decrease in germination as nickel concentration in the soil was increased.

No significant damage was observed when *P. tremuloides* seedlings were treated with 150 mg of Ni per 1 kg of soil (bioavailable amount of Ni), 400 mg/kg, and 800 mg/kg doses. Segregation in the seedling groups treated with 1,600 mg/kg dose was observed with some genotypes being resistant to Ni and others moderately resistant or susceptible. No damage was observed on seedlings treated with the potassium nitrate even at the highest dose of 1, 600 mg/kg.

At the gene level, a decrease of *AT2G16800* expression was observed as the nickel nitrate concentration increased with the lowest level of expression observed in seedlings treated with the 800 mg/kg dose. This supports a previous report that excess Ni affects *AT2G16800* in *B.*

papyrifera. However, no difference was found in the expression level at this dose among susceptible, moderately susceptible and resistant *P. tremuloides* genotypes. This suggests that *AT2G16800* is not directly involved in nickel resistance in *P. tremuloides*.

A significant increase of *NAS3* expression was induced by the 400 mg/kg and 800 mg/kg of nickel nitrate doses compared to water. But no significant difference was observed between the 1, 600 mg/kg dose and the water control. Surprisingly, *NAS3* expression was significantly upregulated at the 400 mg/kg, 800 mg/kg, and 1,600 mg/kg dose of potassium nitrate with a 1.5X, 3X, and 3X fold increase compared to water, respectively. Significant differences between nickel nitrate and potassium nitrate were observed between the two salts at 150 mg/kg, 800 mg/kg and 1,600 mg/kg suggesting that the differential gene expression is likely due to nickel**.**

No significant difference in *NRAMP4* expression was observed for any of the four nickel nitrate doses compared to water. On the other hand, there was an unexpected trend of increased of *NRAMP4* expression when samples were treated with increasing concentrations of potassium nitrate. When nickel nitrate and potassium nitrate were compared, there were a significant upregulation of *NRAMP4* induced by 150 mg/kg of nickel nitrate compared to potassium nitrate used as control; and a significant downregulations for the 800 mg/kg and the 1,600 mg/kg. Overall, only the metal transporter *AT2G16800* gene was clearly affected by nickel while *NAS3* is affected by both nickel and nitrate potassium and *NRAMP4* seem to be affected by nitrate potassium only.

Future studies

To better understand the mechanism underlying the expression of genes in *P. tremuloides* under Ni contamination, a global transcriptome analysis comparing resistant and susceptible *P. tremuloides* should be performed. This investigation could also identify novel candidate genes associated with nickel resistance that are differentially expressed resulting in differences in damage by nickel toxicity like seen in the present study. The expression of genes at a global transcriptome level will be validated if the expression of genes are dose-dependent.

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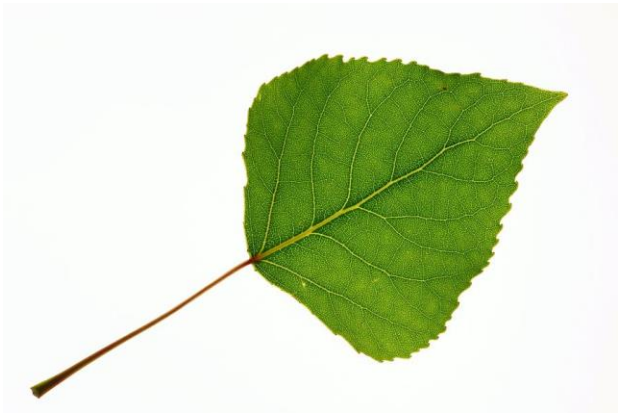
Appendices

Appendix 1: Trembling Aspen (*Populus tremuloides)*



Appendix 2: PCR verifying the amplification of cDNA gene targets. Lane 1- 1kb ladder, Lane 2-5 – cDNA amplified with primers for *AT2G16800, MRP4, NAS3, NRAMP4* in that order. Lane 6-9 – cDNA and control *P.tremuloides* DNA amplified with primers for *AT2G16800, MRP4, NAS3, NRAMP4*. Lane 10-13 – No template controls with primers *AT2G16800, MRP4, NAS3, NRAMP4*.